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Age-related decline in goblet cell numbers and mucin content of the human colon: Implications for lower bowel functions in the elderly

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ABSTRACT

Background & aims: Older people experience a greater incidence of lower bowel disorders, including constipation. Causes can include factors associated with growing older, such as use of medications or disease, but compounded by degenerative changes within the bowel wall. It has been suggested that the latter is exacerbated by loss of an effective mucosal barrier to luminal contents. In human colon, little is known about the impact of ageing on key components of this barrier, namely the goblet cells and mucin content.

Methods: Changes in the number of goblet cells and density of mucin content were investigated in macroscopically normal human ascending (AC; $n = 13$) and descending (DC; $n = 14$) colon from elderly ($\geq$ 67 years) and younger adults (60 years and below). Samples were serially sectioned and stained for haematoxylin and eosin to assess tissue morphology, and alcian blue periodic acid Schiff (ABPAS) and MUC-2 antibody to identify goblet cells producing mucins. New procedures in visualization and identification of goblet cells and mucin contents were employed to ensure unbiased counting and densitometric analysis.

Results: Compared with the younger adults, the numbers of goblet cells per crypt were significantly lower in the elderly AC (72 $\pm$ 1.2 vs 51 $\pm$ 0.5) and DC (75 $\pm$ 2.6 vs. 54 $\pm$ 1.9), although this reduction did not reach statistical significance when assessed per mucosal area (AC: $P = 0.068$; DC: $P = 0.096$). In both regions from the elderly, numerous empty vesicles (normally containing mucins) were observed, and some areas of epithelium were devoid of goblet cells. Thus, the density of mucin content per unit mucosal area were significantly reduced with age.

Conclusions: Ageing could result in a reduced number of goblet cells and development of degenerative changes in mucin production. Together, these have implications for the mucus barrier function in the colon of elderly individuals.

1. Introduction

For humans, the colon of the elderly has undergone several functional and structural changes, compared with younger adults (Baidoo and Sanger, 2024). These include a loss of primary sensory afferent innervation, reducing the ability to sense visceral pain (Yu et al., 2016; Cibert-Goton et al., 2020). Changes, within the wall of the human colon include a loss of pacemaker cells (interstitial cells of Cajal; Gomez-Pinilla et al., 2011), reduced cholinergic motor nerve function (Broad et al., 2019) and numbers of enteric glial cells (Baidoo et al., 2023a), an increase in senescence-like activity within myenteric nerve cell bodies (Palmer et al., 2021), and increased total collagen content within the muscle and submucosal plexus (Baidoo et al., 2022a, 2022b). Mucosal basal resistance, basal short-circuit current, or current evoked by neuronal stimulation appear unchanged (Krueger et al., 2016). Some, but not all the changes within the colonic wall, occur only in the ascending (AC), not descending (DC) colon, raising the possibility that in humans, ageing can influence the physiology of the colon in region-dependent and -independent ways.

It has been suggested that the intestinal wall may become damaged during ageing in a region-dependent manner because the integrity of the mucosal barrier in specific regions has been weakened, allowing potentially noxious stimuli from the lumen to cross into the wall of the intestine (Broad et al., 2019). In one study, the mucosa of terminal ileum from the elderly showed increased permeability to solutes, but not macromolecules (Man et al., 2015). However, in human colon, any

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effects of ageing on the integrity of the mucosal barrier are unknown.

A key component of the mucosal barrier of the intestine is the outer mucus layer. Goblet cells within the epithelial layer of the mucosa produce and maintain the mucus layer by synthesizing and secreting high-molecular-weight glycoproteins called mucins (Specian and Oliver, 1991). The mucus creates a physical barrier and further protects by hosting antimicrobial peptides and key mediators of the immune system (e.g., immunoglobin A; see Lamont, 1992; Johansson et al., 2008; Gill et al., 2010; Kim and Khan, 2013; Yang and Yu, 2021). In addition, the mucus layer lubricates the epithelium surface, to facilitate movement of luminal contents (Cornick et al., 2015; Zhang and Wu, 2020). The development of several GI disorders, including inflammatory bowel disease (Shirazi et al., 2000; Einerhand et al., 2002; Sheng et al., 2012), colorectal cancer (Kufe, 2009; Loktionov, 2022) and enteric infections are influenced by considerable changes in mucin quality and quantity (McGuckin et al., 2011).

Studies with aged mice have generated mixed findings. Shrinkage of the colonic mucus layer (Elderman et al., 2017; Sovran et al., 2019) has been linked to increased bacterial penetration and apoptosis of goblet cells (Sovran et al., 2019). Others report an increased thickness of the mucus layer with increasing age, albeit with a decline in its structural integrity. Further, the number of goblet cells within the mucosal crypts was unchanged, although the proportion of crypts staining for goblet cells was smaller (Sang et al., 2023). Age-related changes in mucosal barrier integrity might also be anticipated in humans, but translational studies are needed because of significant differences in gastrointestinal (GI) tract physiology between humans and rodents, together with marked differences in metabolism and rates of ageing (Sanger et al., 2011).

We have used histochemistry and immunohistochemistry techniques to comprehensively measure the patterns of distribution of goblet cells and mucin content within the human colonic mucosa of the elderly ($\geq$ 67 years) and younger adults (60 years and below). It was important to study different regions of colon separately. The different regions have different functions (e.g., bacterial fermentation of carbohydrates and proteins within ascending colon, extraction of fluid and electrolytes within descending colon), microbiome composition (O'Hara and Shanahan, 2006; Martinez-Guryn et al., 2019; Rodríguez-Romero et al., 2021), and expression of cytoprotective inducible heat shock proteins by surface epithelial cells, regulated in turn by the microbiome (Liu et al., 2022). In addition, as discussed above, ageing can influence colonic functions in a region-dependent manner.

2. Materials and methods

2.1. Subject selection

Macroscopically normal, full thickness, ascending colon from 13 patients (elderly: $n = 9$; 70–89 years and younger adults: $n = 4$; 31–52 years) and descending colon from 14 patients (elderly: $n = 5$; 67–82 years and younger adults: n = 9; 42–60 years; **Supplementary 1**), were obtained from patients at Barts Health NHS Trust following surgery for non-obstructed bowel cancer, after informed, written consent. The sections of colon were obtained at least 5–10 cm away from the tumour and were prospectively collected. None of the patients had previous chemoradiotherapy or diagnosis of active inflammatory colonic disease which may influence epithelial goblet cell numbers and functions (Shirazi et al., 2000; Einerhand et al., 2002; Sheng et al., 2012). Patients with known functional bowel disorder were excluded. This study was approved by East London Ethics Committee (REC 10/H0703/71), the London City Road and Hampstead Research Ethics Committee (REC: 15/LO/21/27) and subsequently by the University of Westminster (ETH2324–1489).

2.2. Procedures

Human ascending and descending colonic tissues (~ 10 × 10 mm) were fixed in 10% neutral buffered formalin, processed overnight and transversely paraffin embedded to demonstrate the mucosal, submucosal, muscularis externa and serosal layers. The tissues were serially sectioned at 4 μm and mounted on super frost-plus glass slides as previously described (Baidoo et al., 2023b). To blind the investigators during analysis to the age of the patients, slides were assigned codes during sectioning. Before staining was performed, sections were deparaffinised and rehydrated.

In routine diagnostic histopathology, Haematoxylin and Eosin (H&E) staining can highlight goblet cells in thin tissue sections (Titford, 2005; Chan, 2014) but is not used for quantification. Instead, analysis of goblet cell number and density within tissue sections have typically been determined by tinctorial or immunohistochemical staining. In the human GI tract, the expression of goblet cells in tissue sections have also been identified using Alcian Blue to identify acidic mucins and periodic acid-Schiff for neutral mucins. However, in human colon, the majority of epithelial goblet cells secrete acidic mucin and very few produce neutral mucins (Danquah et al., 2017). On this basis, we stained for H&E, Alcian Blue Period Acid-Schiff (ABPAS) but also used MUC-2 Immunohistochemistry (IHC).

2.2.1. Haematoxylin and eosin staining

This was primarily performed to look for evidence of significant inflammation, tumour, or structural abnormalities (Feakins and British Society of Gastroenterology, 2013). In brief, all samples were manually stained in Harris haematoxylin and differentiated in 0.5% acid-alcohol. Nuclei were blued in Scott's tap water and counterstained with eosin (Cardiff et al., 2014). The sections were then dehydrated in graded series of industrial methylated spirit (70%, 90% and absolute) and cleared in xylene. Sections were subsequently mounted with Pertex (Sakura), cover slipped with glass slides (Sakura, Tokyo-Japan) and examined. These procedures stained the cell nuclei dark blue, and the cytoplasm a red to pink or orange colour.

2.2.2. Alcian blue periodic acid-schiff

This was performed to look for the presence of acid- and neutral-mucin within the sections, as similarly described (Röhe et al., 2018). Briefly, the sections were stained in 1% alcian blue (pH 2.5) for 30 min. These were rinsed in running tap water for 5 min then briefly in distilled water, followed by oxidation in periodic acid for 5 min. After rinsing again in running tap water for 5 min, the sections were covered with Schiff's reagent for 15 min, rinsed with running tap water for 10 min and then lightly stained with haematoxylin. Subsequently, sections were rinsed in tap water for 5 min, dehydrated, cleared, mounted with Pertex and cover slipped with glass slides (see **Supplementary 2** for Reagent and Solutions Preparation). In brightfield microscopy, acid mucin appeared deep blue-black whilst neutral mucin yielded magenta colour.

2.2.3. MUC-2 immunohistochemistry

Goblet cells in the human colon mostly secrete the MUC-2 mucin, which is highly glycosylated and binds to various O-link oligosaccharides (Arike et al., 2017). In the present experiments, staining for MUC-2 was performed manually as similarly described for thin sections (Zaqout et al., 2020). In brief, sections were treated in antigen retrieval solution (pH 6.0; microwaved at 800 W) for 8 min. After the sections had cooled down, the rim of each slide was marked using a hydrophobic barrier pen (PAP pen; ab2601, Abcam, Cambridge, UK) and placed in a humidity chamber (21,069 – B; Ted Pella, Inc., California, USA). Sections were washed for 10 min in phosphate buffer saline (PBS; replaced with fresh PBS after 5 min) and then for 10 min in cell permeabilization solution (PBS/gelatin/Triton 0.25%; replaced with fresh after 5 min). To block non-specific background staining, sections were incubated in Protein Block (ab64226, Abcam, Cambridge, UK) for at least 30 min at room

temperature. Recombinant Anti-MUC-2 antibody (ab272692, Abcam, Cambridge, UK), diluted in 1% BSA in (PBS/gelatin/Triton 0.25%) solution (1:15,000), was applied to the sections and incubated overnight at room temperature on a flat balanced surface without agitation. MUC-2 immunostaining visualization was based on both a fluorescent and horseradish peroxidase detection system. For fluorescent detection, a fluorophore-conjugated secondary antibody (Goat anti-rabbit IgG; Alexa Fluor 647; ab150079; Abcam, Cambridge, UK), diluted in 1% BSA in (PBS/gelatin/Triton 0.25%) solution (1:1000), was applied on the sections for 1 h. For horseradish peroxidase detection, mouse and rabbit specific HRP/AEC (ABC) detection IHC Kit (ab937905; Abcam, Cambridge, UK) was used, and the rest of the staining was performed following the manufacturer's recommendations (see Supplementary 2 for Reagent and Solutions Preparation). To ensure all MUC-2 immunoreactive (IR) structures were comprehensively visualised, 3,3'diaminobenzidine tetrahydrochloride chromogens (DAB; ab64238, Abcam, Cambridge, UK) and 3-amino-9- ethyl carbazole (AEC; ab93705, Abcam, Cambridge, UK) were used as the preferred coloured end-product. Positive and negative controls were performed using sections of colon with or without primary antibodies. Non-specific structures were counterstained with DAPI (4′,6-diamidino-2-phenylindole; ab104139, Abcam, Cambridge, UK) and Mayer's haematoxylin in fluorescent and horseradish peroxidase, respectively. Stained sections were appropriately cover slipped. MUC-2-IR structures stained brown for DAB, red for AEC and a red signal for immunofluorescence (IMF).

