The pre-lymphatic pathway, the roots of the lymphatic system in breast tissue: A 3D study

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ORIGINAL ARTICLE

The pre-lymphatic pathway, the rooths of the lymphatic system in breast tissue: a 3D study

Sofia Asioli · Vincenzo Eusebi · Laura Gaetano · Luisa Losi · Gianni Bussolati

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Abstract Three-dimensional (3D) visualisation of microscopic structures provides useful information about their configuration and the spatial (hence functional) relationship between different components in tissues. This paper describes 3D dynamic reconstructions of the pre-lymphatic labyrinth in two cases of "normal" breast core biopsies and one case of pseudoangiomatous stromal hyperplasia. Direct anastomoses between pre-lymphatic channels and true lymphatics of the breast were demonstrated. It is concluded that pre-lymphatics are a way of communication between breast epithelial/stromal structures and the main lymphatic system. The present findings suggest that the existence of pre-lymphatics has to be taken in consideration in the intramammary spread of malignant tumours.

Keywords Pre-lymphatics · Lymphatic labyrinth · 3D · Normal breast · PASH

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Introduction

The lymphatic vascular network differs from that of the blood vessels as it is an "open" system to the stromal environment [1]. Lymphatic capillaries are structurally similar to blood capillaries [1], and both are composed of endothelial cells with intercellular junctions, adhesion plates, isolated vesicles, deep caveolae, flat nuclei and a well-formed basal lamina. Nevertheless, in normal tissues, there are spaces within the connective tissue that end directly at a lymphatic junction [2]. The spaces are mostly evident when tracers are injected in tissues and become particularly prominent when the oedema forces the cells and fibre bundles apart [2]. Openings of lymphatic capillaries into stromal spaces have been documented at ultrastructural level by Casley-Smith and Florey [1]. Artefacts were excluded, as no similar openings were seen in neighbouring blood capillaries, and were interpreted as consequent to poorly developed adhesion plates [1]. These spaces were named "pre-lymphatics" by Casley-Smith [2] and in the breast are so numerous and so often interconnected as to deserve the name of "lymphatic labyrinth" [3]. Spaces are lined by flattened attenuated cells that are vimentin- and CD-34-positive. CD 31 and D2-40 are consistently negative [4]. The same cells at ultrastructural level show scattered intermediate filaments and lack basal lamina [4] as to deserve the name of "delimiting fibroblasts" by Ozzello [5]. Pre-lymphatics are present around ducts and within the specialised stroma of lobules, but are hardly visible in normal breast because they are collapsed [4], a phenomenon described by Hartveit as the "missing" lymphatic system [3]. Whenever lymph needs to be drained, especially in inflammatory situations, pre-lymphatics open on demand [3]. Although Vuitch et al. [6] believed that these spaces were artefact, Badve and

Sloane [7] demonstrated that these same spaces are real, as they are visible in frozen sections as well as at ultrastructural level [4]. In addition, neoplastic cells were found to spread through them, highlighting the labyrinth structure of these same spaces [8].

Most of the above observations were obtained indirectly from conventional haematoxylin and eosin staining (H&E) or electron microscopy. Direct visualisation of spaces entering into lymphatics would be obtained only by the use of the 3D reconstruction of histological tissue.

We reconstructed from two cases of normal breast core biopsies and a case of pseudoangiomatous stromal hyperplasia (PASH) through a 3D model, the complex labyrinth arrangement of pre-lymphatics and their connection into lymphatic channels.

