Delineation of the healthy rabbit tonsil by immunohistochemistry - A short communication

Meier Bürgisser, Gabriella ; Heuberger, Dorothea M ; Giovanoli, Pietro ; Calcagni, Maurizio ; Buschmann, Johanna

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Delineation of the healthy rabbit tonsil by immunohistochemistry – A short communication

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ABSTRACT

Situated in the oral cavity, the rabbit palatine tonsils are part of the mucosal immune system and help to defend the body against foreign pathogens. Expressed as two oval protrusions in the wall of the oropharynx, the rabbit palatine tonsils are characterized by excretory ducts and trabeculae. We here compare paraffin embedded and cryosections of the healthy rabbit tonsils. This analysis centers on evaluating the differential outcomes resulting from the application of these fixation methodologies in conjunction with immunohistochemical assays targeting collagen I, collagen III, fibronectin, α-smooth muscle actin (α-SMA), and ki67. Subsequent recommendations are provided based on our findings. Furthermore, we demonstrate the advantage of an antigen retrieval step in immunohistochemical labeling of paraffin sections. Basic classical histological stainings as HE, GT and elastin were also performed. Comparison of different stainings and labelings was furthermore performed in serial sections, showing that adjacent to the excretory ducts, the tonsillar tissue was particularly composed of collagen I and fibronectin, while the vessel walls were predominantly α-SMA positive. Moreover, PAR-2 immunohistochemical staining was performed, where a small fraction of the cells found in the tonsillar connective tissue were PAR-2 positive (probably a subpopulation of mast cells), as well as the lumen of some excretory ducts and trabeculae. Collagen III on the other hand was only weakly expressed in the tonsils. Proliferating ki67 positive cells were rare. This endeavor serves to furnish the scientific community with reference imagery pertinent to researchers opting for the rabbit palatine tonsil model. The diversity of staining techniques employed herein establishes a foundational repository of images, primed for comparative analysis against pathological conditions. Furthermore, these images hold the potential to illustrate inter-species variations. For instance, they can be juxtaposed against murine or rodent tonsils, or even offer insights into the human context.

1. Introduction

The tonsils are primarily important for the defense against foreign antigens. Present as lymph nodules and called lymphoid follicles, the tonsils are part of the mucosal immunology (Casteleyn et al., 2011). While humans have four different tonsils, named after their localization or morphology (adenoid, tubal, palatine, and lingual tonsils (Arambula et al., 2021)), the rabbit species has only palatine tonsils (Casteleyn et al., 2011). They are presented as two oval protrusions in the wall of the oropharynx. Although some lymphoid tissue is present at the bottom of the nasopharynx in rabbits, this tissue is not attributed to nor counted as proper tonsils (Casteleyn et al., 2011), but usually called nasal-cavity-associated lymphoid tissue, briefly NALT.

In comparison with other animals, rabbits exhibit a rather low number of tonsils. Sheep and goats, for example, have six different tonsils (lingual, palatine, paraepiglottic, pharyngeal, tubal, and tonsil of the soft palate, respectively, (Casteleyn et al., 2011)). Other animals like horses, dogs or cats have four to five different tonsils. Hence the rabbit is at the far end of the spectrum, together with the pigeon that has pharyngeal tonsils and NALT. Only the rat has even fewer tonsils than the rabbit – no proper tonsil at all –, exhibiting only NALT (Casteleyn et al., 2011). An explanation for the observation that the rat lacks any tonsils but has a well-developed NALT could be the immunological adaptation to breathing through the nose, which is common in rats (Bienenstock and McDermott, 2005; Samsom et al., 2004), but not in rabbits that also breathe through the mouth.

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Tonsils may also play a role in infectious diseases, as for example *tonsillitis acuta* (Sidell and Shapiro, 2012). Bacterial tonsillitis is mostly caused by *Streptococcus pyogenes*. Polymicrobial and viral infections are also important, and penicillins are usually used for treatment (Georgalas et al., 2014; Munck et al., 2018). Patients with recurrent tonsillitis have been reported to have pericapsular sclerosis, along with thickening of interlobular septa and subepithelial fibrosis (Orlovska et al., 2022). Moreover, brachycephalic airway obstruction syndrome and swelling of the infected tonsils may result in clinical indications for tonsillectomy. Although tonsils are important for antigen defense, tonsillectomy is regarded as safe concerning serious invasive infections later in life. This low risk is in addition attributed to redundant tonsillar lymphoid tissues situated in the pharynx (Casteleyn et al., 2011).

Here, we present the anatomy of the healthy rabbit palatine tonsils from a histological and immunohistochemical perspective. Starting with differential outcomes of formalin fixation versus cryo embedding for immunohistochemical labeling of collagen I and III as well as for fibronectin, α-SMA and ki67, we continue with a recommendation of an antigen retrieval step in case of paraffin sections and immunohistochemistry (IHC). After that, we turn the focus on specific structures, such as the tonsillar crypts, the tonsil epithelium, the excretory ducts, the lymphoid follicles, arteries, veins, and the mucous glands, respectively. Such structures are depicted in serial sections and stained for different important extracellular matrix markers, including elastin, and also for proliferative ki67, among others. Furthermore, PAR-2 IHC staining of the palatine tonsils is shown in detail. As an inflammatory marker PAR-2 is interesting for the comparison in normal healthy tonsils and potential diseased states.

In summary, we here provide representative images of the healthy rabbit tonsils for comparison to a diseased state using the rabbit model in a research approach. Furthermore, these images hold the potential to illustrate inter-species variations. For instance, they can be juxtaposed against murine or rodent tonsils, or even offer insights into the human context. Researchers may get interested and apply this pre-clinical animal model.

2. Materials and methods

2.1. Tonsil extraction

Rabbit tonsils were received from a dead female New Zealand White rabbit that was included in a calvarial bone defect project. This corresponding project was licenced by the Animal Ethics Committee at local authorities (Canton Zurich ZH 115/2015 and 090/2021) (Ghayor and Weber, 2018; Siegenthaler et al., 2020). The whole tonsils were used as received after isolation from this cadaver. Then, following storage on ice for 20 min and sectioning, it was ready for histology. The tonsil was randomly sectioned.

2.2. Histology and immunohistochemistry

The tonsil pieces were cut in 2 parts for either paraffin embedding or cryo embedding. Paraffin embedding included fixation in formalin for one day, dehydration, paraffin-embedding and sectioning into 5-μm-thick slices. Before they were stained, paraffin embedded sections were deparaffinized utilizing xylene and rehydrated (descreasing gradient of ethanol).

As for the cryopreservation samples, they were embedded in Tissue-Tek® O.C.T. (Sakura, Alphen aan den Rijn, The Netherlands, Europe). Then, they were frozen before cryosections of 5 μm thickness were cut with a microtome. Subsequently, they were thawed and fixed with formalin for 10 min, and finally washed with 1xTBS, followed by IHC procedures.