2.2.4. *Quantification of goblet cells and mucin content*

Image acquisition was captured using both brightfield (Nikon Eclipse 80*i,* Melville, United States), and fluorescence microscopes (Olympus BX61, Tokyo, Japan) equipped with a digital camera. Sequential images were captured to obtain at least 80% of the mucosa (i.e., from the apical part of the epithelial cell to the base of the crypt and from the left to right direction of the section). All images from sections stained for histochemistry and immunohistochemical methods were acquired under identical conditions. To maintain image integrity and clarity, all acquired digital images were stored in an uncompressed tagged image file format (TIFF; 24-bit RGB; 7.3 MB and 10.67 × 8.00 in. (1600 × 1200)) for ABPAS and IHC (DAB and AEC visualizations; 8-bit RGB; 1.3 MB;

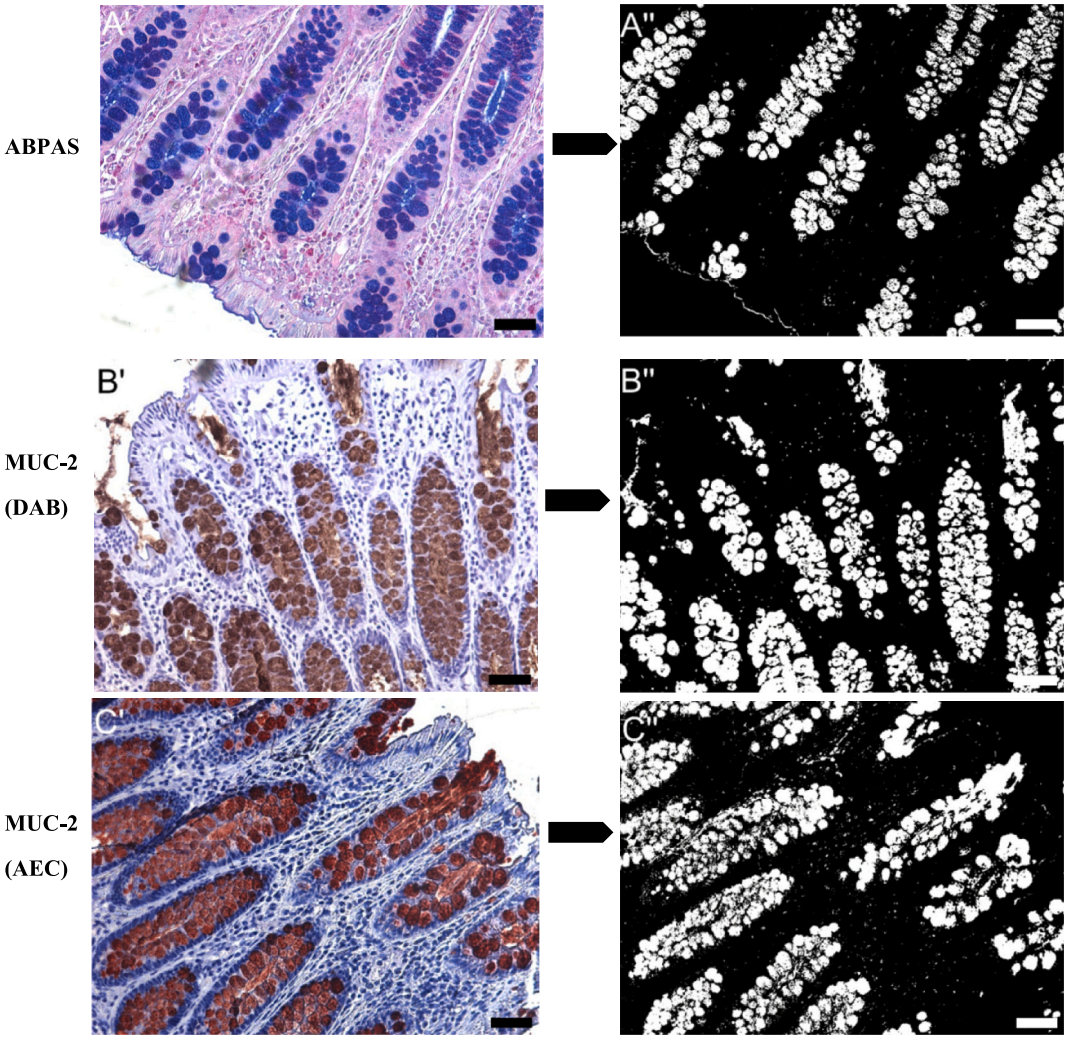

Fig. 1. Analysis of goblet cells producing mucins in the mucosa of formalin-fixed, paraffin-embedded human colon. Human colonic sections were stained in alcian blue periodic acid-Schiff (ABPAS; A') to demonstrate mucins (blue-black and magenta) and MUC-2 mucin by immunohistochemistry method and were visualised by 3,3'diaminobenzidine tetrahydrochloride (DAB; B′) and also by 3-amino-9- ethyl carbazole (AEC; C′). ImageJ processing (version 1.54f) separated colours from background as blue-black and magenta for ABPAS; brown for MUC-2 (DAB) and red for MUC-2 (AEC). Filtered images were then converted to black and white (A"-C″). The density of ABPAS positive cells and MUC-2 immunoreactive structures was obtained from the positive pixel per unit area of the mucosa. The mean density of mucin per unit area of mucosa were 56, 67 and 64 for ABPAS, MUC-2 (DAB) and MUC-2 (AEC) techniques, respectively. All images were taken under identical conditions at 10.67 × 8.00 in. (1600 × 1200) resolutions. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1344 × 1024 pixels) for fluorescence staining. Following digital visualization, the numbers of goblet cells expressing mucins (herein referred to as both acidic and neutral mucins) and stained with ABPAS and MUC-2, were counted using the Cell counter tool in ImageJ (version 1.54f; Schneider et al., 2012) and by direct microscopy examination when blinded to patient age and region of sample. At least 20 crypts per colonic mucosa were examined and the average number of goblet cells was calculated per crypt and by mucosal area. ABPAS positive cells were clearly defined and seen as distinct deep blue-black or magenta structures. For MUC-2 mucin quantitation, images were taken at 20× magnification using the automated setting for focus and exposure. MUC-2 mucin visualizations using DAB and AEC, were defined respectively as brown and red distinct structures with blue nuclei stains. MUC-2 staining of goblet cells, using the fluorescence method depicted blue nuclei (with DAPI) and a red signal for MUC-2-IR cells. Quantitative estimation of goblet cells count in the crypt was based on strict criteria: (1) They must be visible as distinct deep blue-black or magenta and brown or red stain. (2) When an overlapping or continuous region of deep blue-black or magenta and brown or red stain was observed to contain two distinct nuclei these were counted as two cells. (3) Vesicles containing mucin outside the crypt were not counted.

To evaluate the density of ABPAS-positive structures and MUC-2-IR, an area of positive staining was first defined, and then thresholds were systematically set relative to the mucosal region using ImageJ (version 1.54f; Schneider et al., 2012). The density of ABPAS-positive and MUC-2 mucin was determined as the amount of positive pixel per unit mucosal area (see Fig. 1). Similarly, the level of red fluorescence and blue nuclei were evaluated with ImageJ, and the density of MUC-2 mucin per unit area estimated (see Fig. 2).

The use of sequential imaging ensured that tissue sections were systematically analysed, avoiding the possibility that areas of few or many ABPAS-positive cells or MUC-IR structures were inadvertently and preferentially evaluated. At least 3 mm^2 of the crypt were quantified for goblet cell numbers and density in all the methods utilized. Sections with artefacts or poor staining were excluded from quantification. At least five sections (2 per slide) at 16 μm intervals per patient were used. All data were considered and averaged per elderly and younger adult patient.

2.3. Statistics and data analysis

The number of goblet cells and the mucin content within the mucosa were expressed as the mean ± SEM. Shapiro Wilk normality test was used to establish data distribution. Age-related changes in the number and density of goblet cells within the ascending and descending colon of elderly and younger adults were compared by a two-tailed independent student's *t*-test using the Statistical Package for Social Science (IBM Corp. Released 2023. IBM SPSS Statistics for Windows, Version 29.0.1.0 (171). Armonk, NY). A *P*-value of ≤0.05 indicated statistical significance. Unless otherwise specified, *n* represent the number of patients.

3. Results

Examination of H&*E*-stained tissue sections revealed no significant inflammation, tumour growth and structural abnormalities within the mucosa of sections from the ascending and descending colon of both the elderly and the younger adults. Abundant goblet cells featuring basally positioned dark blue nuclei and round pale vacuoles were identified in both groups. Notably, in some regions of the epithelium, goblet cells were absent, a phenomenon observed more frequently in the elderly samples (see Fig. 3).

