



Lymphatic drainage pathways from the cervix uteri: Implications for radical hysterectomy?

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HIGHLIGHTS

- In this study lymphatic drainage pathways from the cervix uteri were immunohistochemically analyzed.
- Two lymphatics pathways were found: a supra-ureteral pathway and a dorsal pathway.
- The vesico-uterine ligament, removed in Okabayashi's radical hysterectomy, did not contain lymph vessel draining from the cervix uteri.

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ABSTRACT

Objective. Radical hysterectomy with pelvic lymphadenectomy is the treatment of choice for early-stage cervical cancer. Wertheim's original technique has been often modified, mainly in the extent of parametrectomy. Okabayashi's technique is considered as the most radical variant regarding removal of the ventral parametrium and paracolpal tissues. Surgical outcome concerning recurrence and survival is good, but morbidity is high due to autonomic nerve damage. While the autonomic network has been studied extensively, the lymphatic system is less understood. This study describes the lymphatic drainage pathways of the cervix uteri and specifically the presence of lymphatics in the vesico-uterine ligament (VUL).

Methods. A developmental series of 10 human female fetal pelvises was studied. Paraffin embedded blocks were sliced in transverse sections of 8 or 10 μ m. Analysis was performed by staining with antibodies against LYVE-1 (lymphatic endothelium), S100 (Schwann cells), alpha-Smooth Muscle Actin (smooth muscle cells) and CD68 (macrophages). The results were three-dimensionally represented.

Results. Two major pathways drained the cervix uteri: a supra-ureteral pathway, running in the cardinal ligament superior to the ureter, and a dorsal pathway, running in the utero-sacral ligament towards the rectal pillars. No lymph vessels draining the cervix uteri were detected in the VUL. In the paracolpal parametrium lymph vessels draining the upper vagina fused with those from the bladder.

Conclusions. The VUL does not contain lymphatics from the cervix uteri. Hence, the favorable survival outcomes of the Okabayashi technique cannot be explained by radical removal of lymphatic pathways in the ventrocaudal parametrium.

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Introduction

Worldwide, 530,000 new cases of cervical cancer leading to 275,000 deaths are being reported annually [1]. In early-stage cervical cancer, where the tumor is limited to the cervix uteri and/or upper vagina, radical hysterectomy with pelvic lymphadenectomy (RHL) is the treatment of choice. The first large series of abdominal RHL was described by

Wertheim, who carried out an en-bloc resection of the uterus, surrounding the parametrium and upper vagina [2,3]. Meigs added pelvic lymphadenectomy to this procedure [4] and since that time the Wertheim–Meigs radical hysterectomy with pelvic lymphadenectomy has been considered worldwide as the standard surgical treatment for International Federation of Gynecology and Obstetrics (FIGO) stage IB and IIA cervical cancer [5]. Okabayashi modified this technique in the early 1920s by removing the deep layers of the vesico-uterine ligament (VUL) enabling complete separation of the bladder with the ureter from the lateral side of the cervix uteri and vagina [6]. Though surgical outcome in terms of local recurrences and survival is very good when performing this radical type of RHL [7,8], morbidity is high. Difficulties

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in or incomplete emptying of the bladder is reported in 45–51% [9], caused by damage of the efferent branches of the inferior hypogastric plexus running in the deep layers of the VUL.

Several modifications of RHL have been reported differing mainly in the extent of parametrectomy. Querleu and Morrow described a classification system based on the extensiveness of different RHL techniques [10] founded on the outdated classification of Piver and colleagues [5]. However, RHL can be very extensive laterally and less extensive dorsally. The Leiden TNM classification has been developed in order to describe the extensiveness of the parametrectomy in all directions [11], based on the well-known TNM system for the description of various tumors in categories for tumor extension (T), nodal disease (N) and distant metastasis (M). “Parametrium” is a universally accepted term to refer to the tissue surrounding the uterus, although it actually belongs to the endopelvic fasciae. However, we will continue to use the term “parametrium” in order to use the most widely accepted nomenclature. Four surgical parametrial extensions can be distinguished (ventral, lateral, caudal and dorsal) based on the Leiden TNM classification (Fig. 1).