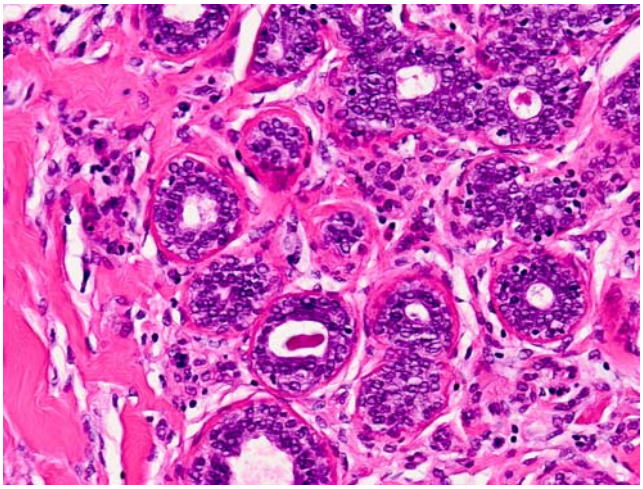

Fig. 1 Case 1: spaces present within the inter acinar stroma. The spaces have elongated shape, are lined by flattened spindle cells and are characteristically devoid of red blood cells

Materials and methods

Cases

Cases 1 and 2 were core biopsies from 48- and 39-year-old women. These were obtained from the files of the Department of Pathology and Forensic Medicine of the University of Modena and Reggio Emilia. Both patients had undergone biopsy as a screening procedure for familiar breast cancer.

Case 3 is of a 51-year-old lady who presented with a 6-month story of a lump in the lower external quadrant of the left breast. The lump was excised. The tissue removed consisted of a 4.5 cm across nodule with smooth surface. The cut surface was homogeneous and grey in colour.

Tissues were embedded in paraffin and stained with H&E as routine.

Histologically, cases 1 and 2 were selected among several others and were constituted by numerous lobules where dilated easily visible spaces were present within the intra-acinar stroma (Fig. 1). Case 3 had the typical feature of PASH [6], being a fibroepithelial lesion in which lobules appeared architecturally distorted, immersed in a stroma showing elongated empty spaces (Fig. 2a,b).

For immunohistochemistry, all cases were stained for D2-40 monoclonal antibody (clone D2-40, diluted 1:100; Signet Laboratories); CD 34 (clone QB-END10, diluted 1:400, Neomarkers) and CD31 (clone yc/70A, diluted 1:20, Cell Marque) employing an automated immunostainer (Ventana BenchMark AutoStainer; Ventana Medical Systems).

The cells that lined the spaces in all cases were CD-34-positive, but negative for CD 31 and D2-40.

For 3D analysis, serial sections obtained from the paraffin blocks were immunohistochemically stained for D2-40. Sections (50 sections, 4 μm thick) then were all photographed. For 3D reconstruction, one lobule from cases 1 and 2 showing numerous widely open spaces were selected (Fig. 1). In case 3, one glandular/stromal area was selected (Fig. 2).

The contours of the spaces of two normal lobules from cases 1 and 2 as well as of one distorted lobule from case 3 were shadowed with the painter tool (red colour) in order to better visualise their shapes. In addition, all the acini of the selected lobule and the perilobular lymphatic channels stained by D2-40 antibody were also shadowed with the painter tool (blue and green colours, respectively).

Three-dimensional procedure

To realign serially cut sections correctly, we created external fiducial markers in the paraffin-embedded specimen using a tissue arrayer instrument (ATA-100; Chemicon International, Temecula, CA, USA) before obtaining serial sections (Fig. 3). Accordingly, four cores removed from an anthracotic lymph node by the tissue arrayer were inserted as reference markers into the ‘recipient’ paraffin block of breast tissue, near the area to be serially cut, and 3D-reconstructed [9]. As already stated, 50 sections, 4 μm thick, were serially cut from the recipient block. Digital pictures of D2-40-stained sections were serially captured at the selected magnification with an OLYMPUS_ DP11 digital camera mounted on an OLYMPUS_ VANOX T microscope. The semi-movable stage of the microscope allowed orientation of the images because the reference markers were set in the same position.