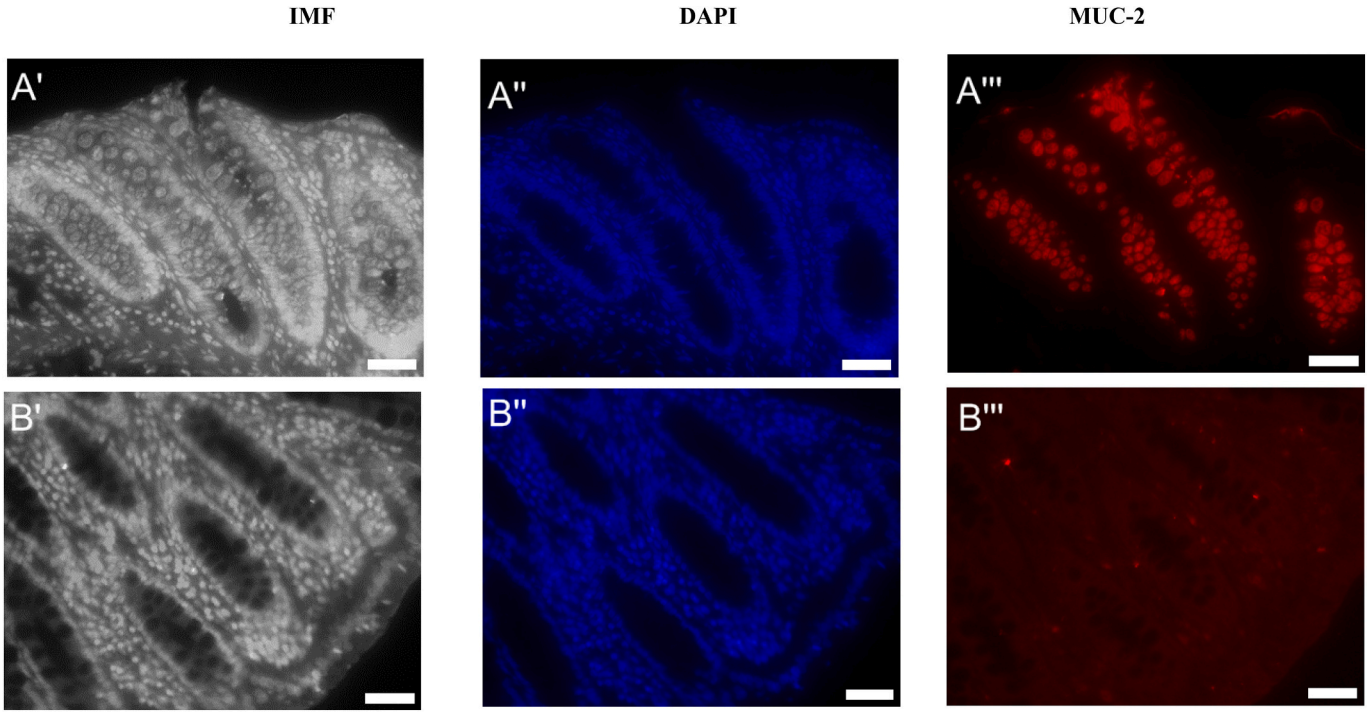

Fig. 2. Quantitation of MUC-2 mucin contents in formalin-fixed, paraffin-embedded human colon using immunofluorescence method. Thresholding was used to identify positive immunostaining. Panels (A'-B′) show the original colonic tissue morphology and panels (A''-B″) identify DAPI (blue nuclei). Image A''' shows MUC-2 immunoreactive structures stained with a fluorophore-conjugated secondary antibody (Goat anti-rabbit IgG; Alexa Fluor 647). To determine the density of MUC-2 immunoreactive cells, the density of total nuclei stain represented by DAPI (A'') was first determined by the level of fluorescence using ImageJ (version 1.54f). Then in the same area, the level of fluorescence obtained by MUC-2 immunoreactivity was measured. Finally, the amount of MUC-2 immunoreactive contents per unit area of the mucosa was expressed per total content of DAPI nuclei. The density of total nuclei identified by DAPI and MUC-2 IR structures were determined to be 76 and 36, respectively. B″' is a negative control without primary antibody. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

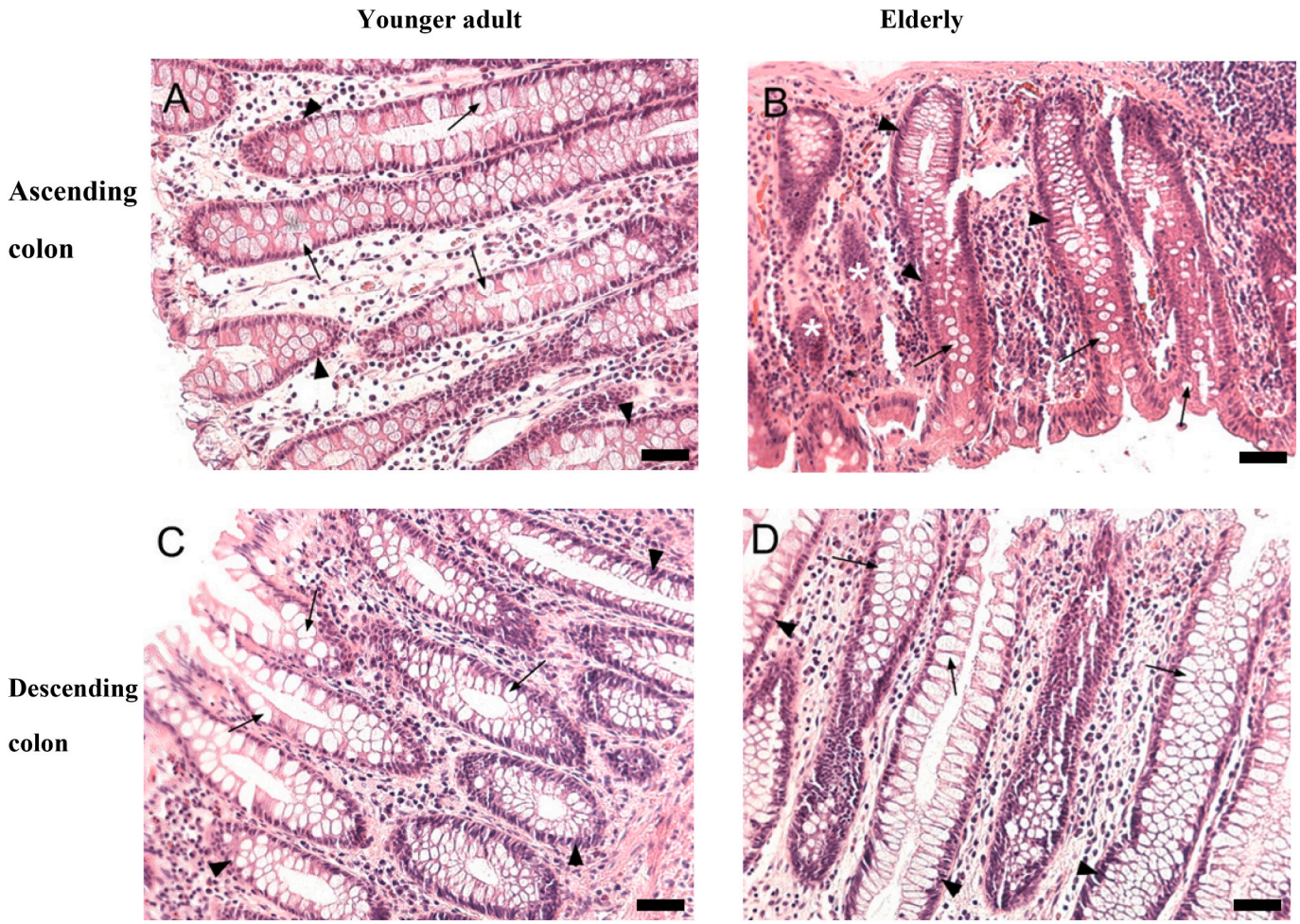

Fig. 3. Representative images of haematoxylin and eosin staining in the mucosa of human ascending and descending colon from younger adult (≤ 60 years) and elderly (≥ 67 years). None of the sections showed inflammation, tumour, or structural abnormalities within the mucosa. Goblet cells with basally located nuclei are stained dark blue (arrowhead) with round pale vacuoles (arrow). For some crypts, goblet cells were absent in the visible regions (*), a phenomenon observed more frequently in the elderly samples. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.1. Numbers of goblet cells and mucin content

The use of ABPAS and MUC-2 antibody staining (see Fig. 5A-H) made it possible to estimate the number of goblet cells and mucin content. The number of ABPAS and MUC-2 positive cells per crypts was quantified in the ascending (elderly: $n = 9$; 70–89 years and younger adults: $n = 4$; 31–52 years) and descending colon (elderly: $n = 5$; 67–82 years and younger adults: n = 9; 42–60 years).

Compared with the younger adults, the number of goblet cells per crypt was significantly smaller in both regions of colon from the elderly, as consistently demonstrated by all employed methods. However, when the number of goblet cells were expressed by mucosal area, the differences between the elderly and the younger adults in both regions did not reach statistical significance (AC: $P = 0.068$; DC: $P = 0.096$; Fig. 4). Microscopic examination revealed the presence of alternating acid mucin (blue) and neutral mucin (magenta) within the same crypts, with a greater abundance of acid mucin in the adult or younger adults compared to neutral mucin (see Fig. 5K, L & M). A qualitative analysis indicated a higher presence of neutral mucins in the elderly samples compared to the younger adults, so that in the elderly, the presence of acid and neutral mucins appeared equally balanced (Table 1).

3.1.2. Effect of age on mucin content in human colon

Studies in mice have demonstrated that the MUC-2 mucin O-glycan pattern varies according to the region of intestine (Holmén Larsson et al., 2013; Arike et al., 2017). Thus, to understand any effects of ageing on mucin production within the human colon, the mean intensity of ABPAS (acid and neutral mucins) and MUC-2 positive structures was comprehensively analysed per mucosal area in both the ascending and descending colon, using immunohistochemistry and immunofluorescence methods. The results showed that when compared with the younger adults, the density of ABPAS positive staining and MUC-2 mucin in the elderly samples of both regions of colon decreased with age, as assessed by all the methods employed (Fig. 5I, J & 6). The use of MUC-2 demonstrated sensitivity in visualizing goblet cells (see above), and no differences were observed in the identification of goblet cells when employing any of the methods utilized in this investigation (see Fig. 6E). Consistent with these observations, a greater number of empty vesicles, normally containing mucins, were observed in the mucosa of the elderly (Fig. 7). Thick, horizontally stratified structures were observed on the surface of the mucosal epithelium, using both ABPAS staining and the MUC-2 antibody (see Fig. 7A-C).