According to general protocols, Elastica van Gieson (EL), Masson Goldner Trichrome (GT) and Haematoxylin&Eosin (HE) stainings were performed. For IHC, an antigen retrieval (AR) step was performed for paraffin sections, using 10 mM citrate buffer (pH 6.0) with 0.05% Tween-20 for 20 min at 95 °C. In some cases, no AR was made in order to generate a control, for the comparison of conditions with and without AR. For certain epitopes, such as ki67 and α-SMA, corresponding sections were permeabilized with 0.5% Triton X-100 in 1xTBS for 10 min, followed by three times washing with 1xTBS. After that, the sections were blocked in 5% donkey serum and 1% BSA in 1xTBS for 1 h (at room temperature (RT)). Next, sections were incubated with mouse monoclonal anti-collagen I antibody (ab90395; Abcam, Lucerne, Switzerland, 1:200 dilution) or mouse monoclonal anti-collagen III antibody (AF5810; Acris, Wettingen, Switzerland, 1:200 dilution) or mouse monoclonal anti-αSMA antibody (A2547; Sigma-Aldrich, Buchs, Switzerland, 1:500 dilution) or mouse monoclonal anti-fibronectin antibody (F0791; Sigma-Aldrich, Buchs, Switzerland, 1:200 dilution) or mouse monoclonal anti-ki67 antibody (NBP2–22112; Novus Biologicals, 1:500 dilution) or mouse monoclonal anti-PAR-2 antibody (Santa Cruz Biotechnology, sc-13504 (SAM11), 1:250 dilution) on Refine-kit (anti-Rabbit-Polymer) and histofine-Mouse Polymer (1:50 dilution) diluted in 3% BSA in 1xTBS overnight at 4 °C (Table 1).

Table 1
Antibodies and conditions for IHC labelings.

Primary antibody	*Supplier*	*Dilution*
Mouse monoclonal anti-collagen I	ab90395; Abcam, Lucerne, Switzerland	1:200
mouse monoclonal anti-collagen III	AF5810; Acris, Wettingen, Switzerland	1:200
Mouse monoclonal anti-αSMA	A2547; Sigma-Aldrich, Buchs, Switzerland	1:500
mouse monoclonal anti-fibronectin	F0791; Sigma-Aldrich, Buchs, Switzerland	1:200
mouse monoclonal anti-ki67	NBP2–22112; Novus Biologicals	1:500
mouse monoclonal anti-PAR-2	Santa Cruz Biotechnology, sc-13504 (SAM11) on Refine-kit (anti-Rabbit-Polymer) and histofine-Mouse Polymer	1:250 1:50
Normal Mouse Serum; Isotype Negative Control	08–6599, Invitrogen	no dilution

Dilutions of antibodies were either taken from technical sheet data, provided by the suppliers or by titration of different concentrations and comparison of staining intensities. The laboratory validation of PAR-2 antibody had been performed before (Meier Bürgisser et al., 2020). In short, the validation consisted of denoting the *specificity* of PAR-2 antibody for rabbit brain tissue as well as the *reproducibility* of PAR-2 labeling, using rabbit Achilles tendon tissues after operation, stained at 3, 6 and 12 weeks, respectively, utilizing different lots of the antibody (Meier Bürgisser et al., 2020). Further information has been published earlier (Meier Bürgisser et al., 2020; Meier Bürgisser et al., 2021; Meier Bürgisser et al., 2022). Normal Mouse Serum Control (08–6599, Invitrogen, no dilution) was used; the corresponding sections were incubated with it (4 °C overnight) and used as a negative control for all antibodies.

Fluorescent IHC was performed for collagen I and III, fibronectin and α-SMA, respectively, and for a control also for ki67. Chromogenic IHC labeling was conducted for collagen I and III, fibronectin, ki67 and PAR-2, respectively, and for a control also for α-SMA. The supernatant primary antibody solution was removed and samples were then washed with 1xTBS for fluorescent IHC before incubation with the secondary donkey anti-mouse Alexa-488 antibody (A-21202; Invitrogen, Basel, Switzerland, 1:500 dilution) and 10 μg/mL 4'6-diamidino-2-phenylindole dilactate (DAPI) (Sigma-Aldrich, Switzerland) diluted in 3% BSA in 1xTBS for 1 h (RT). Finally, the slides were washed in 1xTBS and mounted, utilising Dako Fluorescence Mounting Medium (Agilent, Basel, Switzerland).

For chromogenic IHC, samples were blocked with 3% hydrogen peroxide solution in water for 10 min (RT) and subsequently washed 3

Table 2
Overview of markers, techniques and stainings used in this study. Key: ECM = Extracellular Matrix, Chro = Chromogenic detection, Fluo = Immunofluorescence, AR = antigen retrieval. Note that for Fibronectin and mouse isotype negative control (NC) images with and without AR-step are shown; "AR" and "no AR" is mentioned on these images and AR was only performed in paraffin sections. For all other stainings on paraffin sections there is no mention about AR, but AR was performed.

Marker	Description/ Purpose	Fixation	AR	Detection System
Collagen I (Col I)	Structural protein in ECM	Para + cryo	Yes	Chro + Fluo
Collagen III (Col III)	Structural protein in ECM	Para + cryo	Yes	Chro + Fluo
Fibronectin (Fn)	Glycoprotein for network building of the ECM	Para + cryo	Yes + No	Chro + Fluo
alpha smooth muscle actin (α-SMA)	Contractile smooth muscle fibres in ECM	Para + cryo	Yes	Fluo (+Chro)
ki67	Cell nucleus protein expressed in proliferating cells	Para + cryo	Yes	Chro (+Fluo)
PAR-2	Inflammation-related protein on cell surface	Para	Yes	Chro
Mouse isotype negative control (NC)	(Normal mouse serum)	Para	Yes + No	Chro + Fluo
Elastica van Gieson (EL)	Elastic fibers	Para	(classical histology)	(classical histology)
Masson Goldner Trichrome (GT)	Connective tissue structures	Para	(classical histology)	(classical histology)
HE	Most structures for comprehensive structural overview	Para	(classical histology)	(classical histology)

times with 1xTBS. Primary antibody detection was made with a biotinylated anti-mouse IgG secondary antibody and streptavidin-horseradish peroxidase (HRP) (ZytoChem Plus HRP Kit Mouse; Zytomed Systems, Muttenz, Switzerland). Then, colorimetric detection was conducted according to the manufacturer's protocol with DAB (DAB Substrate Kit High Contrast; Zytomed Systems, Germany). Last, slides were given a wash in tap water and mounted, using Faramount Aqueous Mounting Medium from Agilent.

With a slide scanner (Pannoramic 250 Flash II, 3Dhistech, Budapest, Hungary), images of whole tissue sections were taken. We made snapshots of fields of view (FOVs) with CaseViewer-Sofware v.2.1 or imaged them with a Leica 6000 light microscope (Leica, Basel, Switzerland). The techniques and stainings used in this study are summarized in Table 2.

2. Results and discussion

3.1. Fixation of the tissue

After performing animal experiments and collecting the tissue of interest, researchers have to fix the tissue as a preparation for histological staining. Tissue fixation is needed because cellular components have to be adequately preserved, autolysis and displacements of cell constituents, such as antigens and enzymes, have to be prevented and finally the tissue has to be stabilized against the harmful mechanical effects of the subsequent processing (Ramos-Vara, 2005). Generally speaking, fixatives can be divided into two categories, cross-linking and coagulating fixatives. The most common cross-linking fixative is formalin, which binds to amino groups and reactive hydrogen atoms, resulting sometimes in adversely changing the conformation of macromolecules. This can harm the recognition of the proteins by antibodies, rendering immunohistochemistry almost impossible (complete loss of immunoreactivity). Nevertheless, formalin fixation with subsequent paraffin embedding is one of the most used cross-linking fixation techniques (Sadeghipour and Babaheidarian, 2019).

