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# Commentaries on the publication entitled: "Structure and distribution of an unrecognized interstitium in human tissues" by Benias et al. (2018)

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The claim made in this publication of the existence of a hitherto unknown interstitial space is based on studies with sample-based confocal laser endo-microscopy (pCLM). Due to postings on various web portals (New Cellvizio, EurekAlert, Google Scholar,...) the alleged discovery has found great resonance. Nevertheless, there are several critical issues in this publication, the most important being that this is not the discovery of an "unrecognized" interstitium as it has, in fact, been known for a long time.

Interstitial space is a well-known anatomical entity located between organs and cells and containing loose connective tissue. However, the authors of this paper do not mention this tissue, although in some sections of the work they do describe it in part. An important element of this tissue is the so-called ground substance, which is not mentioned anywhere in this paper. (Bloom and Fawcett (1975, page 158 and following).

The ground substance is a gel-like material consisting of several components of the extracellular matrix and has the ability to bind water. This permits the circulation of products related to cell metabolism etc. and their exchange with the vascular compartment [Fig. 1, see also Krstic (1988)]. In fact, the ground substance corresponds to the so-called tissue fluid in which the fibrous mesh of the connective tissue is immersed.

The fibroblasts are responsible for the synthesis of fibers and ground substance and can be found separately or in contact with fibers, but they never form a lining of any defined spaces (Fig. 2). Under normal conditions no spaces or gaps of any size have been observed in interstitium. However, classical histology books do contain descriptions of the presence of large spaces (lacunae), for example after the artificial injection of air or physiological solutions into the loose connective tissue such as in certain regions of the dermis or hypodermis, these being referred to as a lacunar or cavitary system (Archard, 1922). Already in the XIX century the presence of cavities or enlarged spaces between the fibers of the loose connective tissue were described in edema and also under experimental conditions, created with the aim of dissociating fibrous and cellular components of the loose connective tissue for microscopic observation (Ranvier, 1875).

The pCLM permits the examination in vivo of the wall (surface and deep layers) of hollow and other organs, but requires the application of a fluorescent tracer, in general fluorescein, apparently without negative effects on cells and tissues (Wallace and Fockens, 2009; Wallace et al., 2010). The images published by Wallace et al. show a reticule or lattice of bright and dark areas, depending on the distribution of the tracer and appear to be very similar to those described by Benias et al. (2018).

It is undoubtedly a good idea to interpret the pCLM image according to the particular micro-

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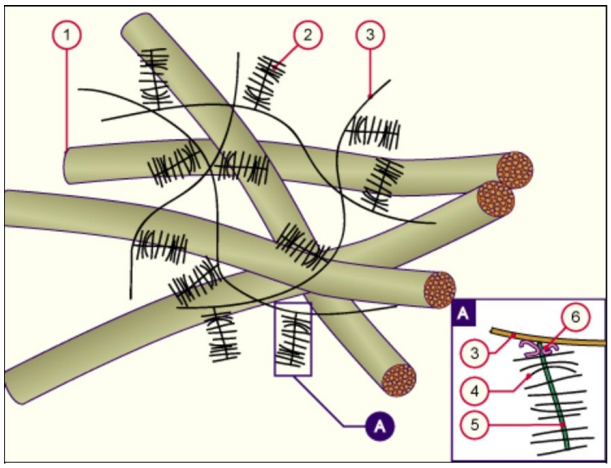


**Fig 1.** Diagram in which the molecular structure of the ground substance is shown. The hyaluronic acid molecules form linear aggregates with many proteoglycans, which form a network with collagen fibrils. Insert A.- shows how GAGs are linked to HA by link proteins. (from Abteilung Medizin. Universität Freiburg (CH); Web search: Lehrplattform Histologie, Grundsubstanz in www3.unifr.ch).

Explanation of numbering:1) Collagen fibrils, 2) Proteoglycans, 3) Hyaluronic acid (HA), 4) Glycosaminoglycan (GAG), 5) core-protein, 6) Link-protein.