3.1.3. Regional differences

The number of goblet cells and the mucin content within the mucosa of the elderly AC were lower than within the DC. There was, however, no

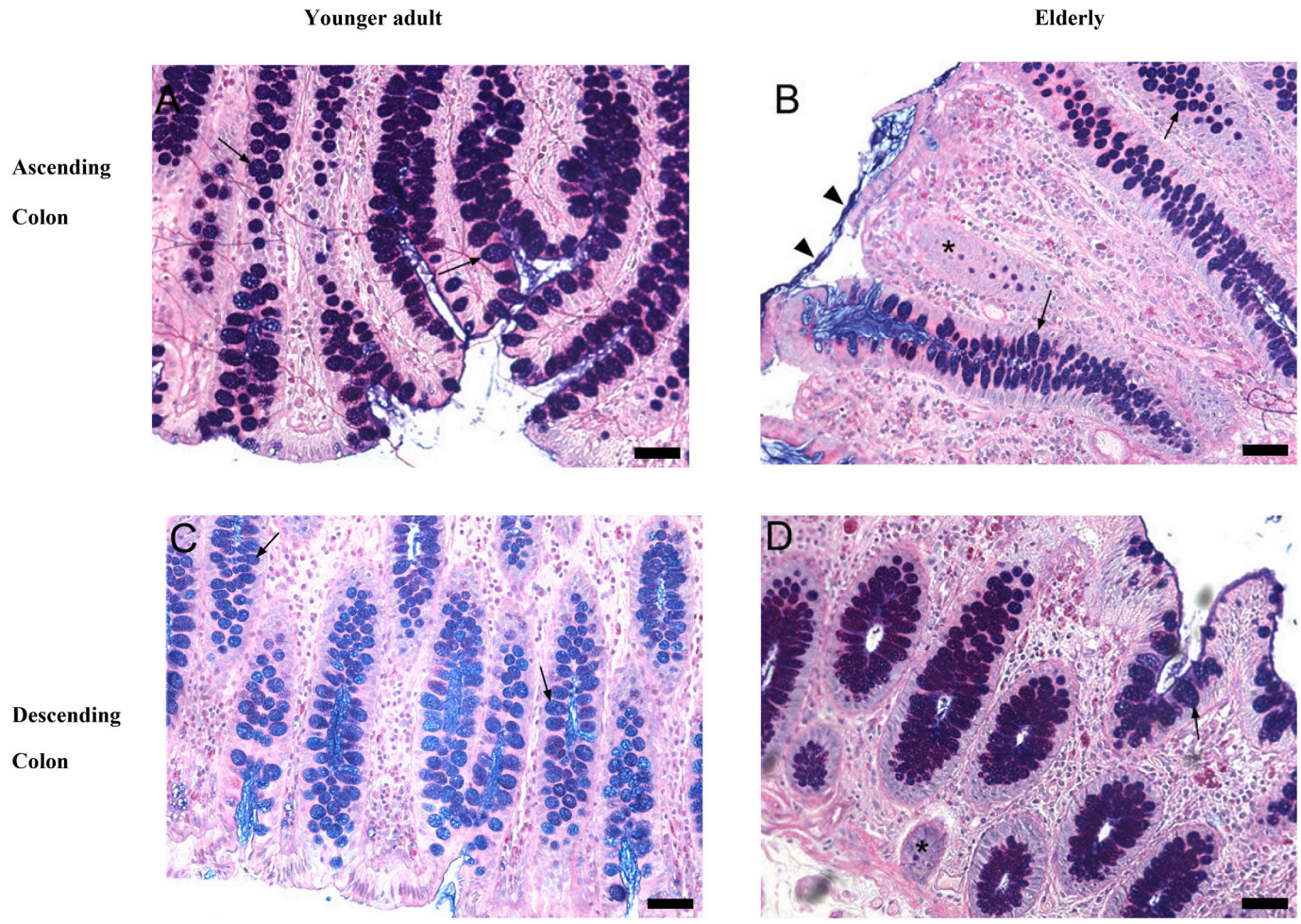

Fig. 4. Effect of age on the number of goblet cells in human colon. Representative images of alcian blue periodic acid-Schiff (ABPAS) staining in the mucosa of human ascending (AC; A-B) and descending (DC; C—D) colon from younger adult (≥ 60 years) and elderly (≥ 67 years) patient. Goblet cells identified using ABPAS staining were blue-black and or magenta cells (arrow). The number of ABPAS (+) cells within the mucosal area appeared to reduce with age but the differences were not statistically significant. Evidence of ABPAS (+) stratified structure on mucosal epithelium were present (arrowhead). In the elderly samples, there were some crypts that did not express ABPAS (*) and these were mostly found deep within the lamina propria. Counting of goblet cells expressing mucins (both acidic and neutral mucins) per crypt and unit area of the mucosa stained with ABPAS and MUC-2 were performed via digital visualization with a Cell counter tool in ImageJ (version 1.54f). At least 20 crypts per colonic mucosa were examined and the average number of goblet cells per crypt and then by mucosal area was calculated. Graphs represent the effect of age on goblet cells number per crypt (E-F) and mucosal unit area (G-H) assessed with ABPAS and MUC-2 antibody visualised with 3,3'diaminobenzidine tetrahydrochloride (DAB), 3-amino-9- ethyl carbazole (AEC) and immunofluorescence (IMF) steps in human ascending and descending colon. The number of goblet cells per crypt (assessed by student's independent *t*-test) decreases significantly with age ($*p < 0.05$). The reduction in goblet cell number per mucosal unit area in the elderly did not reach statistical significance compared with the younger adults, for both regions (respectively $P = 0.068$ and $P = 0.096$ for AC and DC). AC (Adult, n = 4; Elderly n = 9) and DC (Adult, n = 9; Elderly n = 5). The number of goblet cells per crypt and unit mucosal area were expressed as the mean ± SEM. NS: non-significant. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

statistically significance differences in the number of goblet cells per crypt and per mucosal unit area between the elderly AC ($n = 9$) and DC ($n = 5$), similar result was also found when goblet cells number were expressed per mucosal unit area in both regions (see Fig. 7 F & G). Microscopically, the elderly AC showed more areas of crypt devoid of goblets and empty mucin-vesicles features compared to the same in DC.

4. Discussion

Despite the importance of understanding the factors that contribute to age-related disorders of the lower bowel, there is a paucity of studies which investigate the influence of ageing on specific structures and functions within the human bowel (Baidoo and Sanger, 2024). This study is the first demonstration of a reduction in both goblet cell number and mucin content within the mucosa of both the ascending and descending colon from the elderly.

For the goblet cells, the age-related decline achieved statistical significance when analysed per crypt of the mucosa, for both the goblet cells secreting acidic and neutral mucin (ABPAS-positive) and for those expressing MUC-2 proteins. When the number of ABPAS-positive and MUC-2 IR cells were expressed by mucosal area, in both regions of colon, the reductions also tended to be smaller within the elderly population, but the differences did not reach statistical significance. Perhaps the loss of statistical significance can be attributed to variable exposure of all crypts in their entirety during sectioning and/or by age-related increase in crypt height and size (Nalapareddy et al., 2017). Consistent with the age-related decline in numbers of goblet cells, there was also a decline in the mucin content. In healthy individuals, goblet cells fill their secretory vesicles with MUC-2 while migrating from the bottom of crypt to the apical portion (Johansson et al., 2011). However, among the elderly,

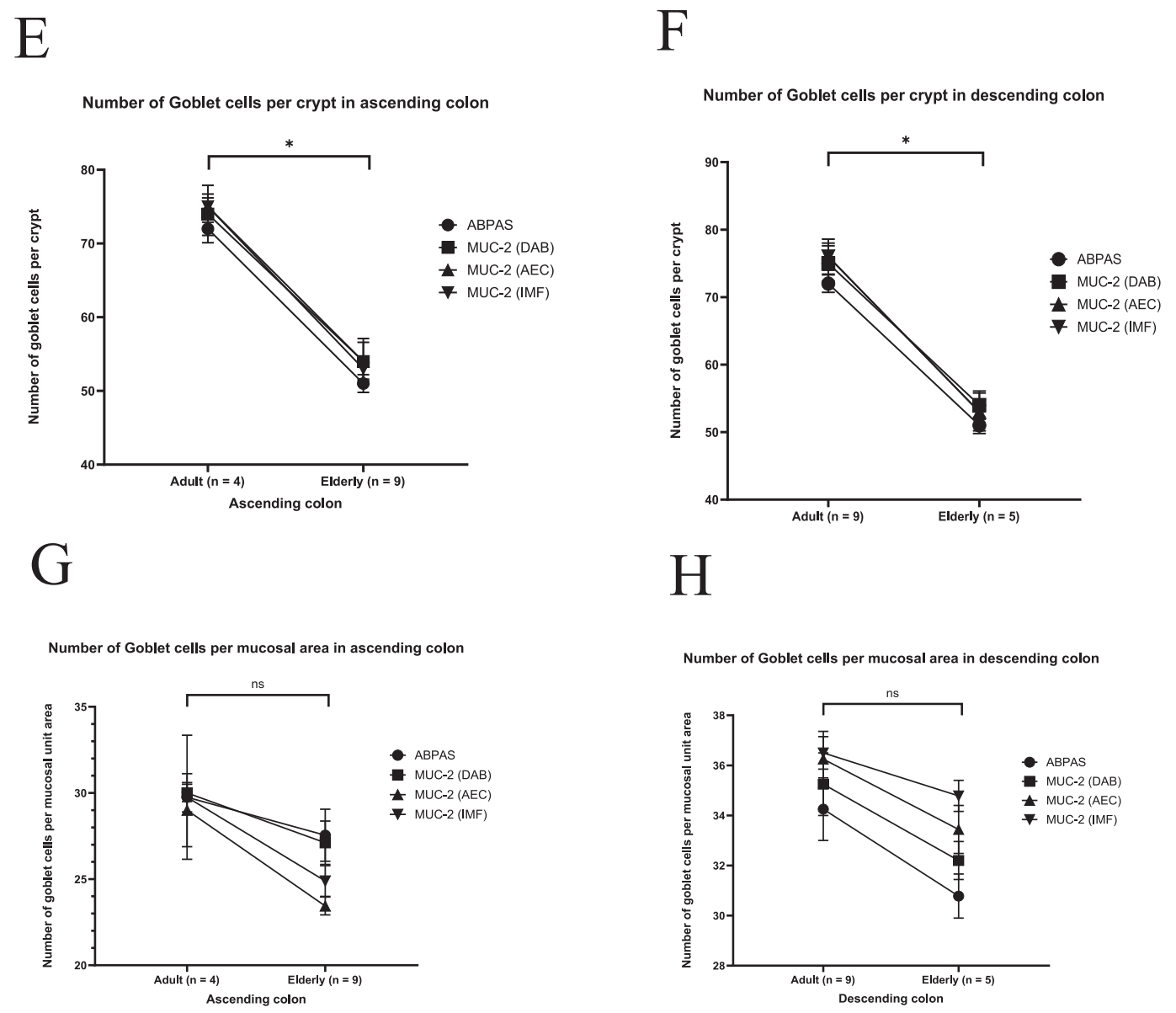

Fig. 4. (*continued*).

there appeared to be more crypts with goblet cells containing vesicles that were not positive to ABPAS staining and the MUC-2 antibody, particularly in the ascending colon. Thick, horizontally stratified structures were observed on the surface of the mucosal epithelium, using both ABPAS staining and the MUC-2 antibody. It is important to note that most of the outer layer of mucus may shrink or not survive the histological preparation process. For this reason, these structures were not quantified due to the use of formalin fixative in preparation of our samples. Nevertheless, it may be speculated that the structures which were observed may be part of the mucus layer which separates the intestinal epithelium from the luminal contents (Zhang and Wu, 2020; Guzmán-Mejía et al., 2021). Whether ageing impacts this mucus layer requires further investigation. Thus, in summary, the present data suggests that advancing age leads to a reduction in number of goblet cells within the colonic mucosa, together with a reduced ability to synthesise and/or store mucin.

Alcian blue and periodic acid-Schiff is a widely used tinctorial staining that, respectively labels acidic (Padra and Lindén, 2022) and neutral (Prasanna, 2016) mucins due to its strong colouration, specificity, and permanence in tissue sections. Indeed, many studies have used ABPAS staining to visualise goblet cells in the gut of humans and animals (Lindén et al., 2008; Osho et al., 2017; Kiliçarslan and Varli, 2024). The present study also used MUC-2 as a marker for goblet cells and to detect mucin presence. Compared with the ABPAS method, there were no significant differences in the ability of either marker to identify goblet cells, confirming the ability to use either marker with FFPE sections as similarly performed in mouse tissues (Gouyer et al., 2011). In order to comprehensively assess and visualise goblet cell numbers and mucin contents in the human colon, we employed both DAB and AEC detection steps in immunohistochemistry as well as immunofluorescence methods. This approach allowed for the thorough visualization of antigen-antibody binding and ensured accurate assessment of goblet cell number and mucin distribution without variations in chromogenic grading (Tsutsumi, 2021).