On the other hand, coagulating fixatives are in use, such as ethanol, which precipitates proteins by breaking hydrogen bonds – without cross-linking the proteins. Disadvantages of ethanol are inadequate cellular preservation and a shift in intracellular immunoreactivity. In contrast to formalin fixation, ethanol fixation has been reported to be much less reliable (Prentø and Lyon, 1997). Particularly, ethanol fixation can lead to histological distortion, cell shrinkage and vacuolization, which occur only to a minor degree after formalin fixation (Prentø and Lyon, 1997). A further fixation technique is the cryopreservation (cryo embedding). This technique includes the freezing of freshly harvested tissue after embedding in Tissue-Tek®. Cryo embedding has been reported to potentially lead to morphological changes in the tissue and the crystallized water may damage the tissue to some extent (Katoh, 2017). Noteworthy to mention is that any kind of fixation technique may inactivate enzymes to some degree and still allow proper morphological analysis (Fowler et al., 2008), but full denaturation should be in any case avoided (Tsutsumi, 2021).

Further fixation techniques have been reported, such as paraformaldehyde-lysine-periodate, the Bouin solution, or acetic formalin (Salguero et al., 2001). In addition, antigen retrieval (AR) steps are used to retrieve the loss of antigenicity and to theoretically reach the state of proteins in their prefixation conformation. Furthermore, enzymatic AR and heat-induced epitope retrieval have been described with the help of a microwave oven, a pressure cooker or a steamer (Ramos-Vara, 2017).

To access antigens inside cells, permeabilization chemicals are required, such as the detergents TritonX-100, Tween20, glycine, and hydrogen peroxide (Rosas-Arellano et al., 2016).

In this report on rabbit tonsil anatomy, we have compared formalin fixation with a buffered 4% formalin solution to cryo embedding. The superiority of the cryo embedding method was hypothesized. During the comparison, it however turned out that neither cryo embedding nor formalin fixation was always better, but it depended on the immunohistochemical target structure and on the detection system (chromogenic versus fluorescent detection). Recommendations for specific fixation techniques will be given after the discussion of several antibodies and the comparison of paraffin sections with cryosections.

Fig. 1 shows healthy rabbit palatine tonsil sections and immunohistochemical labeling for collagen I, collagen III, fibronectin, ki67 and α-SMA, and paraffin section and cryosection are compared. We chose DAB for the chromogenic detection system, as amino ethyl carbazole (AEC) has been reported to be inferior (Hira et al., 2019), which we could confirm. Whereas in the paraffin sections the collagen I positive areas can be distinctively seen as dark brown areas with a light brown background, the cryosections present these areas also as dark brown zones, however, with a bit more slurriness and hence less precisely compared to the paraffin sections. Nevertheless, as the cryosections have a white background, the contrast between collagen I positive areas and the background is more prominent than in the paraffin sections.

Collagen I rich areas in the rabbit tonsils are particularly found at the layer facing the lumen of the excretory ducts, which can be nicely seen at the highest magnifications for both, the paraffin and the cryosections, respectively (Fig. 1). Moreover, there is a collagen I rich zone around the excretory ducts. At the highest magnification, there are also two collagen I positive lymphoid follicles in the tonsillar tissue, as shown in the paraffin sections. In contrast, the connective tissue of the tonsil does not show collagen I protein expression in the paraffin sections – while in the cryosections, this is not clear because of the very dark brown stained tonsillar cells and the slightly less brown colour attributed to the extracellular collagen I.

As for the collagen III expression, there is hardly any in the paraffin section (Fig. 1). However, due to strong brown background staining in the cryosections, the collagen III rich areas are difficult to delineate and

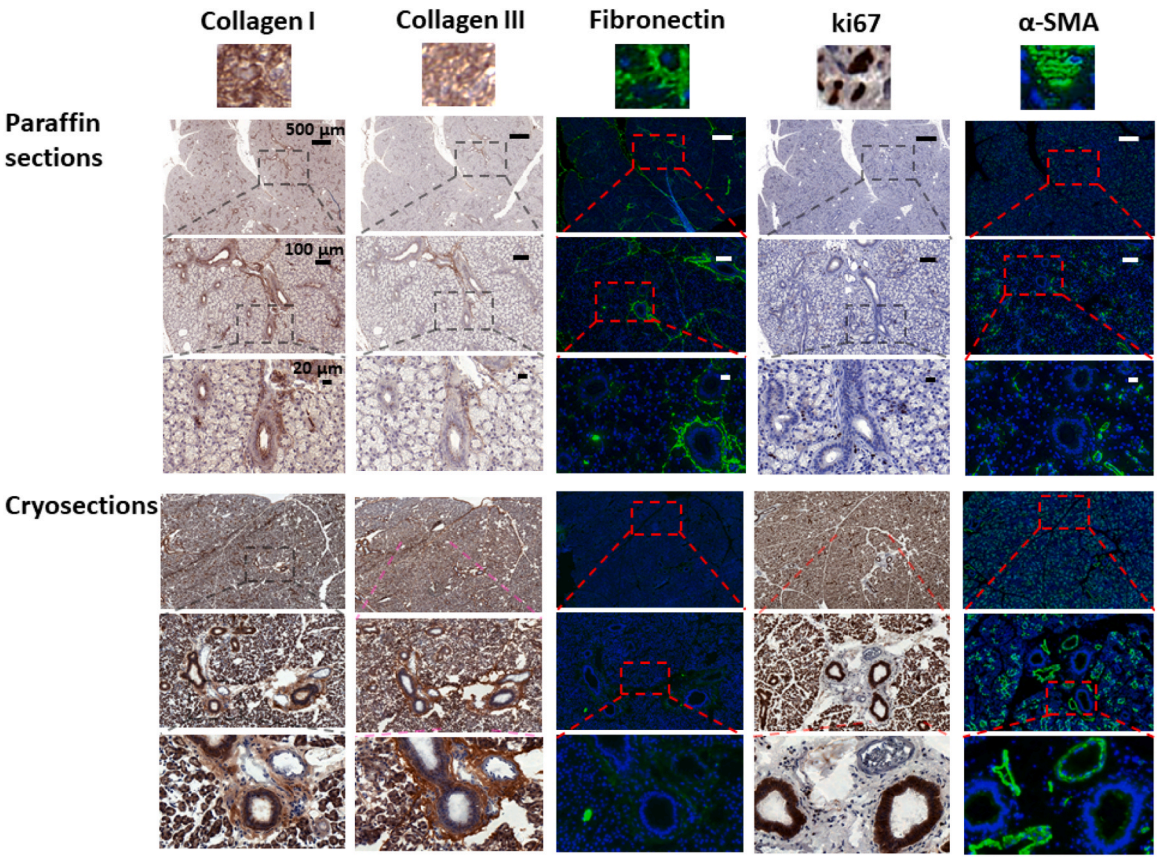

Fig. 1. Collagen I, collagen III, fibronectin, ki67 and α-SMA immunohistochemical labeling with DAB as chromogen (dark brown colour for collagen I, collagen III and ki67) and immunofluorescence detection (light green for fibronectin and α-SMA); with cell nuclei DAPI labeling, blue) for paraffin sections with antigen retrieval (AR, upper rows) and cryosections (lower rows). Corresponding inserts (dashed line) depict the area used for images at the next lower level.

to consider separately. If any collagen III is present, then it is found between the tonsillar cells, lining up with the outer layer of the excretory ducts. This can be seen better in the paraffin sections than in the corresponding cryosections (any magnification).