Nonetheless, the clinical role of the extent of parametrectomy in cervical cancer is still a matter of debate. The fact that cervical cancer mainly spreads via local tumor growth and lymphatics underlines the importance of accurately removing the draining lymph vessels besides pelvic lymph nodes. Several studies have been conducted in order to

reveal locations for parametrial and pelvic lymph node metastasis [12–15]. However, detailed descriptions based on microscopic analysis about specific organ-draining lymphatic pathways are missing.

The human lymphatic system starts to develop out of lymph sacs that arise from developing veins by the end of the 5th embryonic week. The main lymphatics are formed by a number of separate primordia: the paired jugular and axillary lymph sacs and paratracheal, internal thoracic, lumbar and iliac lymph plexuses. These primordia lose their venous connections, rapidly enlarge, fuse with another and send out sprouts into peripheral regions. By the end of the embryonic period (11th week), the definitive pattern of the major lymphatics can be recognized and formation of new primordia has stopped [16].

We hypothesized that the good survival rates of the Okabayashi technique might be explained by accurate removal of lymphatic pathways in the ventrocaudal parametrium draining the cervix uteri. A developmental series of human fetal pelvises was studied in order to reveal the lymphatic drainage pathways of the cervix uteri and their connections with those of the bladder and rectum during different stages of development. Specifically, we focused on the presence of lymph vessels in the VUL. Our results are represented in a 3D reconstruction.

Methods

Material

A developmental series of ten human female fetuses with an embryonic stage of 10, 10.5, 11, 12, 14, 15, 16, 19, 20 and 24 weeks from collections of the Department of Anatomy and Embryology of Leiden University Medical Center, the Department of Anatomy and Embryology of the Academic Medical Center and the University of Warsaw in Poland was studied. We specifically chose to study this material as the usage of embryos allowed accurate exploration of the 3D anatomy of the human pelvic lymphatic system. The fetuses were obtained with informed consent after legal abortion or miscarriage and no congenital macroscopic malformations of the pelvis were observed. The bony pelvis was manually removed in the fetuses aged 14 and 15 weeks. The remaining fetuses were decalcified in an ethylenediaminetetraacetic acid (EDTA) 10% solution for 72 h.

Immunohistochemistry

Paraffin sections of 8 or 10 μ m were alternately stained with hematoxylin and eosin, and azan. Selected sections were alternately stained with antibodies against LYVE-1 (ReliaTech), S100 (DAKO), alpha-Smooth Muscle Actin (Sigma-Aldrich) and CD68 (DAKO). LYVE-1 was used to detect lymphatic endothelial cells. S100 was used to stain Schwann cells in order to reveal the peripheral neural network. Alpha-Smooth Muscle Actin was used to detect vascular and visceral smooth muscle fibers and applied to distinguish between blood and lymph vessels. CD68 is a general marker of various lineages of macrophages and was used to show immature and mature macrophages. Between all steps, sections were rinsed twice with phosphate-buffered saline (PBS) and once with PBS/0.05% Tween-20 unless indicated otherwise. After deparaffinization and rehydration in graded ethanol, antigen retrieval was performed by heating the sections (12 min to 98 °C) in citric acid buffer (0.01 mol/l, pH 6.0), except for CD68 where a Tris/EDTA buffer (pH 9.0) was used. Inhibition of endogenous peroxidase was performed by incubating the sections for 20 min in PBS with 0.3% H₂O₂. Sections were incubated overnight with primary antibodies LYVE-1 (1/500 μ l), S100 (1/10,000 μ l), alpha-Smooth Muscle Actin (1/10,000 μ l) and CD68 (1/1000 μ l) diluted in PBS-BSA/Tween-20. Hereafter, sections were incubated for 45 min with biotin-labeled secondary antibody (goat-anti-rabbit, BA-1000, Vector Labs) and additionally incubated with Vectastain ABC staining kit (PK-6100, Vector Labs) for 45 min. The sections stained with alpha-Smooth Muscle Actin were treated only with a secondary antibody (rabbit-