Three-dimensional reconstruction

Using the serial images obtained, 3D models of selected channels, lymphatics and acini were obtained employing

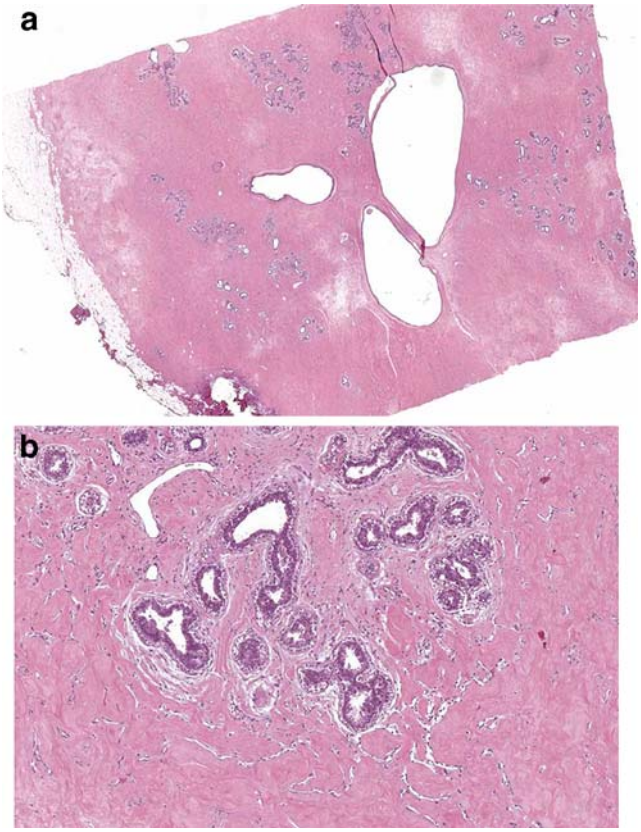

Fig. 2 Case 3 shows typical features of pseudoangiomatous stromal hyperplasia (**a**) in which lobules appear architecturally distorted, immersed in a stroma showing elongated empty spaces (**b**)

Amira 4.0, advanced 3D visualisation and volume modelling software (TGS Template Graphics Software, http://www.tgs.com). In order to have the studied structures unchanged through sections, we previously included the anthracotic lymph node in four points of the paraffin-embedded sample as fiducial tissue markers (see above) [9]. Then, we could manually align serial sections by matching these reference points with the software tools. Finally, AMIRA allowed to segment automatically the regions of interest which were interpolated and finally 3D-reconstructed.

Results

At low magnification, as seen at H&E level, the spaces situated among the acini and around the lobules have an elongated shape, were characteristically devoid of red blood cells and lined by flattened spindle cells (Figs. 1 and 2). Serial sections, stained for D2-40, highlighted lymphatics that were observed around the lobules, whilst the empty spaces were consistently unstained (Fig. 4). Depending on the plane of sectioning, spaces appeared round or ovoid in shape, with some of them laying very close to lymphatic vessels (positive for D2-40) present in the perilobular stroma (Fig. 5).

Three-dimensional modelling

The spatial organisation of the empty spaces (channels), as reconstructed on serial sections, was very complex, but similar in all three cases. The channels were of various size, shape and length and were frequently interconnected. Most spaces were tortuous, elongated and showed small calibre. A minority was short, had irregular profile and large calibre. Intermediate combinations were seen. In general, the caliber of the channels of the PASH case was larger than that seen in normal lobules. Some spaces were linear and followed almost parallel planes of growth as seen in modelling field (level) 31 of case 1 (Fig. 6). Other

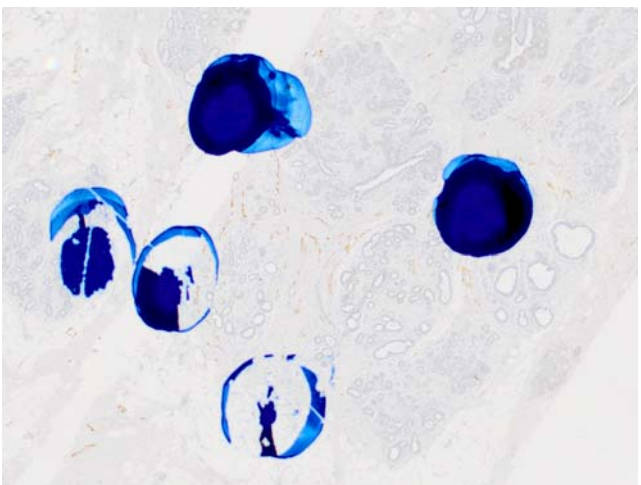

Fig. 3 In order to align serial sections, antracotic lymph node arrays were included as fiducial markers in four points of the paraffin-embedded tissue block