While the tonsil tissue immunohistochemically stained for fibronectin exhibits clear green areas in the paraffin sections, the cryosections do not show such areas or are only very weak (Fig. 1). The superiority of the paraffin sections is obvious: they have a much higher green-to-black contrast. There are clear fibronectin-positive areas, shown as fine lines at low magnification, better visible at higher magnifications and particularly found around the excretory ducts – coexistent with collagen I (Fig. 1). In addition, these fibronectin positive lines connect the excretory ducts, resembling filigree streets from duct to duct.

The proliferation status of cells with the dark brown, practically black, ki67 positive cell nuclei in the connective tissue, were clearly and well visible for both paraffin and cryosections. Due to stronger brownish background staining found in the cryosections, the paraffin sections had nevertheless to be favoured when the chromogenic DAB detection system was used. Proliferative cells were found in the connective tissue of the rabbit tonsils, with approximately 2.57 ± 1.22% ki67 positive cells (cryosections) and in tonsillar tissue 2.51 ± 1.17% (paraffin sections). Among these proliferating cells of the tonsillar connective tissue, there has been reported to be a fraction of stem cells (Janjanin et al., 2008). Such tonsil-derived stem cells (TDSCs) have been compared to bone-marrow-derived stem cells (BMSCs). They have been found to proliferate with significantly shorter doubling times (37.1 ± 3.4 h versus 58.2 ± 2.3 h) than the BMSCs. It has to be noted, however, that TDSCs had been gained after tonsillectomy and extracted from rest tissue of quite young patients (aged 4 – 15 y), while the BMSCs were from patients aged 39 – 58 y (Janjanin et al., 2008) – and it is well-known that senescence of stem cells has an impact on their proliferation capacity, besides other implications (Turinetto et al., 2016). Nevertheless, an advantageous feature of TDSCs is their ability to differentiate easily into various tissue types from all three germ layers, whereas the use of other adult stem cells is limited by their differentiation capacity restricted to tissues derived from only one distinct germ layer (Cho et al., 2019).

Table 3
Recommendations which technique is favoured for which purpose.

Antibody	*Detection system*	*Recommendation*
Collagen I	Chromogenic	Cryosections that have less background staining.
Collagen III	Chromogenic	Cryosections despite more background staining: distinction of collagen III positive areas easier (higher contrast).
Ki67	Chromogenic	Paraffin sections favoured over cryosections, because of stronger background staining in cryosections.
Fibronectin	Fluorescent	Paraffin sections favoured due to higher green-to-black contrast
α-SMA	Fluorescent	Paraffin sections because of lower slurryness and better confinement of α-SMA positive areas.

Moreover, the comparison of α-SMA staining with a fluorescent detection system (Fig. 1) revealed similar staining intensities of green colour in paraffin sections and cryosections. Nevertheless, paraffin sections might be favoured over cryosections, because the α-SMA positive areas in vessel walls of arteries and veins are a bit less slurry than in the corresponding cryosections.

Table 3 gives an overview of the recommendations to use either formalin fixation or cryo embedding for the previously discussed

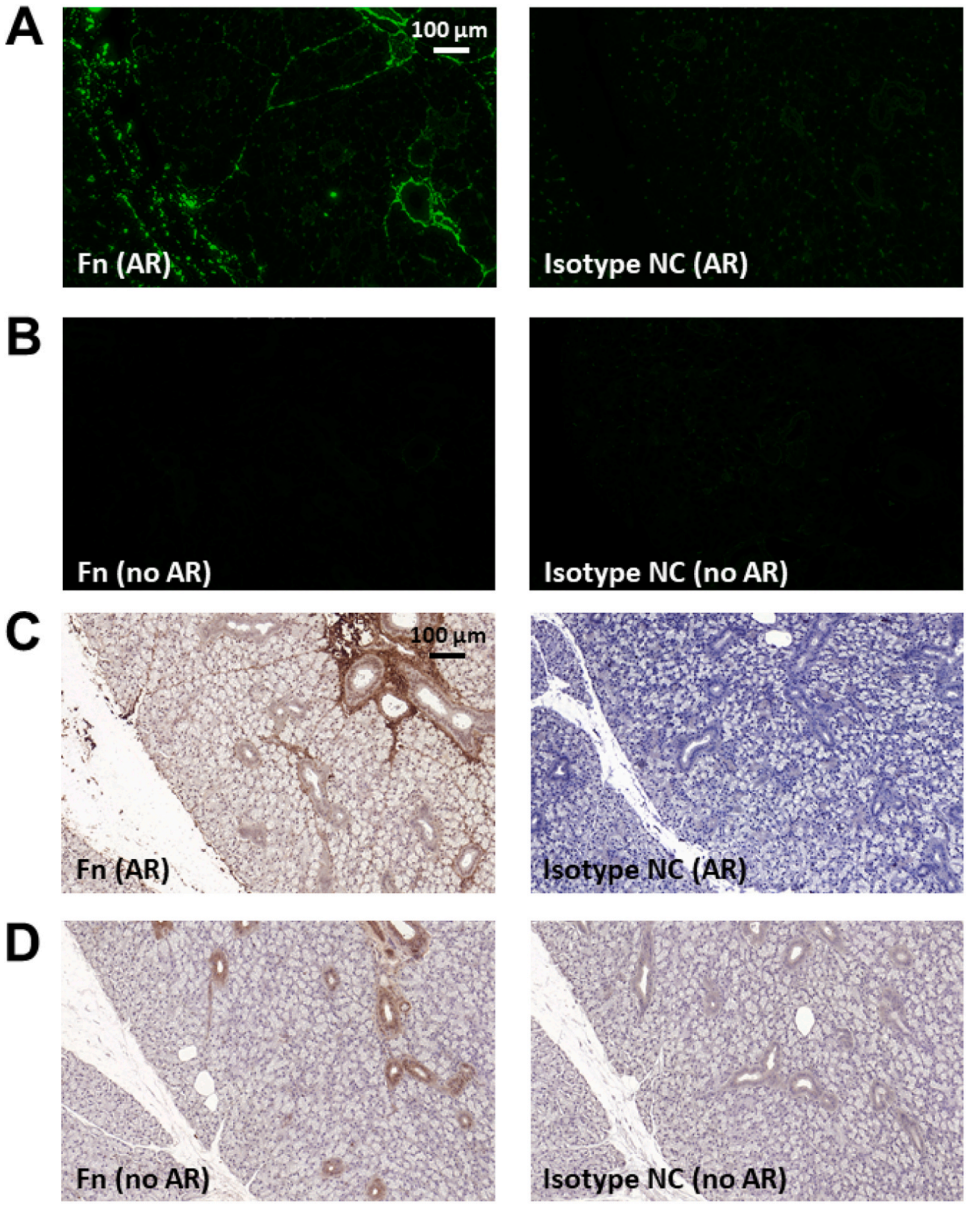

Fig. 2. Comparison of fibronectin and Isotype negative controls in paraffin sections with immunofluorescence (**A** and **B**) and chromogenic labeling (**C** and **D**), with antigen retrieval (AR; **A** and **C**) or without AR (no AR; **B** and **D**) for tonsil tissue.

antibodies of Fig. 1.