The present findings, demonstrating a reduced number of goblet cells in human colon from the elderly, are in part, similar to those obtained in colonic mucosal crypts of 19-month-old mice (Sovran et al., 2019), in the small intestinal mucosa of 17–24-month-old mice (Gebert et al., 2020) and in the colonic mucosa of 24-month-old mice (Sang et al., 2023). In other rodent studies, an increase in the number of goblet cells

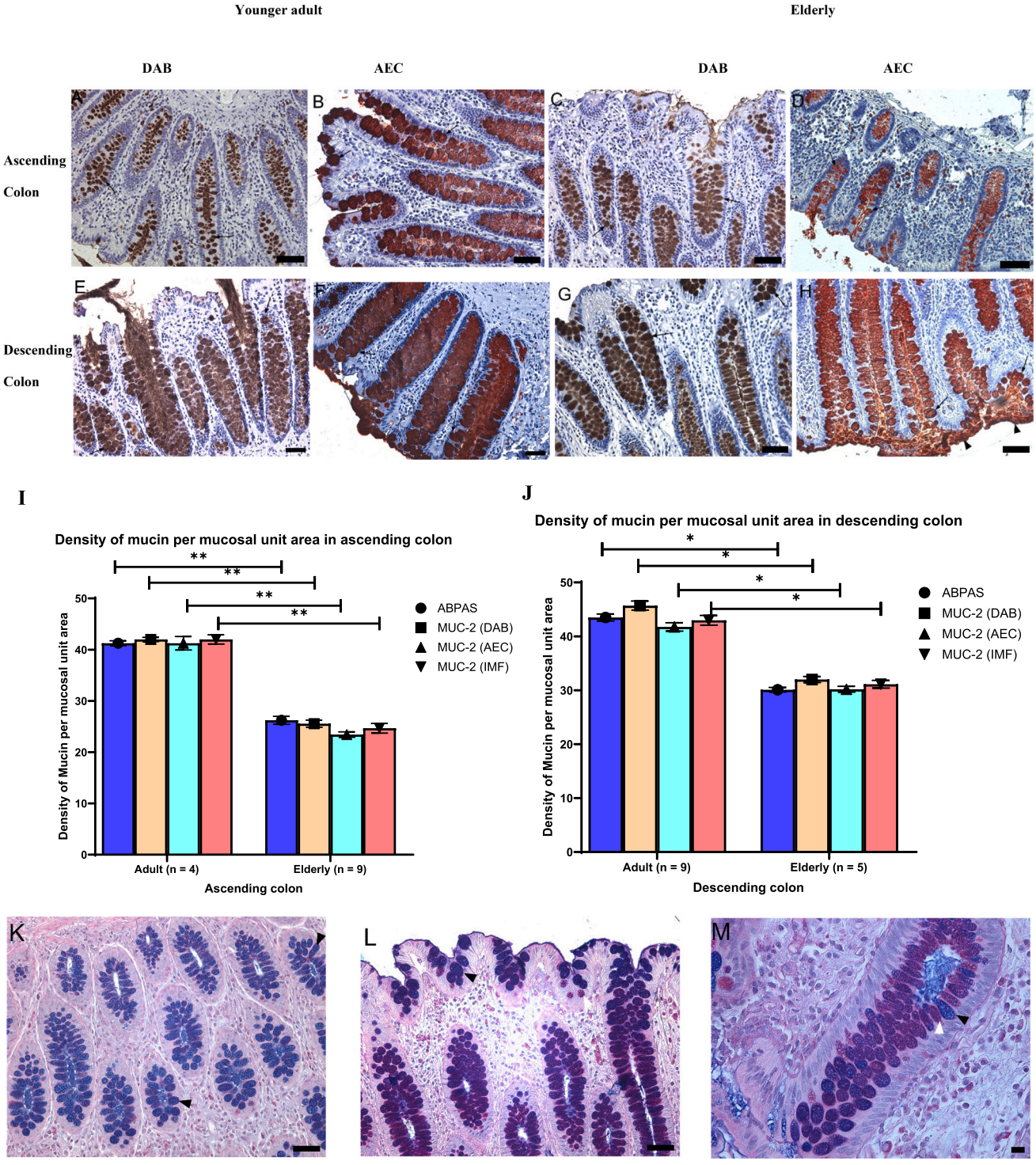

Fig. 5. Characterisation of goblet cells secreting mucins using the MUC-2 antibody in formalin-fixed paraffin-embedded sections of younger adult (≥ 60 years) and elderly (≥ 67 years) mucosa of human ascending and descending colon. Individual MUC-2-immunoreactive (IR) cells were clearly visualised with 3,3'diaminobenzidine tetrahydrochloride (DAB, brown; A, C, E, G) and 3-amino-9- ethyl carbazole (AEC, red; B, D, F, H) chromogens and nuclei were stained with Mayer's haematoxylin. MUC-2 IR structures (arrow) were morphologically detected in the epithelium when using both DAB and AEC methods, in all samples. The densities of alcian blue periodic acid-Schiff (ABPAS) -positive and MUC-2 mucin were determined as the amount of positive pixel per unit mucosal area using ImageJ. Panels I (ascending colon) and J (descending colon) show an age-related decline in goblet cell secreting mucin density per mucosal unit area assessed by histochemistry and immunohistochemistry methods. AC (Adult, n = 4; Elderly n = 9) and DC (Adult, n = 9; Elderly n = 5). Panels K, L and M are representative images of ABPAS staining in the mucosa of human colon. Goblet cells identified using ABPAS staining were blue-black (black arrowhead) and or magenta cells (white arrowhead). Microscopic examination revealed the presence of alternating acid mucin (blue) and neutral mucin (magenta) within the same crypts, with a greater abundance of acid mucin in adult sample compared to neutral mucin. Pattern of acid and neutral mucin distribution between adult (K) and elderly (L & M). The density of mucin content within the mucosa were expressed as the mean ± SEM. * $P \leq 0.05$; ** $P \leq 0.01$. A – H, K & L -Scale bar 50 μm; M – Scale bar 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Histochemical expression of alcian blue periodic acid-Schiff (ABPAS) in formalin-fixed, paraffin embedded human colonic sections. Macroscopically normal, full thickness, ascending colon from 13 patients (adults: $n = 4$; 31–52 years and elderly: $n = 9$; 70–89 years) and descending colon from 14 patients (adults: n = 9; 42–60 years and elderly: $n = 5$; 67–82 years) samples were stained for ABPAS stain to identify goblet cells in the mucosa. The sections were microscopically assessed by the presence of high (+++), moderate (++) and low (+) acid mucin (dark blue) and neutral mucin (magenta) in the ascending and descending colon for both adult and elderly samples.

	Ascending colon		Descending colon	
	Acid mucin	Neutral mucin	Acid mucin	Neutral mucin
Younger adult	+++	+	+++	+
Elderly	++	++	++	++

per crypt was found in the duodenum but not the distal ileum (Gebert et al., 2020) and in the small intestine of 20–22-month-old mice (Nalapareddy et al., 2017). No changes in goblet cell number per mucosal crypt, as assessed by alcian blue staining, were reported in the jejunum of 18–22-month-old mice (Moorefield et al., 2017), but these results differ from other non-human studies reporting significant reduction of goblet cells in the GI tract during ageing (Sang et al., 2023; Saleem et al., 2023). The reasons for any differences between the present findings with human colon and these previous animal studies are not clear. Perhaps the discrepancies are caused by species-dependent anatomical differences and differences in the microbiota composition. Humans, for example, have a greater number of goblet cells within the ileum, compared with the mouse and rat (Ermund et al., 2013). Further, the microbiota has been shown to influence the development and function of goblet cells, thus potentially influencing the numbers of goblet cells in a species dependent manner (Kim and Ho, 2010). Notably, older people also have reduced diversity in microbiota species and phyla (Rampelli et al., 2013; Lakshminarayanan et al., 2014; O'Toole and Jeffery, 2015; An et al., 2018; Paone and Cani, 2020) compared to healthy adult population. Further investigations are needed to elucidate the specific mechanisms underlying these differences and their impact on goblet cells populations.

In the aged colon, we qualitatively observed an approximately equal balance of acid and neutral mucins, whereas in the younger adults the acid mucins appeared to dominate. Neutral mucins have been found to play a role in facilitating the absorption of water-soluble nutrients across the colonic epithelium (Pelaseyed et al., 2014), whereas the primary function of acid mucins in the colon is to act as a physical barrier, preventing direct contact between noxious luminal contents and the epithelial cells (Johansson et al., 2011) and thereby reducing the risk of damage to the mucosal lining (Hansson and Johansson, 2010). Thus, our study suggests that a reduction in the relative content of acid mucins within the ageing human colon, compared with neutral mucins, may contribute to a mucosal barrier defect. Further investigations are now required to determine the specific roles and potential impacts of this changed balance in type of mucins within the ageing human colon. Interestingly, higher neutral mucin content has also been reported in human specific colonic cancer (Ionilă et al., 2011; Danquah et al., 2017). Perhaps, studies are needed to investigate the relationship between increased neutral mucin content in aged colon and the risk of developing colonic cancers (Milosevic et al., 2015).

The mechanism by which increasing age leads to a reduction in numbers of goblet cells and their mucin content are unclear. Notably, the age-related structural changes were predominantly observed towards the basal area of the crypt, where interestingly, major colonic stem cells are situated (see Choi and Augenlicht, 2024). Whether or not, ageing of goblet cells and associated mucin synthesis has an impact on the activity of stem cells or vice versa in human colon, requires further investigation. Another possibility is that the diminished production of mucin content can be ascribed to apoptosis-induced death of goblet cells (Sovran et al., 2019), rendering them unable to replenish the dying goblet cells. Further, the impact of senescence on goblet cells in the elderly individuals, may result in a decline in the quantity of mucin production (McHugh and Gil, 2018). Finally, since goblet cells at the base of colonic mucosal crypts may contain a particular subtype of mucin composition (e.g. abundance of MUC-5B; Burclaff et al., 2022), it becomes a possibility that the consequence of ageing preferentially impacts the production of one type of mucin over another.

The current study has limitations which should be acknowledged. One limitation is the relatively small sample size that was examined. To establish a more clinically relevant outcome, it may be necessary to include larger sample that provides a more detailed understanding of patient demographics. Additionally, mucus is known to be highly sensitive and often undergoes changes during the fixation process (Cohen et al., 2012). Therefore, it would be preferable to use fresh colonic tissues to preserve the full mucus content, in order to accurately study the mucus barrier during the process of natural ageing.