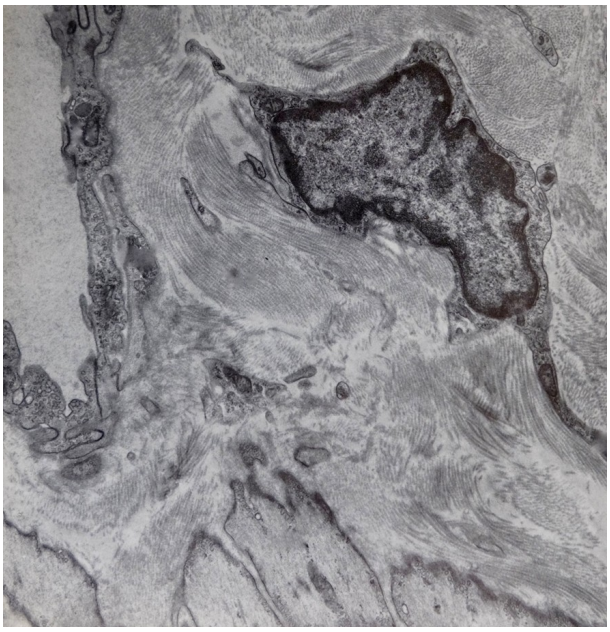

**Fig 2.** Electron micrograph of the oesophagus lamina propria. On the upper right side a fibroblast with nucleus and thin cytoplasm processes closely associated with bundles of collagen fibers can be seen. It is of note that no relevant spaces or gaps exist between the collagen formations. In the lower part of the image four smooth muscle cells of the muscle layer of the mucosa can be seen. On the left a blood vessel can be recognized. Magnification of the negative: 15.000X.

scopic structure, however the loss of tracer during preparation makes it difficult for the observer to identify structures and spaces. It is regrettable that other tracers more easily detectable with optical microscopy have not been tested, leaving this aspect of the correlation disregarded. The "holes" shown under intravital microscopy and in cryofixed samples are huge (over 20 µm)! However, in the images of fixed material (LM, EM), they are significantly smaller (1 µm and less). This is an indication that the cavities vary depending on the method of preparation. Unfortunately, this aspect is not discussed.

In the case of the spaces described after cryofixation, the suspicion arises that they are not real structures. This is based firstly on their large size and secondly on the apparent complete lack of ground substance. With the method of aerosol-based freezing spray it is not possible to immobilize water fast enough in order to prevent the formation of ice crystals: the origin of tissue disruptions and many artifacts. In view of this, new experiments applying special cryofixation methods also adapted for biopsies and which fulfill the requirements could be recommended (Hohenberg et al., 1996; Vanhecke et al., 2008). Proof needs to be provided that the spaces described in the submucosa after cryofixation are genuine (Fig. 1G of Benias paper).

In the 3D diagram (Fig. 1 I of Benias paper) the arrangement of cells and fibers of the connective tissue obviously indicates a misinterpretation. Firstly, the CD34-positive cells may be undifferentiated fibroblasts but they could also be completely different cell types, suggesting that this may not have been healthy tissue. Furthermore, there are more appropriate markers than CD34 to typify fibroblasts.

Secondly, as stated above, the cells do not cover the surface of hypothetical spaces, but are attached or at least in close relation to the fibers that they themselves form and maintain (Fig. 2). The TEM image in Benias paper (it is the same image repeated at slightly larger magnification) indicates deficits in tissue preparation and the optically empty spaces of considerable size around the collagen fibers could be due to compression during tissue dissection or further handling.

It can also be argued that, even if the postulated tissue spaces collapsed, any cell lining still intact to some degree could be identified with certainty in histological sections and this is not the case.

As far as the discussion is concerned, it is already established that the interstitium is upstream of the lymphatic and vascular pathways; the observations made here are already well known and contain hardly any new information, although they are well presented.

In summary, the attempt to correlate the pCLM images of the submucosa of the bile duct to the microscopic structure of the tissue is a good idea but it has not been proven in this study.

And, finally, the claim to the discovery of a hitherto unknown interstitial space and the assertion that the study of the interstitium has so far been neglected are grossly exaggerated and cannot remain uncriticised.

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