3.2. Advantages of antigen retrieval (AR) for paraffin sections

In order to counteract the deleterious effects of formalin treatment, antigen retrieval (AR) is optional, but in many cases a necessary step to overcome the compromised immunoreactivity of the antigens in paraffin embedded sections (Ramos-Vara, 2005). Several techniques have been developed to realize AR, such as heating the sections to approximately 100 °C in a microwave oven (Shi et al., 1991) or in steamers and pressure cookers (Ramos-Vara, 2017). AR is not restricted to crosslinking fixatives like formalin, but has been adapted to other fixation techniques, such as PAXgene tissue fixation (Stumptner et al., 2019). Noteworthy to consider, buffers having specific pH values and the exact time frames for microwave processing have to be tested for each tissue, fixative and antibody under view (Stumptner et al., 2019).

After an AR step, tissue proteomics can be applied, bridging classical histology and molecular histology. To realize tissue proteomics and enable not only the validation of specific immunohistochemical labeling but also a more in-depth analysis of components, a matrix assisted laser desorption/ionization (MALDI) mass spectrometer can be used, which allows for differential mapping of hundreds of compounds at a distinct location of a section to further analyse the tissue (Longuespée et al., 2014).

To visualize the hugely positive effect of an AR step before immunohistochemical staining in paraffin sections, we present fibronectin and isotype negative control labelled sections, with or without AR (Fig. 2). As can be seen in the fluorescent detection system, fibronectin positive tonsillar tissue was only distinguishable (green) with an AR step, while the sections without AR were just black (no apparent signal) (Fig. 2 AB). Accordingly, some weak signals were seen in the Isotype negative control with AR, while again no signal is detectable without AR. As for the chromogenic detection system with DAB, tissue around tonsillar excretory ducts was stained brown with AR, while they exhibited only weak brown intensity without AR (Fig. 2CD); and correspondingly, the isotype negative control (no AR) (Fig. 2D). Therefore, it is advisable to perform AR before IHC to guarantee access to the corresponding epitopes.

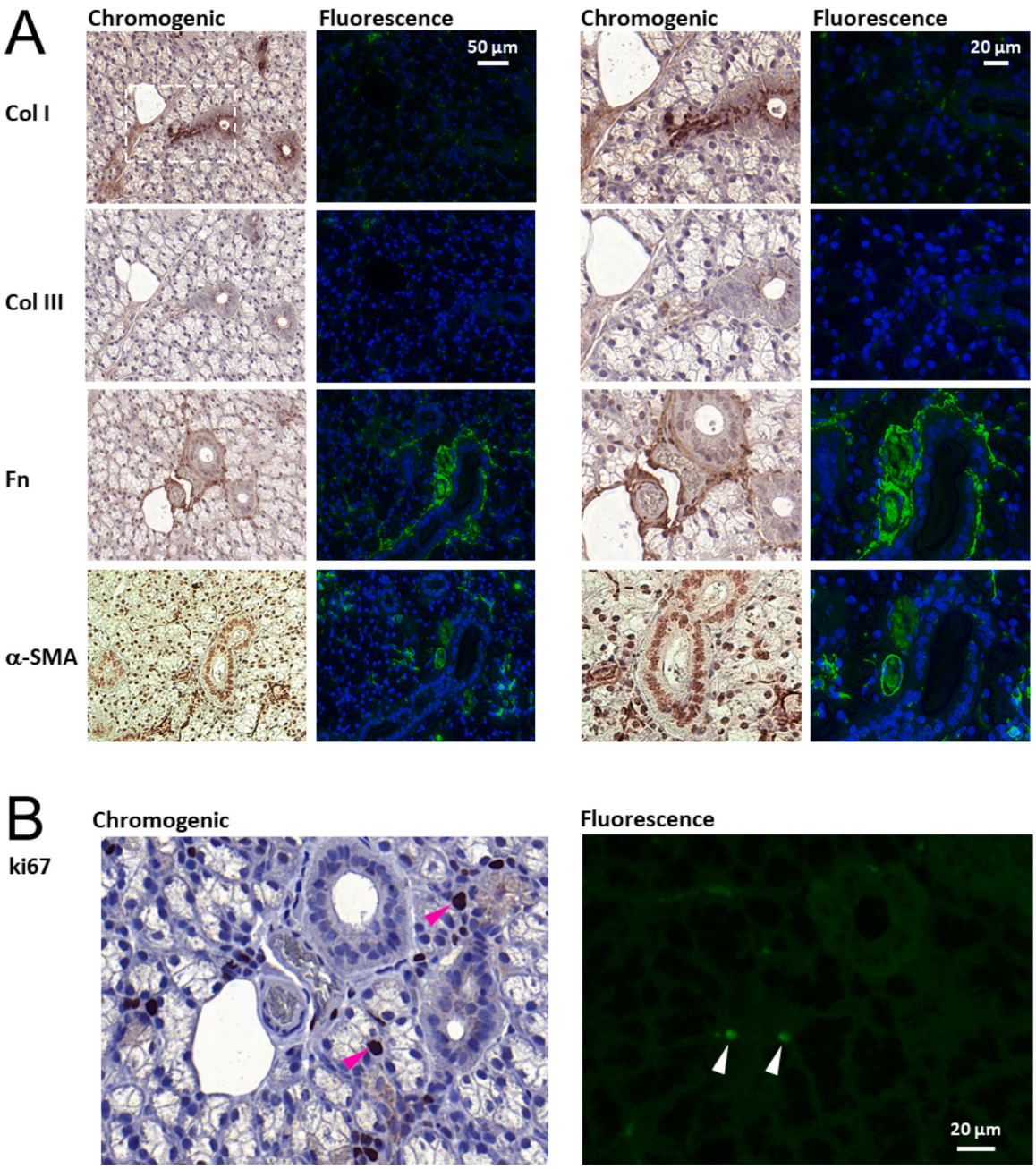

Fig. 3. Immunohistochemical labeling for **collagen I**, **collagen III**, **fibronectin**, **a-SMA (A)** and **ki67 (B)** in tonsil tissue, for chromogenic staining with subsequent bright field imaging (left; DAB as chromogen) and immunofluorescence staining and imaging (right). All samples were paraffin embedded and labelings performed with AR. Arrowheads show proliferating cells in (**B**).

3.3. Chromogenic staining versus fluorescent detection system

For collagen I, collagen III, fibronectin and α-SMA as well as ki67 immunohistochemical labeling in serial sections, we have compared chromogenic DAB/brightfield imaging with fluoresecent detection system in paraffin sections with AR (Fig. 3). While the extracellular markers collagen I, collagen III and fibronectin exhibited the excretory ducts in the rabbit palatine tonsils better with DAB staining than the corresponding fluorescent detections system, for α-SMA, the distinct borders of the vessels were slightly better visible (with more contrast) in the fluorescent detection system. We also compared ki67 labeling: in the chromogenic staining, the dark brown cell nuclei of the proliferating cells were very well visible, embedded in the connective tissue between the excretory ducts (Fig. 3B), while in the corresponding fluorescent detections system, the proliferating cells were visible, however, all the surrounding tissue was ki67 negative and therefore quite dark. We conclude that for ki67 chromogenic staining has to be favoured over the fluorescent detection system.