Finally, the present study adds to the accumulating evidence for structural changes within the lower bowel of the elderly. One idea (Broad et al., 2019; Baidoo and Sanger, 2024) is that such changes may have only small effects on the gross physiological functions of the bowel, but they will, nonetheless, exacerbate the effects of other changes which occur as we age. Thus, ageing affects how the intestine copes with other age-related challenges. Consequently, therapeutic strategies to minimise the effects of ageing on bowel functions could have wide-ranging consequences on quality of life. The need to assess specific regions and sublayers of the GI tract in ageing studies has been recently elaborated (Baidoo, 2023) and affirmed in this present study, suggesting that it is important to assess key components in individual sublayers, so that in the future, a precision therapy can be targeted.

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Contributors

NB critically reviewed, designed and conducted the experiments, analysed the data, cowrote the manuscript and supervised the overall project. **GJS** co-wrote the manuscript and facilitated the identification, collection and governance of human tissue collection for this study, all authors participated in its construction and refinement.

Patient consent

All patients provided written informed consent for the donation of tissue (REC 10/H0703/71; East London ethics committee).

Ethics approval

Approved by the East London Research Ethics Committee (REC: 10/HO703/71), the London City Road and Hampstead Research Ethics Committee (REC: 15/LO/21/27) and subsequently by the University of Westminster (ETH2324-1489).

CRediT authorship contribution statement

Nicholas Baidoo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gareth J. Sanger:** Writing – review & editing, Validation, Project administration, Funding acquisition, Conceptualization.

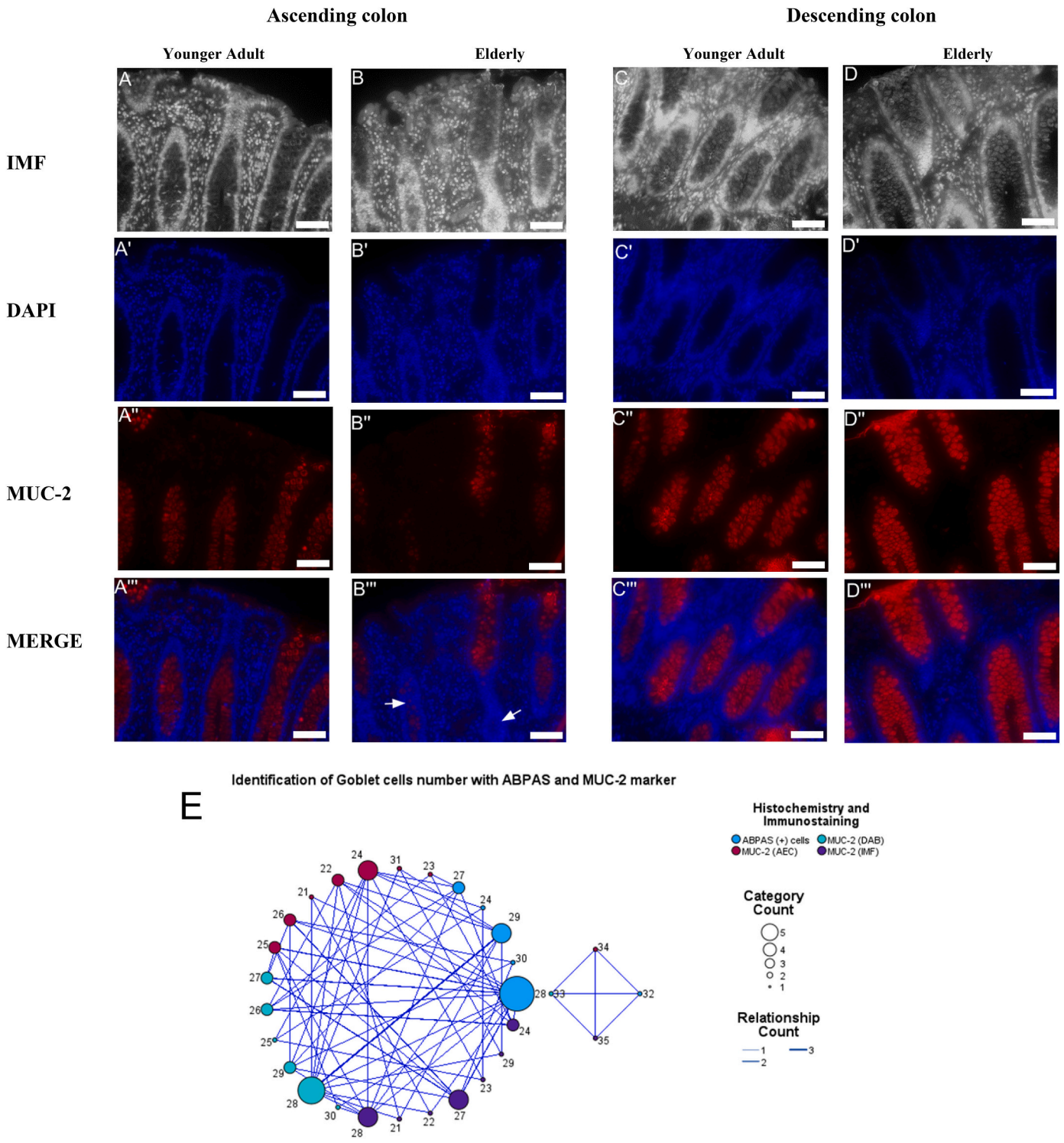

Fig. 6. MUC-2 mucin content in formalin-fixed paraffin-embedded sections of younger adult (≤ 60 years) and elderly (≥ 67 years) mucosa of human ascending and descending colon. Immunofluorescence staining of human colonic tissues with recombinant anti-MUC-2 antibody (ab272692, Abcam, Cambridge, United Kingdom) and a fluorophore-conjugated secondary antibody (Goat anti-rabbit IgG; Alexa Fluor 647; ab150079; Abcam, Cambridge, United Kingdom). Panels (A-D) are representative of mucosa morphology observed with immunofluorescence technique. Panels (A'-D') showed blue nuclei stain with 4′,6-diamidino-2-phenylindole. Panels (A"-D") demonstrate MUC-2 immunopositive cells in red colour. Panels (A"'-D"') are the merge images of positive structures (in red) and nuclei (in blue). White arrows indicate areas of the epithelium without goblet cells. **E** shows the relationship pattern of data from histochemistry, and immunohistochemistry methods plotted with Statistical Package for Social Science (IBM Corp. Released 2023. IBM SPSS Statistics for Windows, Version 29.0.1.0 (171). AC (Adult, n = 4; Elderly n = 9) and DC (Adult, n = 9; Elderly n = 5). ABPAS: Alcian Blue Periodic Acid-Schiff; MUC-2 DAB: 3,3'diaminobenzidine tetrahydrochloride; MUC-2 AEC: 3-amino-9-ethyl carbazole; IMF: Immunofluorescence. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

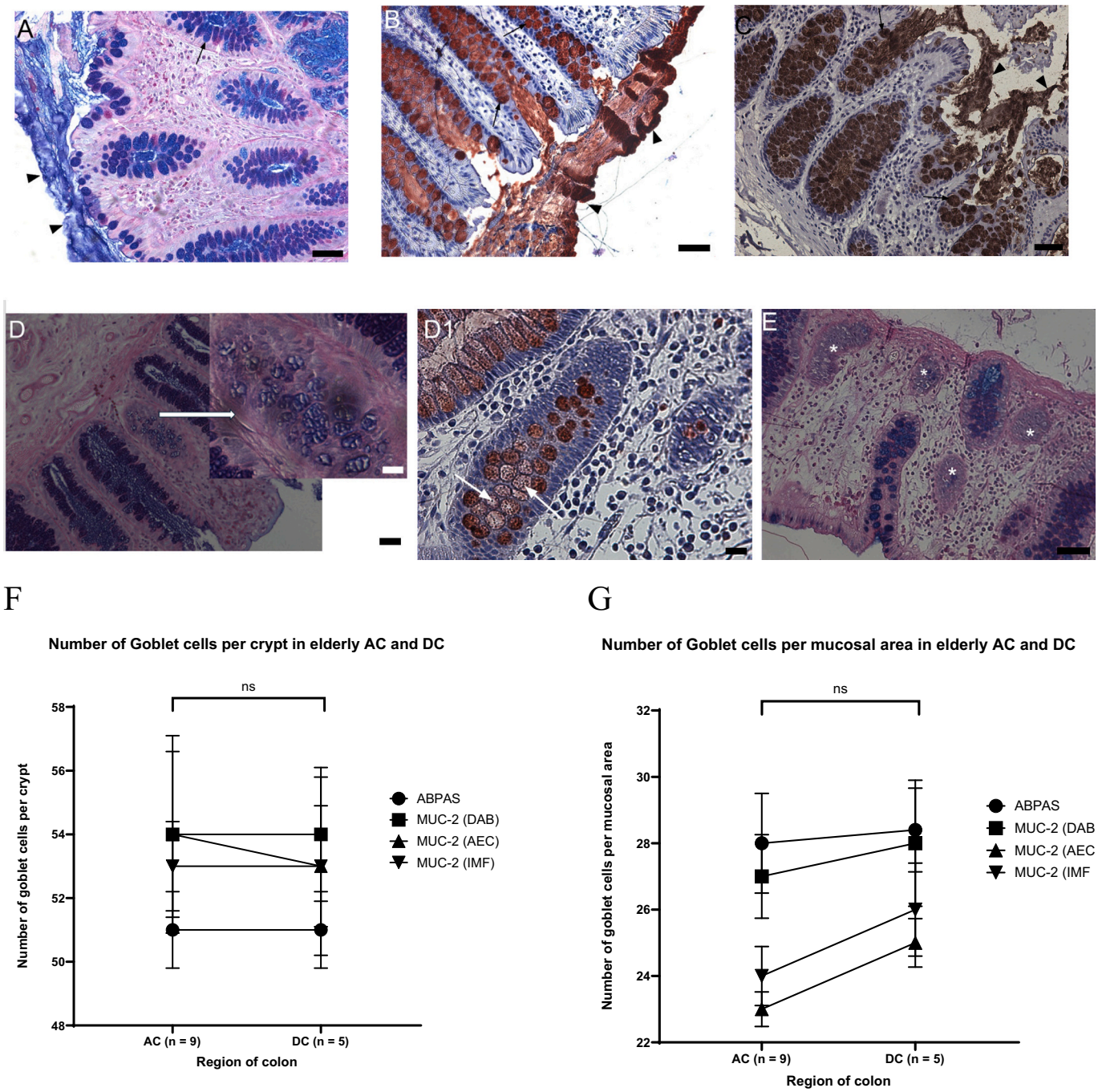

Fig. 7. Histochemical and immunohistochemical expression of goblet cells producing mucins in human colonic sections. Using alcian blue periodic acid-Schiff (ABPAS; A, D, E) and by MUC-2 antibodies visualised by chromogen AEC (B, D1) and DAB (C), goblet cells were identified (arrow) in all the sections and staining methods (ABPAS: deep blue-black and magenta; MUC-2 AEC: red; MUC-2 DAB: brown). Empty mucin-vesicles were observed more often in the mucosa of the elderly (D & D1); Scale bar 25 μm, inset scale bar 10 μm. Panel (E) shows some crypts devoid of goblet cells (*). Panels A, B and C show evidence of horizontal stratified structure positive to ABPAS and MUC-2 antibody and adhered and suspending onto the epithelia (arrowhead). In graphs F & G, there was no statistical difference in goblet cell number per crypt or mucosal unit area between the AC and DC of the elderly. Data are given as means +/− SEM. NS: non-significant; * $P \leq 0.05$; ** $P \leq 0.01$. AC: n = 4; DC: $n = 6$. AC (Adult, n = 4; Elderly n = 9) and DC (Adult, n = 9; Elderly n = 5). (A-C, & E; Scale bar 50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

All data supporting the findings of this study are available from the corresponding author upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2024.104923.