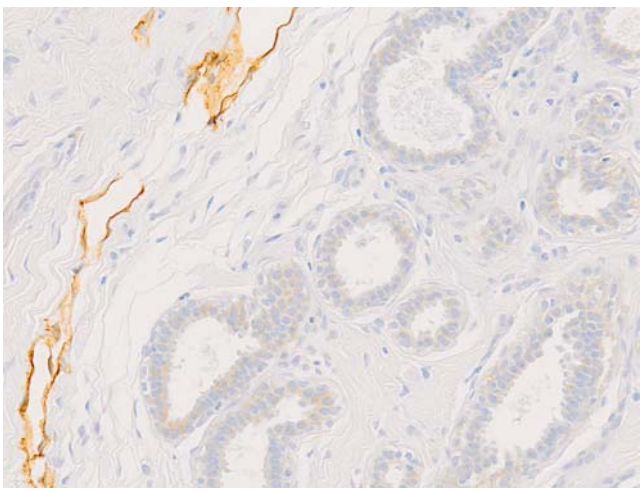

Fig. 4 Stain for D2-40 highlights occasional lymphatics around the lobules, whilst the empty spaces scattered among the acini are consistently unstained

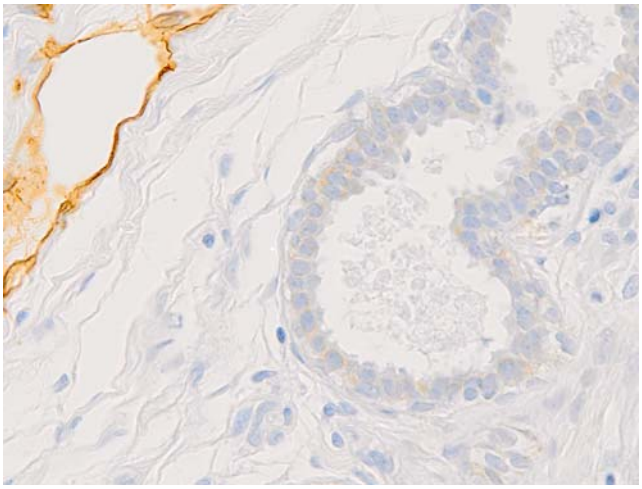

Fig. 5 Spaces appear round to ovoid in shape. One of them is very close to a lymphatic vessel (positive for D2-40) present in the perilobular stroma

modelling fields (levels), especially in PASH, showed spaces with convoluted cavities. Most of the spaces were present within the acinar stroma, but others, especially in the case of PASH, were observed in the perilobular stroma as to build up a complex scaffolding around the lobule (Fig. 7a, modelling field 27, case 2, and b, modelling field 46, case 3). Spaces present within the lobule frequently showed a circumferential distribution around acini (Fig. 8, modelling field 15, case 1).

In no fewer that seven modelling fields, spaces in different cases connected directly to the D2-40-stained perilobular lymphatic vessel (Figs. 8 and 9a, modelling field 10, case 3, and b, modelling field 5, case 3). The