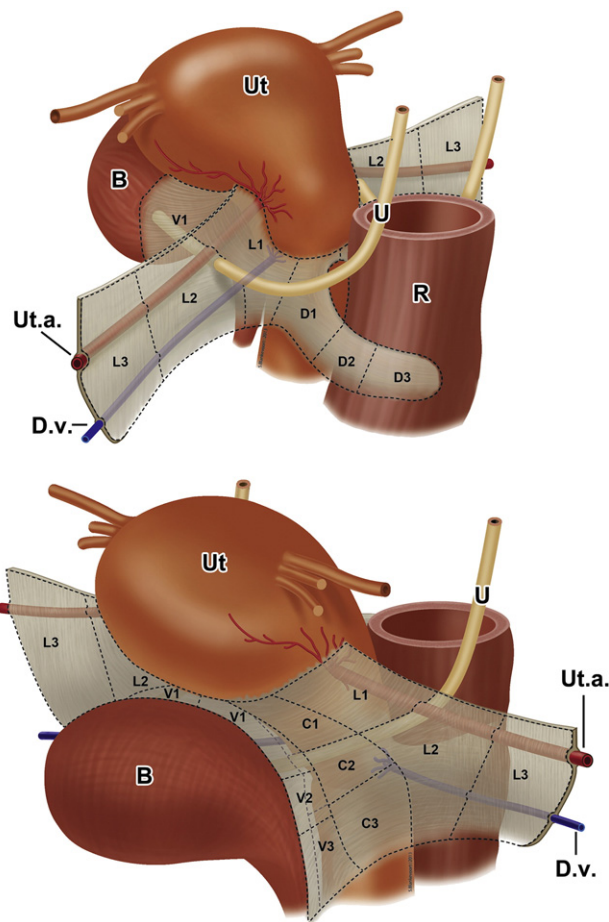


Fig. 1. The parametrium can be divided into four surgical directions based on the Leiden TNM classification. Ventral (V) 1: superficial layer of VUL; V2: medial part of the deep layer of VUL; V3: lateral part of the deep layer of VUL. Lateral (L) 1: medial to the ureter; L2: between ureter and pelvic side wall; L3: until the pelvic side wall. Caudal (C) 1: above the ureter; C2: above the deep uterine vein; C3: underneath the deep uterine vein. Dorsal (D) 1: between the uterus and rectum; D2: at the anterior rectal border; D3: halfway the rectal circumference. Note: the ureter (U) passes through the lateral, ventral and upper caudal parametrium. The uterine artery (Ut.a.) runs in the lateral parametrium, whereas the deep uterine vein (D.v.) proceeds in the lateral and caudal parametrium. Ut: uterus, B: bladder, R: rectum.

anti-mouse, P-0260, DAKO). Before visualization, these sections were rinsed twice with PBS and once with Tris/maleate (pH 7.6). Visualization was performed with 3-3'-diaminobenzidine tetrahydrochloride as a chromogen and hematoxylin was used as a counterstain. These sections were mounted with Entellan (1.07961.0100, Merck). Selected sections were double-stained with CD68 and LYVE-1 and incubated for 1 h with Alexa Fluor® 488 (donkey anti-rabbit, A-11055, Invitrogen) and Alexa Fluor® 647 (donkey anti-mouse IgG, A-31571, Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and sections were mounted in ProLong Gold (Molecular Probes, Life Technologies) after rinsing twice in PBS.

Image processing

A 3D reconstruction was made of the pelvic lymphatics and autonomic nerves, based on the fetus aged 14 weeks. Micrographs were made using an Olympus AX70-microscope with an Olympus D12 camera. One in twenty sections of the investigated area was used for the reconstruction, creating a cross-sectional interval of 160 µm. Amira® software package version 5.3.3 (Template Graphics Software; Visage Imaging, San Diego, California, USA) was used for the 2D labeling of anatomical structures and DeVIDE software [17] was used to create the actual 3D reconstruction. Fluorescent images were taken with an Olympus IX70 microscope equipped with a Leica DFC340 FX camera using LAS AF software (Leica).

Results

Development of the pelvic lymphatics

We recognized lymph vessels by endothelial LYVE-1 positivity and the absence of red blood cells and smooth muscle fibers.

In all fetuses, prominent lymphatic plexuses were seen around the common, internal and external iliac arteries and the obturator arteries. In the fetuses aged 10 and 10.5 weeks, extensive lymphatic plexuses were seen at the pelvic side walls, but real pathways were not detectable. In the 11th week, organ-draining pathways could be identified and developed rapidly into clear visible pathways. Lymph vessels from the cervix uteri were connected with those from the bladder and rectum before draining into the plexuses. In the 12th week, the ventro-dorsal connection between lymph vessels of the cervix uteri and rectum disappeared with the developing mesorectum and mesorectal fascia. From the 14th week onwards, an apparent horseshoe-shaped fascia was noticeable extending from the ventrolateral cervical angle towards the lateral parametrium (Fig. 2). It formed a continuous border between the bladder and cervix, while keeping the ureter and major nerve bundles branching from the IHP on its lateral side.