Generally, optical colour loss in immunofluorescence is a disadvantage over the stable DAB chromogenic system, so from this point of view, DAB is recommended rather than fluorescence. In addition, it has to be noted that during scanning the slides with digital microscopic devices, all slides have to be treated adequately in order to achieve full information concerning immunohistochemical labeling of target structures.

3.4. Anatomy of the rabbit palatine tonsil

The rabbit tonsil tissue including the epithelium is furthermore

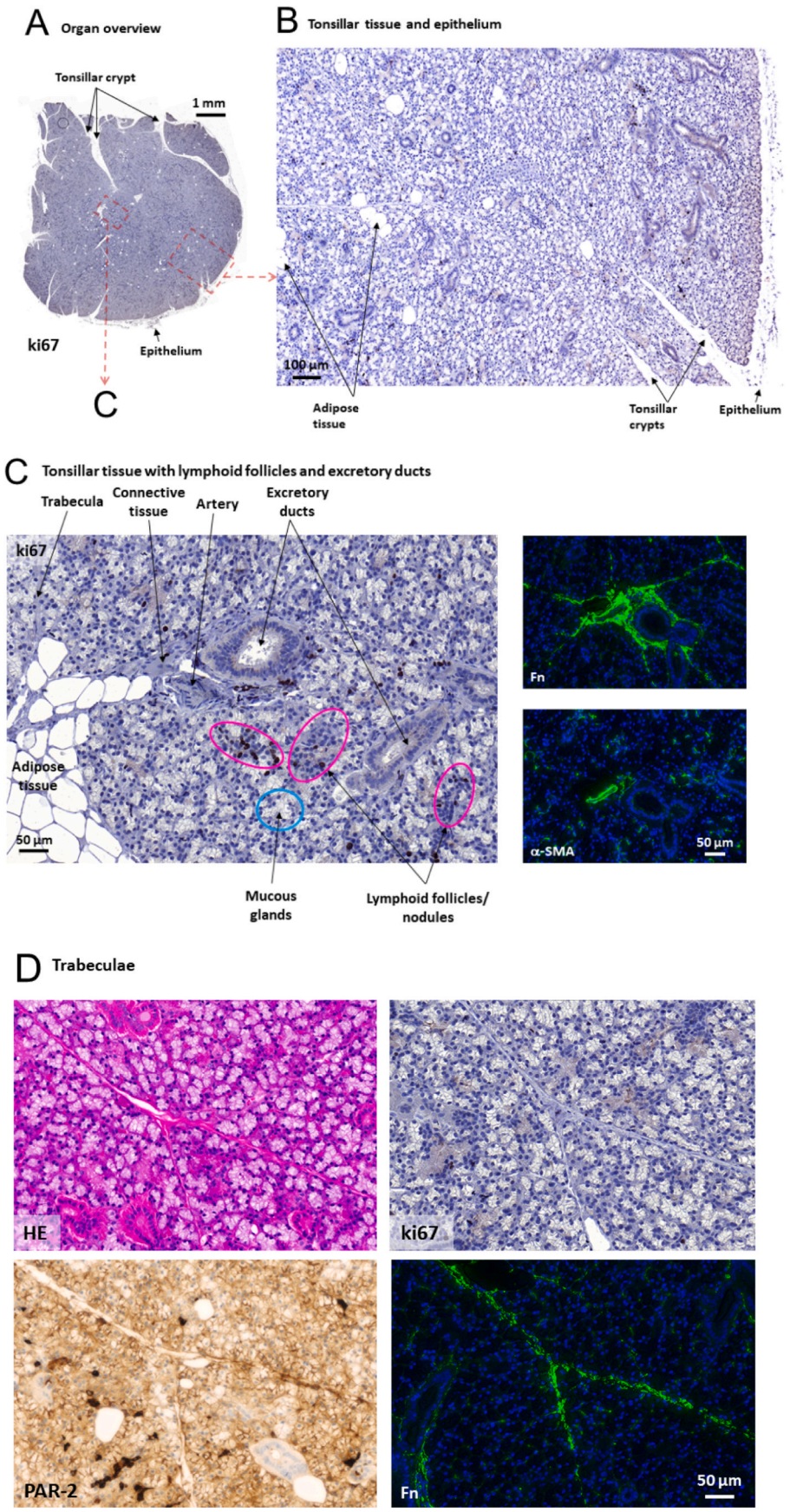

Fig. 4. Specific structures and tissue in a healthy rabbit tonsil section stained for ki67, fibronectin (Fn), α-SMA, PAR-2 and HE. Overview (**A**), tonsillar tissue and epithelium (**B**), tonsillar tissue with lymphoid follicles and excretory ducts (**C**) and trabeculae (**D**).