References

An, R., Wilms, E., Masclee, A.A.M., Smidt, H., Zoetendal, E.G., Jonkers, D., 2018. Age-dependent changes in GI physiology and microbiota: time to reconsider? Gut 67 (12), 2213–2222. https://doi.org/10.1136/gutjnl-2017-315542.

Arike, L., Holmén-Larsson, J., Hansson, G.C., 2017. Intestinal Muc2 mucin O-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases. Glycobiology 27 (4), 318–328. https://doi.org/10.1093/glycob/cww134.

Baidoo, N., 2023. The Effect of Ageing on Innervation and Total Collagen Content in Human Colon. https://doi.org/10.13140/rg.2.2.31758.74560.

Baidoo, N., Sanger, G.J., 2024. The human colon: evidence for degenerative changes during aging and the physiological consequences. Neurogastroenterol. Motil., e14848. Advance online publication. https://doi.org/10.1111/nmo.14848.

Baidoo, N., Crawley, E., Knowles, C.H., Sanger, G.J., Belai, A., 2022a. Total collagen content and distribution is increased in human colon during advancing age. PLoS One 17 (6), e0269689. https://doi.org/10.1371/journal.pone.0269689.

Baidoo, N., Sanger, G.J., Belai, A., 2022b. The vulnerability of the human taenia coli to alterations in total collagen within the colon of the elderly. Acta Histochem. 124 (8), 151958 https://doi.org/10.1016/j.acthis.2022.151958.

Baidoo, N., Sanger, G.J., Belai, A., 2023a. Effect of old age on the subpopulations of enteric glial cells in human descending colon. Glia 71 (2), 305–316. https://doi.org/10.1002/glia.24272.

Baidoo, N., Sanger, G.J., Belai, A., 2023b. Histochemical and biochemical analysis of collagen content in formalin-fixed, paraffin embedded colonic samples. MethodsX 11, 102416. https://doi.org/10.1016/j.mex.2023.102416.

Broad, J., Kung, V.W.S., Palmer, A., Elahi, S., Karami, A., Darreh-Shori, T., Ahmed, S., Thaha, M.A., Carroll, R., Chin-Aleong, J., Martin, J.E., Saffrey, M.J., Knowles, C.H., Sanger, G.J., 2019. Changes in neuromuscular structure and functions of human colon during ageing are region-dependent. Gut 68 (7), 1210–1223. https://doi.org/10.1136/gutjnl-2018-316279.

Burclaff, J., Bliton, R.J., Breau, K.A., Ok, M.T., Gomez-Martinez, I., Ranek, J.S., Bhatt, A. P., Purvis, J.E., Woosley, J.T., Magness, S.T., 2022. A proximal-to-distal survey of healthy adult human small intestine and colon epithelium by single-cell transcriptomics. Cell. Mol. Gastroenterol. Hepatol. 13 (5), 1554–1589. https://doi.org/10.1016/j.jcmgh.2022.02.007.

Cardiff, R.D., Miller, C.H., Munn, R.J., 2014. Manual hematoxylin and eosin staining of mouse tissue sections. Cold Spring Harb Protoc 2014 (6), 655–658. https://doi.org/10.1101/pdb.prot073411.

Chan, J.K., 2014. The wonderful colors of the hematoxylin-eosin stain in diagnostic surgical pathology. Int. J. Surg. Pathol. 22 (1), 12–32. https://doi.org/10.1177/1066896913517939.

Choi, J., Augenlicht, L.H., 2024. Intestinal stem cells: guardians of homeostasis in health and aging amid environmental challenges. Exp. Mol. Med. 56 (3), 495–500. https://doi.org/10.1038/s12276-024-01179-1.

Cibert-Goton, V., Kung, V.W.S., McGuire, C., Hockley, J.R.F., Tranter, M.M., Dogra, H., Belai, A., Blackshaw, L.A., Sanger, G.J., Knowles, C.H., Araujo, E.J.A., Winchester, W.J., Bulmer, D.C., 2020. Functional and anatomical deficits in visceral nociception with age: a mechanism of silent appendicitis in the elderly? Pain 161 (4), 773–786. https://doi.org/10.1097/j.pain.0000000000001764.

Cohen, M., Varki, N.M., Jankowski, M.D., Gagneux, P., 2012. Using unfixed, frozen tissues to study natural mucin distribution. J. Visual. Exp.: JoVE 67, e3928. https://doi.org/10.3791/3928.

Cornick, S., Tawiah, A., Chadee, K., 2015. Roles and regulation of the mucus barrier in the gut. Tissue Barriers 3 (1–2), e982426. https://doi.org/10.4161/21688370.2014.982426.

Danquah, K.O., Adjei, E., Quayson, S., Adankwah, E., Gyamfi, D., Ossei, P.P.S., Dzikunu, G., Mensah, P., Lepkor, C., 2017. Mucin expression patterns in histological grades of colonic cancers in Ghanaian population. Pan Afr. Med. J. 27, 267. https://doi.org/10.11604/pamj.2017.27.267.9793.

Einerhand, A.W., Renes, I.B., Makkink, M.K., van der Sluis, M., Büller, H.A., Dekker, J., 2002. Role of mucins in inflammatory bowel disease: important lessons from experimental models. Eur. J. Gastroenterol. Hepatol. 14 (7), 757–765. https://doi.org/10.1097/00042737-200207000-00008.

Elderman, M., Sovran, B., Hugenholtz, F., Graversen, K., Huijskes, M., Houtsma, E., Belzer, C., Boekschoten, M., de Vos, P., Dekker, J., Wells, J., Faas, M., 2017. The effect of age on the intestinal mucus thickness, microbiota composition and immunity in relation to sex in mice. PLoS One 12 (9), e0184274. https://doi.org/10.1371/journal.pone.0184274.

Ermund, A., Gustafsson, J.K., Hansson, G.C., Keita, A.V., 2013. Mucus properties and goblet cell quantification in mouse, rat and human ileal Peyer's patches. PLoS One 8 (12), e83688. https://doi.org/10.1371/journal.pone.0083688.

Feakins, R.M., British Society of Gastroenterology, 2013. Inflammatory bowel disease biopsies: updated British Society of Gastroenterology reporting guidelines. J. Clin. Pathol. 66 (12), 1005–1026. https://doi.org/10.1136/jclinpath-2013-201885.

Gebert, N., Cheng, C.W., Kirkpatrick, J.M., Di Fraia, D., Yun, J., Schädel, P., Pace, S., Garside, G.B., Werz, O., Rudolph, K.L., Jasper, H., Yilmaz, Ö.H., Ori, A., 2020. Region-specific proteome changes of the intestinal epithelium during aging and dietary restriction. Cell Rep. 31 (4), 107565 https://doi.org/10.1016/j.celrep.2020.107565.

Gill, N., Wlodarska, M., Finlay, B.B., 2010. The future of mucosal immunology: studying an integrated system-wide organ. Nat. Immunol. 11 (7), 558–560. https://doi.org/10.1038/ni0710-558.

Gomez-Pinilla, P.J., Gibbons, S.J., Sarr, M.G., Kendrick, M.L., Shen, K.R., Cima, R.R., Dozois, E.J., Larson, D.W., Ordog, T., Pozo, M.J., Farrugia, G., 2011. Changes in interstitial cells of cajal with age in the human stomach and colon. Neurogastroenterol. Motil. 23 (1), 36–44. https://doi.org/10.1111/j.1365-2982.2010.01590.x.

Gouyer, V., Gottrand, F., Desseyn, J.L., 2011. The extraordinarily complex but highly structured organization of intestinal mucus-gel unveiled in multicolor images. PLoS One 6 (4), e18761. https://doi.org/10.1371/journal.pone.0018761.

Guzmán-Mejía, F., Godínez-Victoria, M., Vega-Bautista, A., Pacheco-Yépez, J., Drago-Serrano, M.E., 2021. Intestinal Homeostasis under Stress Siege. Int. J. Mol. Sci. 22 (10), 5095. https://doi.org/10.3390/ijms22105095.

Hansson, G.C., Johansson, M.E., 2010. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Gut Microbes 1 (1), 51–54. https://doi.org/10.4161/gmic.1.1.10470.

Holmén Larsson, J.M., Thomsson, K.A., Rodríguez-Piñeiro, A.M., Karlsson, H., Hansson, G.C., 2013. Studies of mucus in mouse stomach, small intestine, and colon. III. Gastrointestinal Muc5ac and Muc2 mucin O-glycan patterns reveal a regiospecific distribution. Am. J. Physiol. Gastrointest. Liver Physiol. 305 (5), G357–G363. https://doi.org/10.1152/ajpgi.00048.2013.

Ionilă, M., Mărgăritescu, C., Pirici, D., Mogoantă, S.S., 2011. Mucinous adenocarcinoma of the colon - a histochemical study. Romanian J. Morphol. Embryol. = Revue Roumaine de Morphologie et Embryologie 52 (3), 783–790.

Johansson, M.E., Phillipson, M., Petersson, J., Velcich, A., Holm, L., Hansson, G.C., 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc. Natl. Acad. Sci. USA 105 (39), 15064–15069. https://doi.org/10.1073/pnas.0803124105.

Johansson, M.E., Ambort, D., Pelaseyed, T., Schütte, A., Gustafsson, J.K., Ermund, A., Subramani, D.B., Holmén-Larsson, J.M., Thomsson, K.A., Bergström, J.H., van der Post, S., Rodriguez-Piñeiro, A.M., Sjövall, H., Bäckström, M., Hansson, G.C., 2011. Composition and functional role of the mucus layers in the intestine. Cell. Molecular Life Sci.: CMLS 68 (22), 3635–3641. https://doi.org/10.1007/s00018-011-0822-3.

Kiliçarslan, A., Varli, G., 2024. Should special staining (AB/PAS) be routinely performed in gastric biopsies for the detection of intestinal metaplasia? Int. J. Surg. Pathol. 32 (2), 217–222. https://doi.org/10.1177/10668969231169050.

Kim, Y.S., Ho, S.B., 2010. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr. Gastroenterol. Rep. 12 (5), 319–330. https://doi.org/10.1007/s11894-010-0131-2.

Kim, J.J., Khan, W.I., 2013. Goblet cells and mucins: role in innate defense in enteric infections. Pathogens (Basel, Switzerland) 2 (1), 55–70. https://doi.org/10.3390/pathogens2010055.