At the time this fascia was formed, there were no more connections detectable between bladder, cervix uteri and rectum. Instead, primarily medial-lateral pathways were detected that drained from the individual organs into the laterally situated plexuses. This fascia did not define any surgical parametrial extension, as it ran from the ventral parametrium (V1) throughout the lateral parametrium (L1) and disappeared in the latter.

In the fetuses aged 14 weeks or more, single cells with LYVE-1 positivity were seen. Double-staining with LYVE-1 and CD68 showed that these single cells were macrophages (Supplementary Fig. S3). Hence, we rejected the hypothesis that these cells could be early precursors of a lymphatic capillary network.

Lymphatic drainage pathways in the parametrium

Two major lymphatic pathways from the cervix uteri could be recognized in all embryonic stages from week 14th onwards. In the lateral parametrium (L1), a clear medial-lateral pathway draining the cervix uteri was visible crossing the ureter superiorly, being referred as the

supra-ureteral pathway (Fig. 4). This pathway was located within the superior part of the CL and ran along the uterine artery (L2). At the lateral side of the ureter, it fused with lymph vessels from the bladder just before draining into the plexuses located at the obturator fossa (L3).

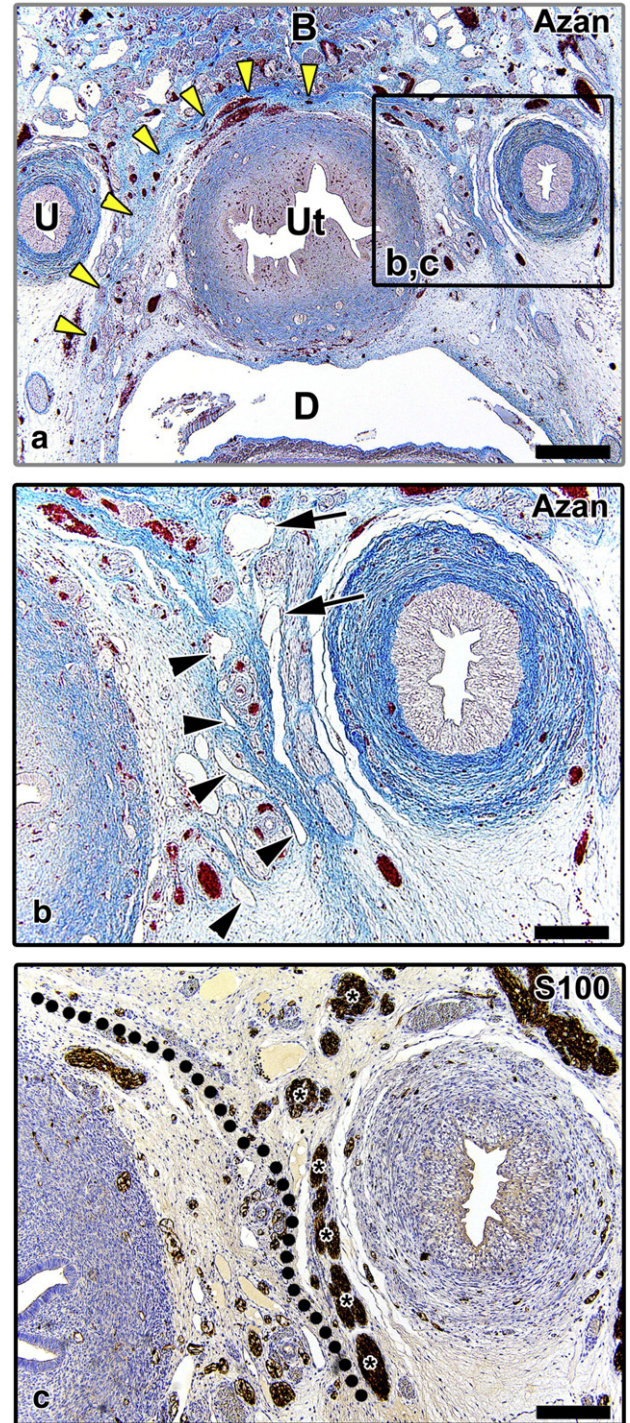


Fig. 2. Transverse azan stained (a, b) and S100 stained (c) adjacent sections of a female fetus aged 14 weeks at the level where the ureters (U) are lateral to the uterus (Ut). The yellow arrowheads (a) point out the horseshoe-shaped fascia. The black arrowheads in detail window (b) point out lymph vessels from the cervix uteri, whereas the black arrows show lymph vessels from the bladder. The fascia forms a border and keeps the nerves on its lateral side (dotted line in detail window (c)). Nerves are depicted by stars, D: pouch of Douglas, B: bladder. Scale bar overview 500 µm; detail 200 µm.