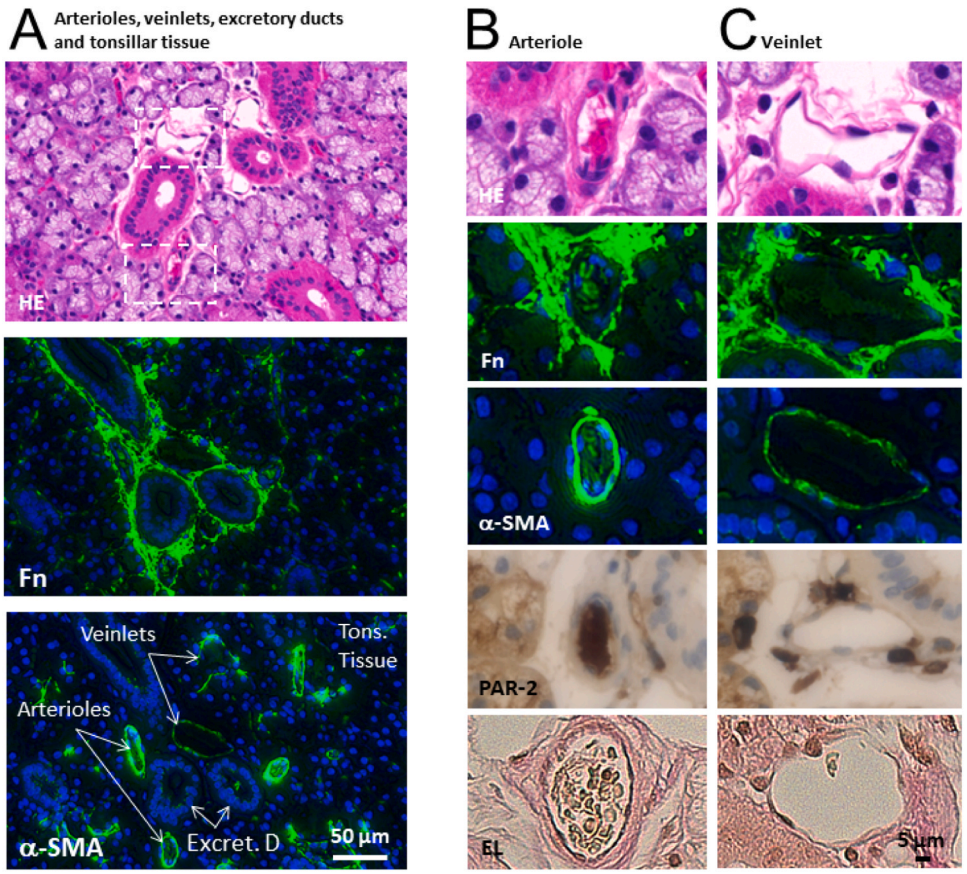

Fig. 5. Arterioles, veinlets, excretory ducts and tonsillar tissue (**A**) in rabbit tonsil immunohistochemically labelled for a series of different markers as well as HE and Elastica van Gieson for elastin (in paraffin sections with AR) and enlarged for arterioles (**B**) and veinlets (**C**): *Key*: HE = Haematoxylin&Eosin, Fn = Fibronectin, α-SMA = alpha smooth muscle actin, PAR-2 = protease activated receptor-2, EL = Elastica van Gieson staining for elastin.

shown in ki67 labelled sections (Fig. 4A), where the tonsillar crypts are prominently depicted in the overview image as well as in the higher magnification (Fig. 4B). Furthermore, some adipose tissue is reflected by the lipid droplets within the tonsillar connective tissue.

In addition, Fig. 4C shows the trabeculae, some mucous glands as well as the lymphoid follicles, also called lymphoid nodules. While cells of the excretory ducts exhibit low (approximately zero) numbers of proliferating ki67 positive cells, there is a larger fraction of such cells in the lymphoid follicles (14.6 ± 5.7%). The adipose tissue parts of the tonsil can be very well seen here. The connective tissue surrounding the excretory ducts has furthermore been visualized by fibronectin staining, emphasizing the predominance of the extracellular matrix component near the ducts. In contrast, α-SMA areas are restricted to vessel walls, as shown in the smaller images of Fig. 4C, and are sparsely found in the

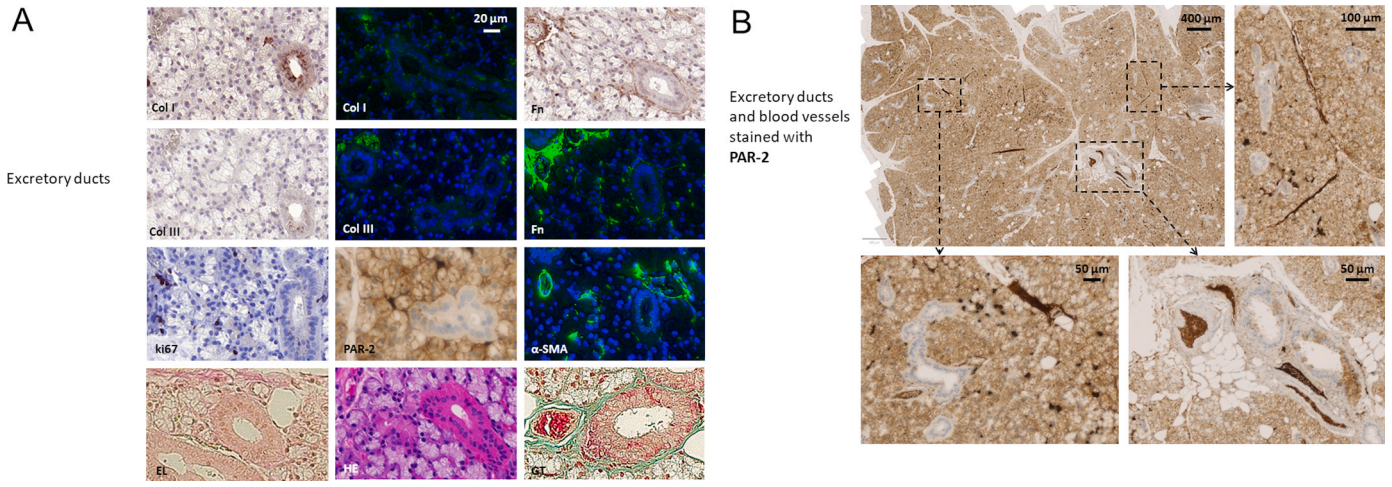

Fig. 6. Excretory ducts and vessels for serial paraffin sections of healthy rabbit tonsillar tissue stained with collagen I, collagen III, fibronectin, α-SMA, ki67, PAR-2, elasin, HE and GT, respectively (**A**) and selected structures with PAR-2 immunohistochemical labeling (**B**): excretory ducts and blood vessels. *Key*: Col = Collagen, Fn = Fibronectin, α-SMA = alpha smooth muscle actin, ki67 = proliferation marker ki67, PAR-2 = protease activated receptor-2, EL = Elastica van Gieson staining for elastin, HE = Haematoxylin&Eosin, GT = Masson Goldner Trichrom. All stainings were performed after antigen retrieval.