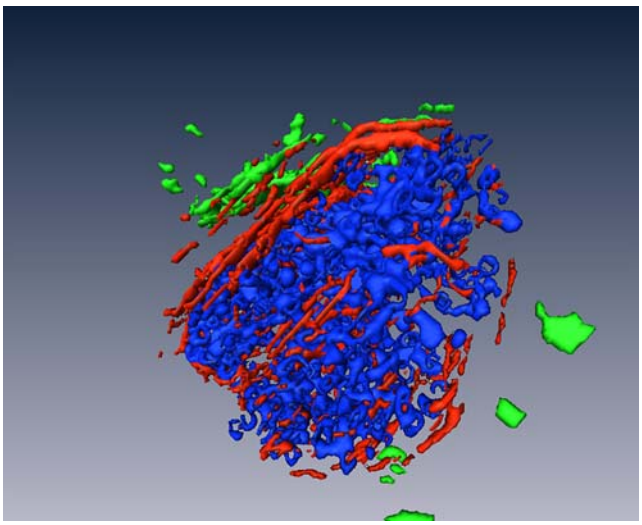

Fig. 6 The spatial organisation of the empty spaces (channels), as reconstructed on serial sections, is very complex, but similar in all three cases. Some spaces are linear and follow almost parallel planes of growth as seen in modelling field (level) 31 of case 1 (*red*: channels; *blue*: acini; *green*: lymphatics)

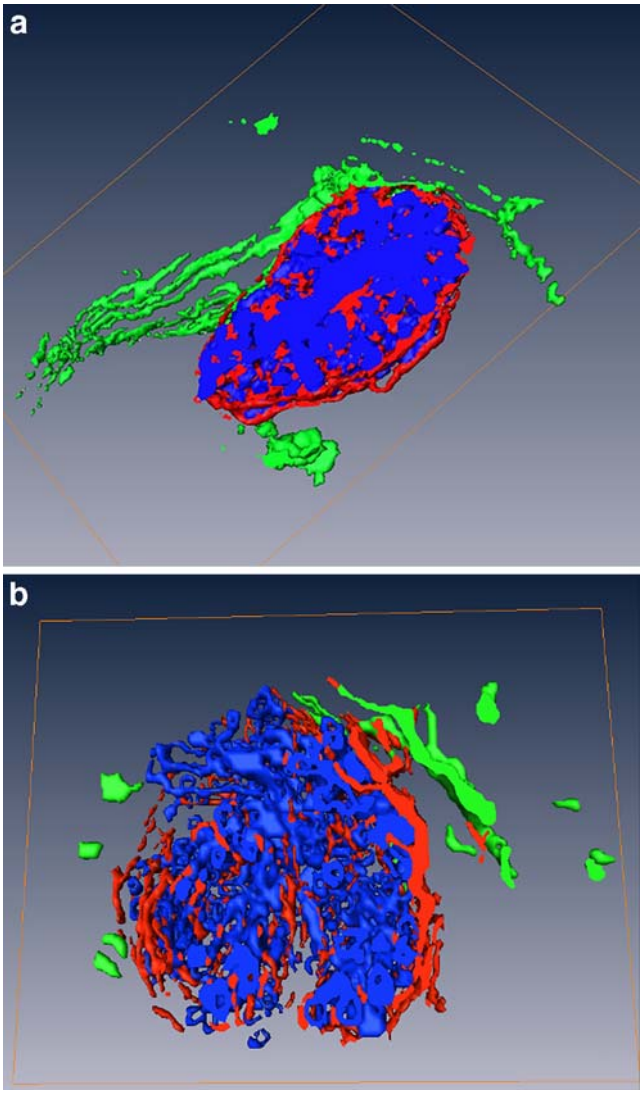

Fig. 7 Most of the spaces are localised within the lobular stroma. When in perilobular location, they build up a complex scaffolding around the lobule. **a** Modelling field 27, case 2. **b** Modelling field 15, case 1. In **b**, perivascular channels connect with a lymphatic in the upper outer corner

3D profile of the three cases can be directly visualised in the motion images stored in the web site of this journal (www.sprigerlink.com).