Krueger, D., Michel, K., Zeller, F., Demir, I.E., Ceyhan, G.O., Slotta-Huspenina, J., Schemann, M., 2016. Neural influences on human intestinal epithelium in vitro. J. Physiol. 594 (2), 357–372. https://doi.org/10.1113/JP271493.

Kufe, D.W., 2009. Mucins in cancer: function, prognosis and therapy. Nat. Rev. Cancer 9 (12), 874–885. https://doi.org/10.1038/nrc2761.

Lakshminarayanan, B., Stanton, C., O'Toole, P.W., Ross, R.P., 2014. Compositional dynamics of the human intestinal microbiota with aging: implications for health. J. Nutr. Health Aging 18 (9), 773–786. https://doi.org/10.1007/s12603-014-0549-6.

Lamont, J.T., 1992. Mucus: the front line of intestinal mucosal defense. Ann. N. Y. Acad. Sci. 664, 190–201. https://doi.org/10.1111/j.1749-6632.1992.tb39760.x.

Lindén, S.K., Florin, T.H., McGuckin, M.A., 2008. Mucin dynamics in intestinal bacterial infection. PLoS One 3 (12), e3952. https://doi.org/10.1371/journal.pone.0003952.

Liu, H.Y., Gu, F., Zhu, C., Yuan, L., Zhu, C., Zhu, M., Yao, J., Hu, P., Zhang, Y., Dicksved, J., Bao, W., Cai, D., 2022. Epithelial heat shock proteins mediate the protective effects of *Limosilactobacillus reuteri* in dextran sulfate sodium-induced colitis. Front. Immunol. 13, 865982 https://doi.org/10.3389/fimmu.2022.865982.

Loktionov, A., 2022. Colon mucus in colorectal neoplasia and beyond. World J. Gastroenterol. 28 (32), 4475–4492. https://doi.org/10.3748/wjg.v28.i32.4475.

Man, A.L., Bertelli, E., Rentini, S., Regoli, M., Briars, G., Marini, M., Watson, A.J., Nicoletti, C., 2015. Age-associated modifications of intestinal permeability and innate immunity in human small intestine. Clin. Sci. (Lond.) 129 (7), 515–527. https://doi.org/10.1042/CS20150046.

Martinez-Guryn, K., Leone, V., Chang, E.B., 2019. Regional diversity of the gastrointestinal microbiome. Cell Host Microbe 26 (3), 314–324. https://doi.org/10.1016/j.chom.2019.08.011.

McGuckin, M.A., Lindén, S.K., Sutton, P., Florin, T.H., 2011. Mucin dynamics and enteric pathogens. Nat. Rev. Microbiol. 9 (4), 265–278. https://doi.org/10.1038/nrmicro2538.

McHugh, D., Gil, J., 2018. Senescence and aging: causes, consequences, and therapeutic avenues. J. Cell Biol. 217 (1), 65–77. https://doi.org/10.1083/jcb.201708092.

Milosevic, V., Vukmirovic, F., Zindovic, M., Krstic, M., Milenkovic, S., Jancic, S., 2015. Interplay between expression of leptin receptors and mucin histochemical aberrations in colorectal adenocarcinoma. Roman. J. Morphol. Embryol. =Revue Roumaine de Morphologie et Embryologie 56 (2 Suppl), 709–716.

Moorefield, E.C., Andres, S.F., Blue, R.E., Van Landeghem, L., Mah, A.T., Santoro, M.A., Ding, S., 2017. Aging effects on intestinal homeostasis associated with expansion and dysfunction of intestinal epithelial stem cells. Aging 9 (8), 1898–1915. https://doi.org/10.18632/aging.101279.

Nalapareddy, K., Nattamai, K.J., Kumar, R.S., Karns, R., Wikenheiser-Brokamp, K.A., Sampson, L.L., Mahe, M.M., Sundaram, N., Yacyshyn, M.B., Yacyshyn, B., Helmrath, M.A., Zheng, Y., Geiger, H., 2017. Canonical Wnt signaling ameliorates aging of intestinal stem cells. Cell Rep. 18 (11), 2608–2621. https://doi.org/10.1016/j.celrep.2017.02.056.

O'Hara, A.M., Shanahan, F., 2006. The gut flora as a forgotten organ. EMBO Rep. 7 (7), 688–693. https://doi.org/10.1038/sj.embor.7400731.

Osho, S.O., Wang, T., Horn, N.L., Adeola, O., 2017. Comparison of goblet cell staining methods in jejunal mucosa of Turkey poults. Poult. Sci. 96 (3), 556–559. https://doi.org/10.3382/ps/pew324.

O'Toole, P.W., Jeffery, I.B., 2015. Gut microbiota and aging. Science (New York, N.Y.) 350 (6265), 1214–1215. https://doi.org/10.1126/science.aac8469.

Padra, J.T., Lindén, S.K., 2022. Optimization of Alcian blue pH 1.0 histo-staining protocols to match mass spectrometric quantification of sulfomucins and circumvent false positive results due to sialomucins. Glycobiology 32 (1), 6–10. https://doi.org/10.1093/glycob/cwab091.

Palmer, A., Epton, S., Crawley, E., Straface, M., Gammon, L., Edgar, M.M., Xu, Y., Elahi, S., Chin-Aleong, J., Martin, J.E., Bishop, C.L., Knowles, C.H., Sanger, G.J., 2021. Expression of p16 within myenteric neurons of the aged Colon: a potential marker of declining function. Front. Neurosci. 15, 747067 https://doi.org/10.3389/fnins.2021.747067.

Paone, P., Cani, P.D., 2020. Mucus barrier, mucins and gut microbiota: the expected slimy partners? Gut 69 (12), 2232–2243. https://doi.org/10.1136/gutjnl-2020-322260.

Pelaseyed, T., Bergström, J.H., Gustafsson, J.K., Ermund, A., Birchenough, G.M., Schütte, A., van der Post, S., Svensson, F., Rodríguez-Piñeiro, A.M., Nyström, E.E., Wising, C., Johansson, M.E., Hansson, G.C., 2014. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immunol. Rev. 260 (1), 8–20. https://doi.org/10.1111/imr.12182.

Prasanna, L.C., 2016. Analysis of the distribution of mucins in adult human gastric mucosa and its functional significance. J. Clin. Diagn. Res. 10 (2), AC01–AC04. https://doi.org/10.7860/JCDR/2016/12323.7162.

Rampelli, S., Candela, M., Turroni, S., Biagi, E., Collino, S., Franceschi, C., O'Toole, P.W., Brigidi, P., 2013. Functional metagenomic profiling of intestinal microbiome in extreme ageing. Aging 5 (12), 902–912. https://doi.org/10.18632/aging.100623.

Rodríguez-Romero, J.J., Durán-Castañeda, A.C., Cárdenas-Castro, A.P., Sánchez-Burgos, J.A., Zamora-Gasga, V.M., Sáyago-Ayerdi, S.G., 2021. What we know about protein gut metabolites: implications and insights for human health and diseases. Food chemistry: X 13, 100195. https://doi.org/10.1016/j.fochx.2021.100195.

Röhe, I., Hüttner, F.J., Plendl, J., Drewes, B., Zentek, J., 2018. Comparison of different histological protocols for the preservation and quantification of the intestinal mucus layer in pigs. Eur. J. Histochem : EJH 62 (1), 2874. https://doi.org/10.4081/ejh.2018.2874.

Saleem, W., Ren, X., Van Den Broeck, W., Nauwynck, H., 2023. Changes in intestinal morphology, number of mucus-producing cells and expression of coronavirus receptors APN, DPP4, ACE2 and TMPRSS2 in pigs with aging. Vet. Res. 54 (1), 34. https://doi.org/10.1186/s13567-023-01169-7.

Sang, X., Wang, Q., Ning, Y., Wang, H., Zhang, R., Li, Y., Fang, B., Lv, C., Zhang, Y., Wang, X., Ren, F., 2023. Age-related mucus barrier dysfunction in mice is related to the changes in Muc2 mucin in the Colon. Nutrients 15 (8), 1830. https://doi.org/10.3390/nu15081830.

Sanger, G.J., Holbrook, J.D., Andrews, P.L., 2011. The translational value of rodent gastrointestinal functions: a cautionary tale. Trends Pharmacol. Sci. 32 (7), 402–409. https://doi.org/10.1016/j.tips.2011.03.009.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH image to Imagej: 25 years of image analysis. Nat. Methods 9 (7), 671–675. https://doi.org/10.1038/nmeth.2089.

Sheng, Y.H., Hasnain, S.Z., Florin, T.H., McGuckin, M.A., 2012. Mucins in inflammatory bowel diseases and colorectal cancer. J. Gastroenterol. Hepatol. 27 (1), 28–38. https://doi.org/10.1111/j.1440-1746.2011.06909.x.

Shirazi, T., Longman, R.J., Corfield, A.P., Probert, C.S., 2000. Mucins and inflammatory bowel disease. Postgrad. Med. J. 76 (898), 473–478. https://doi.org/10.1136/pmj.76.898.473.

Sovran, B., Hugenholtz, F., Elderman, M., Van Beek, A.A., Graversen, K., Huijskes, M., Boekschoten, M.V., Savelkoul, H.F.J., De Vos, P., Dekker, J., Wells, J.M., 2019. Age-associated impairment of the mucus barrier function is associated with profound changes in microbiota and immunity. Sci. Rep. 9 (1), 1437. https://doi.org/10.1038/s41598-018-35228-3.

Specian, R.D., Oliver, M.G., 1991. Functional biology of intestinal goblet cells. Am. J. Phys. 260 (2 Pt 1), C183–C193. https://doi.org/10.1152/ajpcell.1991.260.2.C183.

Titford, M., 2005. The long history of hematoxylin. Biotech. & Histochem.: Off. Pub. Biol. Stain Comm. 80 (2), 73–78. https://doi.org/10.1080/10520290500138372.

Tsutsumi, Y., 2021. Pitfalls and caveats in applying chromogenic immunostaining to histopathological diagnosis. Cells 10 (6), 1501. https://doi.org/10.3390/cells10061501.

Yang, S., Yu, M., 2021. Role of goblet cells in intestinal barrier and mucosal immunity. J. Inflamm. Res. 14, 3171–3183. https://doi.org/10.2147/JIR.S318327.

Yu, Y., Daly, D.M., Adam, I.J., Kitsanta, P., Hill, C.J., Wild, J., Shorthouse, A., Grundy, D., Jiang, W., 2016. Interplay between mast cells, enterochromaffin cells, and sensory signaling in the aging human bowel. Neurogastroenterol. Motil. 28 (10), 1465–1479. https://doi.org/10.1111/nmo.12842.

Zaqout, S., Becker, L.L., Kaindl, A.M., 2020. Immunofluorescence staining of paraffin sections step by step. Front. Neuroanat. 14, 582218 https://doi.org/10.3389/fnana.2020.582218.

Zhang, M., Wu, C., 2020. The relationship between intestinal goblet cells and the immune response. Biosci. Rep. 40 (10), BSR20201471 https://doi.org/10.1042/BSR20201471.