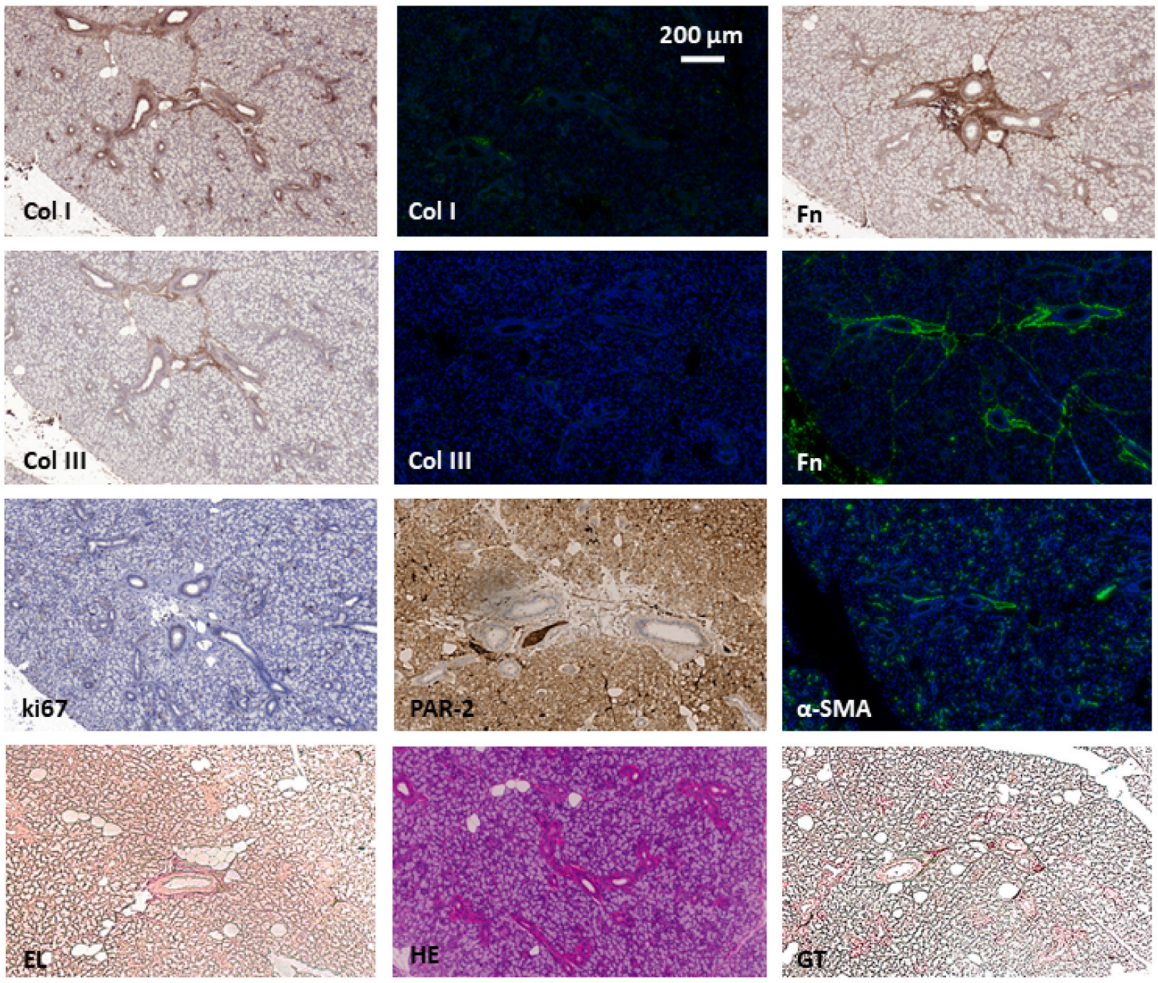

Fig. 7. Serial paraffin sections of vessels and excretory ducts labelled for different markers. *Key*: Col = Collagen, Fn = Fibronectin, α-SMA = alpha smooth muscle actin, ki67 = proliferation marker ki67, PAR-2 = protease activated receptor-2, EL = Elastica van Gieson staining for elastin, HE = Haematoxylin&Eosin, GT = Masson Goldner Trichrome. All stainings were performed after AR.

connective tissue.

The trabeculae are furthermore stained with HE, ki67, PAR-2 and fibronectin, respectively (Fig. 4D). As can be seen, they are fibronectin positive, but do not exhibit any proliferating cells. Furthermore, they do not exhibit PAR-2 positive areas in this particular trabecula. In the HE stained sections, the dark violet cell nuclei are depicted best and allow us to detect the cellular distribution of all cells. As can be seen, trabeculae lack of any cells, but are filled with connective tissue, most prominently filled with fibronectin.

Furthermore, we provide more detailed images of arterioles and veinlets of the tonsillar tissue, with HE, fibronectin, α-SMA, PAR-2 and EL (Elastica von Gieson) for elastin stainings (Fig. 5). The artery walls were characterized by strong and dominant α-SMA expression, while the veinlets exhibited a much lower α-SMA content. Fibronectin rich tissue was adjacent and around to the arterioles and veinlets. Elastin was only weakly expressed in the artery walls facing the lumen, while it was not expressed in the veinlets. Interestingly, we found a strong PAR-2 expression in arterioles and cells situated around veinlets with PAR-2 positive expression (Fig. 5B and C).

Serial sections of excretory ducts are shown in Fig. 6A. Stainings for collagen I, collagen III, fibronectin, α-SMA, ki67, PAR-2, HE, elastin and Masson Goldner Trichrome (all collagen in green) allow a direct comparison of the distribution of the extracellular matrix components. While there was some collagen I expression around the excretory ducts, the collagen III expression was low. On the other hand, fibronectin was found around the excretory ducts and also to some extent in the connective tissue, whereas α-SMA positive areas were predominantly found in the vessel walls, but not in the excretory ducts. Interestingly, in contrast to the excretory ducts of the tongue tissue (paper submitted), excretory ducts in the tonsillar tissue were PAR-2 negative, but there were zones in the connective tissue that exhibited a strong PAR-2 (over) expression. We have therefore provided more PAR-2 stained structures in Fig. 6B. As can be seen, PAR-2 expression was prominent in these particular trabeculae (together with fibronectin, Fig. 4D) and also in the lumen of some arterioles (Fig. 6B).

PAR-2 expression for the healthy rabbit palatine tonsils has not been examined experimentally so far. For human tonsils, however, it has been shown that extracted tonsillar mast cells (a subpopulation) responded to PAR-2 agonists by a dose-dependent release of tryptase (He et al., 2005). The authors reported an activation of tonsillar mast cells by PAR-2 agonists, such as SLIGKV or tc-RIGRLO, indicating a self-amplification mechanism of mast cell degranulation after an inflammatory stimulus (He et al., 2005). In another study focusing on allergic airway disorders, induction of INF-γ2 mRNA expression has been reported when human tonsil cells were stimulated with agonist peptides of PAR-2 in vitro (Wang et al., 2016). We therefore speculate that the dark brown PAR-2 positive cells in Fig. 6B could be attributed to tonsillar mast cells, particularly considering the consistent cellular fraction: it has been reported that roughly 7% of the tonsillar cells in humans were mast cells (Wang et al., 2016).

In order to demonstrate vessels and excretory duct bundles, serial sections of the rabbit tonsils are shown in Fig. 7. While excretory ducts

were fibronectin and collagen I positive, α-SMA was restricted to vessel walls. PAR-2 positive areas were found in the lumen of some vessels. Elastin was only weakly expressed and can be found in the excretory duct walls. In the HE stained sections, different shades of pink enable the vessels and excretory ducts to be visualized well.

3.5. Conclusive remarks

We have shown that histological staining for elastin, haematoxylin&eosin, Masson Goldner Trichrome and immunohistochemical labeling for collagen I and III, α-SMA, fibronectin, ki67 and PAR-2 is feasible in the healthy rabbit palatine tonsil tissue. The main structures (excretory ducts, the vessels and the trabeculae), are well depicted. Recommendations are given on whether paraffin sections or cryosections may serve specific purposes better. Moreover, we show in a side-by-side comparison that an antigen retrieval step for formalin fixation and paraffin embedding is extremely valuable. Finally, as PAR-2 expression has not been described in the New Zealand white rabbit tonsils so far, we showed the PAR-2 distribution and found a predominant PAR-2 protein expression in some trabeculae at their tiniest branches and also at the lumen of some arterioles.

Several limitations have to be addressed. We only deal with the healthy state of the tonsils and not the diseased or inflamed state. In addition, we have dealt with the tonsils of only one rabbit – and therefore are not able to elucidate potential inter-rabbit variability in anatomical palatine tonsil structures. However, as New Zealand white rabbits have all the same genetic background, we judge the anatomy of the healthy rabbit to be very similar and coherent over many New Zealand white rabbits.

To conclude, histological and immunohistochemically stained sections of the healthy rabbit tonsil are presented to be used for a comparison with the pathological situation when tonsil researchers have decided to use this animal model.

Authorship contribution statement

Gabriella Meier Bürgisser performed IHC stainings (except for PAR-2), imaged all sections and composed all figures. Dorothea M. Heuberger gave valuable input on PAR-2 staining. Maurizio Calcagni, Pietro Giovanoli and Johanna Buschmann supervised the study. Johanna Buschmann provided funding, wrote the manuscript and supervised the study.

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

Data will be made available on request.

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