Discussion

Three-dimensional reconstruction provides useful information about the configuration of microscopic structures, and it offers a useful tool to examine the spatial (hence functional) relationship between different components in tissues. Three-dimensional studies, especially in breast tissues, were based on diafanisation of paraffin blocks of large sections that were examined directly at stereomicroscopic level [10–13]. More recently, the use of the computer that allows the 3D

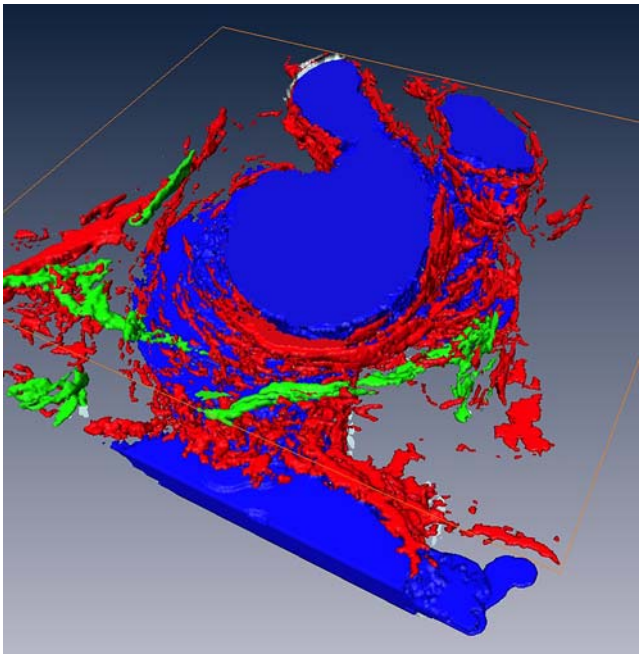

Fig. 8 Spaces frequently show a circumferential distribution around acini in PASH and appear of large caliber (modelling field 46, case 3)

reconstruction of normal structures and of specific lesions from the analysis of multiple histological sections (static 3D) has proven to be a valuable technique in different areas of pathology such as normal distribution of microcirculation of retina [14], para-trabecular pattern of infiltration of non-Hodgkin's lymphoma in bone marrow [15], angiogenesis in the bone marrow of children with leukemia [16], distribution of microvessels in prostate cancer [17], evaluation and reconstruction of spinal cord injury [18], relationship between functional and structural changes of glomerular capillary networks in normal kidney [19], distribution and organisation of microvascular structure of cardiac coronary vascular tree [20], distribution of the vessels in normal and neoplastic thyroid [10] and distributions of vessels in oligodendrogliomas [21]. In breast, Ohtake et al. [22, 23], in a 3D reconstruction of intraductal extension of invasive breast carcinoma, have highlighted the presence of occasional intralobar and extralobar anastomoses which allowed the spread of in situ duct carcinoma through different lobes. These same data were not confirmed by Foschini et al. [11] and Going and Moffat [24]. The similarity between tubular and tubulo-lobular carcinomas has been defined [25], and the size and extent of lobes has been illustrated [24].

A novel approach to the use of computerised 3D techniques is the dynamic reconstruction of specific areas in which the area itself rotate and can be visualised from many angles. In this way, the convoluted shape of the nuclei of papillary thyroid carcinoma was highlighted [26].

In the present study, the lobules that were selected contained several spaces within the specialised stroma. Spaces were seen also in perilobular location where lymphatics were also visible, and these features were consistent with the description of the pre-lymphatic labyrinth depicted by Hartveit [3]; in fact, spaces appeared round to ovoid, some were elongated, devoid of red blood cells, lined by flat "attenuated" cells that were negative for D2-40 stain and invested the acini. Lymphatics were present only around the lobules, showed dilated lumens that were also devoid of red blood cells and lined by flattened cells that were positive for D2-40. The difference between normal lobules and PASH consisted in the larger

