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

Neil D. Theise[1], Petros C. Benias[2], David A. Carr-Locke[3], Rebecca G. Wells[4]

[1]Department of Pathology, New York University School of Medicine, New York New York, USA, [2]Division of Gastroenterology, Zucker School of Medicine at Hofstra/Northwell, Northwell Health System, Manhasset, New York, USA, [3]Division of Gastroenterology and Hepatology, Weil Cornell School of Medicine, New York Hospital, New York, New York, USA, [4]Departments of Medicine and Bioengineering, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

We appreciate the time and attention paid to our paper by Prof. Mestres-Ventura and similarly appreciate the opportunity to respond to his concerns. We would like to address what we believe are several fundamental misunderstandings in his commentary.

1. Scale: The most significant misunderstanding is one of scale. The schematic (Fig. 1) provided by Prof. Mestres-Ventura is (per personal communication) at the nano scale, while in vivo microscopy of extrahepatic bile duct and dermis shows that the collagen bundles we report are at the micron scale, each containing many individual fibrils at the nanometer scale. Indeed, examining the tissues described in our paper – submucosae, dermis and subcutaneous fascia – fresh in resected specimens or intraoperatively, we find that the structures we describe are visible at the macroscopic level (if one leans in closely enough). In other words, they are macroscopic, not microscopic.

Prof. Mestres-Ventura, in summarizing the prior pCLE work of Wallace and Fockens, which he notes is similar to ours, states "the 'holes' shown under intravital microscopy and in cryofixed samples are huge (over 20 μm)!" This is exactly our point - we were surprised as well at the scale of these structures, as this has not been well appreciated in the past.

2. Was this space recognized previously: Prof. Mestres-Ventura is correct that the interstitium has been known for decades, as have the fascia, the submucosal compartments, and the dermis. What is new, however, is the understanding that these large spaces support fluid flow, and that there is a structural similarity – and potentially physical connection – between them. (Note, however, that this space is not the lamina propria, as shown in Prof. Mestres-Ventura's Fig. 2. Our data do not bear on the structures of any lamina propria of the body and the illustration is not relevant, although it emphasizes the confusion and that the "interstitium" he is describing is not the "interstitium" we are describing. Our digestive tract images, including esophagus, are all submucosae.) That a reticular structure in the bile duct submucosa was observed but not fully appreciated or understood previously is seen in the commentator's own statement summarizing the prior pCLE work of Wallace and Fockens, which he notes is similar to ours. While it is indeed established that the interstitium is upstream of lymphatic and vascular pathways, we continue to hold that the full nature of that interstitium has been only vaguely defined. We make it very clear

Corresponding author: Neil D. Theise, MD. Dept. of Pathology, NYU Langone Health, 550 First Avenue, New York NY 10016, USA.

Rebecca G. Wells, MD. 905 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104, USA.

in our published paper that what we propose is a revision of existing anatomical concepts.

3. Fixation and spaces: A fundamental issue highlighted by Prof. Mestres-Ventura's commentary is that tissue may look different under the microscope than in vivo, and it is difficult to distinguish artifact from "real" and "real" from artifact, particularly when the artifacts (of staining, of fixation) become so routine as to make them nearly invisible. It should be acknowledged that any technical intervention in tissue creates artifact - there are no methods of rendering tissue stiff enough to sample for microscopy (e.g. freezing, chemical fixation) that do not introduce artifacts. The goal of all histologists, however, must be to visualize tissue as close to its living state as possible. Our in vivo imaging is at least closer to the state of living tissue than any fixed or frozen tissue cited in the commentary. It is bewildering that Prof. Mestres-Ventura holds in vivo microscopic imaging to be less real and more artifactual than the work cited in his commentary, none of which involves in vivo microscopy. We reveal not virtual spaces, as he implies by reference to the work of Archard or Ranvier, in which injected air or edema fluid expand the spaces, but actual spaces that are fluid filled in healthy living tissue, and thus can expand with air or fluid in the certain experimental or pathologic settings.

The commentary also states: "[…] 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." We agree that the use of multiple tracers would be optimal; however, although fluorescein may not elucidate all the properties of this space, it is unfortunately the only approved contrast agent safe for use in humans at this time.

4. Ground substance: The nature of the material filling the spaces we describe was not a primary focus of our paper, other to observe that this substance permits the free flow of fluid. We agree that proteoglycans and glycosaminoglycans (the ground substance) are key components of the interstitium and have many functions, including the control of fluid flow and, likely, the organization of collagen in the interstitial space. In work currently under revision, we have begun to characterize the features of the soluble and non-collagenous matrix components of the interstitial space, but this is a complex topic beyond the scope of our original report. While the water-binding abilities of proteoglycans and glycosaminoglycans are well known, the ability of such a space to support fluid flow as we have described has not been.

5. The nature of the cells: Prof. Mestres-Ventura writes that our schematic diagram of the arrangement of cells and fibers "obviously indicates a misinterpretation." He suggests that while the CD34-positive cells may be a type of fibroblast (as we claimed), they "could also be completely different cell types, suggesting that this may not have been healthy tissue." The tissues were in fact healthy; the diagnostic pathologist and endoscopists have cumulatively more than a century of clinical practice evaluating tissues for disease vs. health. We agree that CD34 is not a standard fibroblast marker, and that was exactly our point – this is an unusual population of cells with positivity for both CD34 and vimentin. Since publication of our original work, we have carried out further immunostains and isolated these cells from the submucosa of the mouse extrahepatic bile duct. While the cells are heterogeneous, a significant percentage express both CD34 and standard fibroblast markers including vimentin and PDGFRb (manuscript in revision).

We do not know why Prof. Mestres-Ventura writes that "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." We agree; this is what we have claimed, and is what is shown in our TEM photographs and schematic. We have carried out additional high resolution imaging examining the relationship between cells and collagen bundles; this confirms that the cells are in close apposition to collagen bundles. We look forward to the publication of these data in the future.

In summary, in our paper we made initial observations regarding the interstitial space and its supporting collagen bundles at a scale that has not been consistently described before. We observed the flow of fluid through large interstitial spaces between and surrounding tissues, and further specified aspects of the fibroblast-like cells lining the collagen bundles, in particular their discontinuity through the spaces and their expression of CD34. The use of in vivo microscopy demonstrated these macroscopic structures and indicated that there were gaps in our knowledge, not just in the tissues. As Prof. Mestres-Ventura also emphasizes, the non-collagenous, non-cellular components of the interstitial space, while not a focus of our initial publication, is an important and fascinating area of study; we look forward to collegial associations with other investigators with shared interests to pursue such investigations.

REFERENCES

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