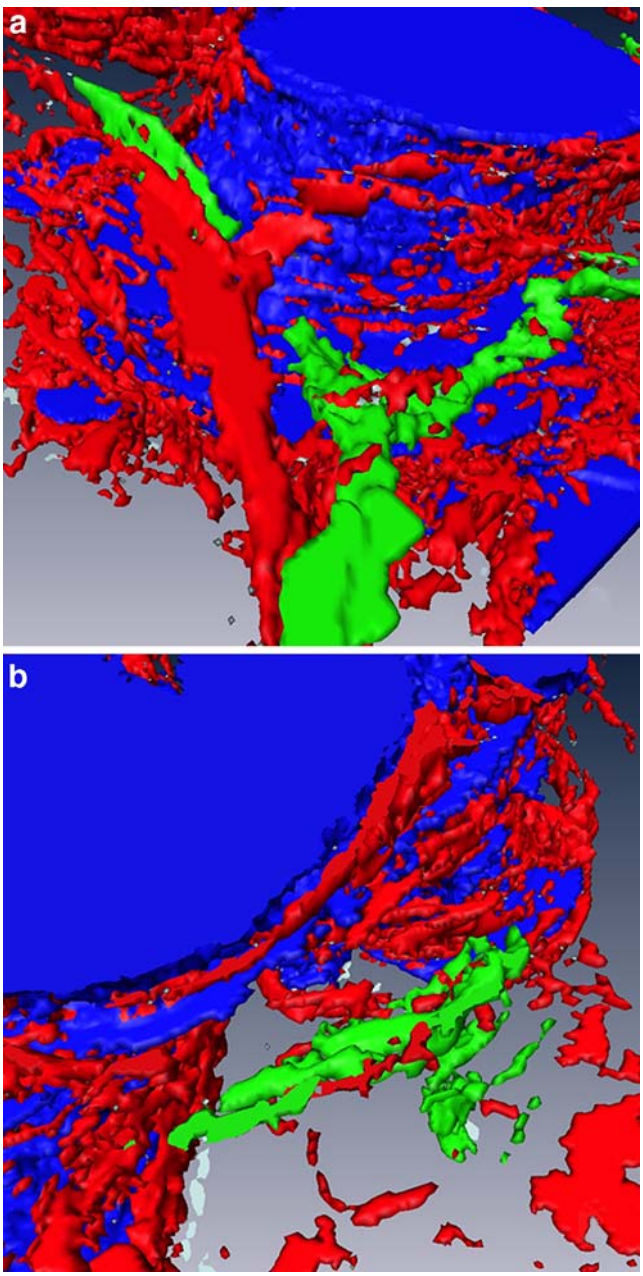

Fig. 9 Channels connect directly to the perilobular lymphatic vessel. **a** Modelling field 10, case 3. **b** Modelling field 5, case 3

caliber of channels D2-40-negative that were characteristic of pseudoangiomatous stromal hyperplasia and by the larger number of channels in perilobular stroma.

The dynamic 3D reconstruction of the lobules from all cases showed evidence that the spaces were intimately interconnected and proved the existence of an intralobular labyrinth, and the distribution of the spaces around the lobules indicated the existence of a sort of scaffolding. Shape, distribution and connection of the interstitial spaces, as traced in serial sections, concur in defining the nature of such pre-lymphatic spaces as real structures, and not as shrinkage artifacts. It was also found that at least one space in each lobule anastomosed directly with a lymphatic, also proving the previous theories of the existence of a pre-lymphatic network of which most of the credit goes to Casley-Smith and Florey and to Hartveit [1, 3]. Whether larger caliber of channels as mostly seen in PASH is the result of higher pressure in the stroma than lymphatics is still open to question.

The pre-lymphatic network cannot certainly any longer be disregarded as a way of spread of inflammatory and neoplastic cells. As the channels appear located around the acini, it is possible [4] that they can be the easiest way of spreading non-cohesive neoplastic cells such as lymphomas and invasive lobular carcinomas [4, 8]. It is pertinent to remind that one of the cases reported by Damiani et al. [8] consisted of a lymphoma that spread along the channels of PASH.

Conflict of interest statement We declare that we have no conflict of interest.

References

1. Casley-Smith JR, Florey HW (1961) The structure of normal small lymphatics. Q J Exp Physiol Cogn Med Sci 46:101–106
2. Casley-Smith JR (1973) The lymphatic system in inflammation. In: Zweifach BW, Grant L, McCluskey RT (eds) The inflammatory process. Academic, New York, pp 161–203
3. Hartveit F (1990) Attenuated cells in breast stroma: the missing lymphatic system of the breast. Histopathology 16:533–543
4. Eusebi V, Tavassoli FA (2008) Tumors of the breast. American Registry of Pathology/AFIP, Washington
5. Ozzello L, Sanpitak P (1970) Epithelial-stromal junction of intraductal carcinoma of the breast. Cancer 26:1186–1198
6. Vuitch MF, Rosen PP, Erlandson RA (1986) Pseudoangiomatous hyperplasia of the mammary stroma. Hum Pathol 17:185–191
7. Badve S, Sloane JP (1994) Pseudoangiomatous hyperplasia of male breast. Histopathology 26:463–466
8. Damiani S, Peterse JL, Eusebi V (2002) Malignant neoplasms infiltrating "pseudoangiomatous" stromal hyperplasia of the breast: an unrecognized pathway of tumor spread. Histopathology 41:208–215
9. Bussolati G, Marchiò C, Volante M (2005) Tissue array as fiducial markers for section alignment in 3-D reconstruction technology. J Cell Mol Med 9:438–445
10. Foschini MP, Papotti M, Parmeggiani A et al (2004) Three-dimensional reconstruction of vessel distribution in benign and malignant lesions of thyroid. Virchows Arch 445:189–198
11. Foschini MP, Righi A, Cucchi MC et al (2006) The impact of large sections and 3D technique on the study of lobular in situ and invasive carcinoma of the breast. Virchows Arch 448:256–261
12. Foschini MP, Tot T, Eusebi V (2002) Large-section (macro-section) histologic slides. In: Silverstein MJ (ed) Ductal carcinoma in situ of the breast. Lippincott, Philadelphia, pp 249–254
13. Wellings SR, Jensen HM (1973) On the origin and progression of ductal carcinoma in the human breast. J Natl Cancer Inst 50:1111–1118
14. Foreman DM, Bagley S, Moore J et al (1996) Three-dimensional analysis of the retinal vasculature using immunofluorescent staining and confocal laser scanning microscopy. Br J Ophthalmol 80:246–251
15. Salisbury JR, Deverell MH, Seaton JM et al (1997) Three-dimensional reconstruction of non-Hodgkin's lymphoma in bone marrow trephines. J Pathol 181:451–454
16. Perez-Atayde AR, Sallan SE, Tedrow U et al (1997) Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukaemia. Am J Pathol 150:815–821
17. Kay PA, Robb RA, Bostwick DG (1998) Prostate cancer micro-vessels: a novel method for three-dimensional reconstruction and analysis. Prostate 37:270–277
18. Duerstock BS, Bajaj CL, Pascucci V et al (2000) Advances in three-dimensional reconstruction of the experimental spinal cord injury. Comput Med Imaging Graph 24:389–406
19. Antiga L, Ene-Iordache B, Remuzzi G et al (2001) Automatic generation of glomerular capillary topological organization. Microvasc Res 62:346–354
20. Brey EM, King TW, Johnston C et al (2002) A technique for quantitative three-dimensional analysis of microvascular structure. Microvasc Res 63:279–294
21. Cassoni P, Gaetano L, Senetta R et al (2008) Histology far away from Flatland: 3D roller-coasting into grade-dependent angiogenetic patterns in oligodendrogliomas. J Cell Mol Med 12:564–568
22. Ohtake T, Abe R, Kimijima I et al (1995) Intraductal extension of primary invasive breast carcinoma treated by breast conservative surgery. Cancer 76:32–45
23. Ohtake T, Kimijima I, Fukushima T et al (2001) Computer-assisted complete three-dimensional reconstruction of the mammary ductal/lobular systems. Cancer 91:2263–2272
24. Going JJ, Moffat DF (2004) Escaping from flatland: clinical and biological aspects of human mammary duct anatomy in three dimensions. J Pathol 203:538–544
25. Marchiò C, Sapino A, Arisio R et al (2006) A new vision of tubular and tubulo-lobular carcinomas of the breast, as revealed by 3-D modelling. Histopathology 48:556–562
26. Papotti M, Manazza AD, Chiarle R et al (2004) Confocal microscope analysis and tridimensional reconstruction of papillary thyroid carcinoma nuclei. Virchows Arch 444:350–355