A second lymphatic pathway from the cervix uteri emerged in the dorsal parametrium (D1), coursing directly medial to the IHP and running in the USL towards the rectal pillars (D2, D3; Fig. 5). This pathway was no longer connected with the rectal lymphatic pathways. More superiorly, this so-called dorsal pathway had two directions: it fused with the supra-ureteral pathway, and in the most superior part of the USL some lymph vessels merged with lymph vessels draining the ureter (Supplementary Fig. S6).

In all studied fetuses from the 14th week onwards ($n = 6$), the VUL did not contain lymph vessels draining the cervix uteri (Supplementary Fig. S7). The lymph vessels found in the superficial (V1) and deep (V2, V3) layers of the VUL were all of bladder origin. A difference was seen in the number of lymph vessels in the VUL; the fetus of 15 weeks hardly contained lymph vessels in the superficial and deep layers of the VUL, whereas in the fetus aged 14 weeks clearly more lymph vessels were found. Drainage from the bladder occurred from its total circumference in a dorsolateral direction and lymph vessels mainly passed the ureters laterally. A minority passed the ureters medially, but did not connect with lymph vessels from the cervix in the VUL. These findings are represented three-dimensionally (Fig. 8) and an interactive PDF of the same model can be explored online: <http://graphics.tudelft.nl/pub/3Dreconstruction.pdf>.

The deep layers of the VUL contained a few lymph vessels from the upper vagina as well. These lymph vessels became less prominent during embryonic development. In the 11th and 12th week, a small pathway was found running from the anterolateral upper vagina towards the lymph plexuses at the pelvic side wall. When the VUL gained more volume in later embryonic stages, this pathway was diminished to a small lymph vessel directly attached to the ventrolateral angle of the upper vagina (C2) running in a dorsolateral direction and fusing with the supra-ureteral pathway. Moreover, the most important drainage pathway from the upper vagina ran from its dorsolateral angle towards the USL. In the lateral paracolpal parametrium (C3), this

pathway fused with lymph vessels from the bladder forming a ventro-dorsal connection (Fig. 9). Superiorly, such a connection was not detectable, because the above described fascia formed a boundary.

Discussion

So far, studies on patterns of lymphatic spread of cervical cancer mainly focused on detecting solid parametrial and pelvic lymph nodes. One of the studies that contributed the most was conducted by Benedetti-Panici and colleagues, who microscopically examined 109 giant section specimens of patients with early-stage and locally advanced cervical cancer [14]. The study demonstrated the presence of metastatic and non-metastatic parametrial lymph nodes in the superficial and deep layers of the VUL, the USL and the distal part of the lateral parametrium. Although this confirmed that paracervical tissue forms a major route for lymphatic spread of cancer, the study did not reveal any specific organ-draining lymphatic pathways. Hence, the clinical question on the extent of parametrectomy cannot be resolved by their study.

Ercoli and colleagues recently studied the paracervical lymphatic pathways by injection of Lipiodol dye into 18 cadaveric cervixes and distinguished a supra-ureteral, infra-ureteral and neural pathway in respectively 96%, 22% and 7% of the cases [18]. We confirmed microscopically the presence of a supra-ureteral pathway. The neural pathway could be in agreement with the lymph vessels draining from the ventro- and dorsolateral angle of the upper vagina, but we could not identify an infra-ureteral pathway in all fetuses. In the fetus aged 12 weeks, a pathway was observed running inferior to the ureter to the obturator fossa. However, in the elder fetuses this could not be confirmed. The developing mesoureter expanding between the ureter and USL could function as a border where lymphatics in a certain embryonic stage cannot run through anymore. Perhaps this pathway could be an anatomical variation considering the identification with Lipiodol dye

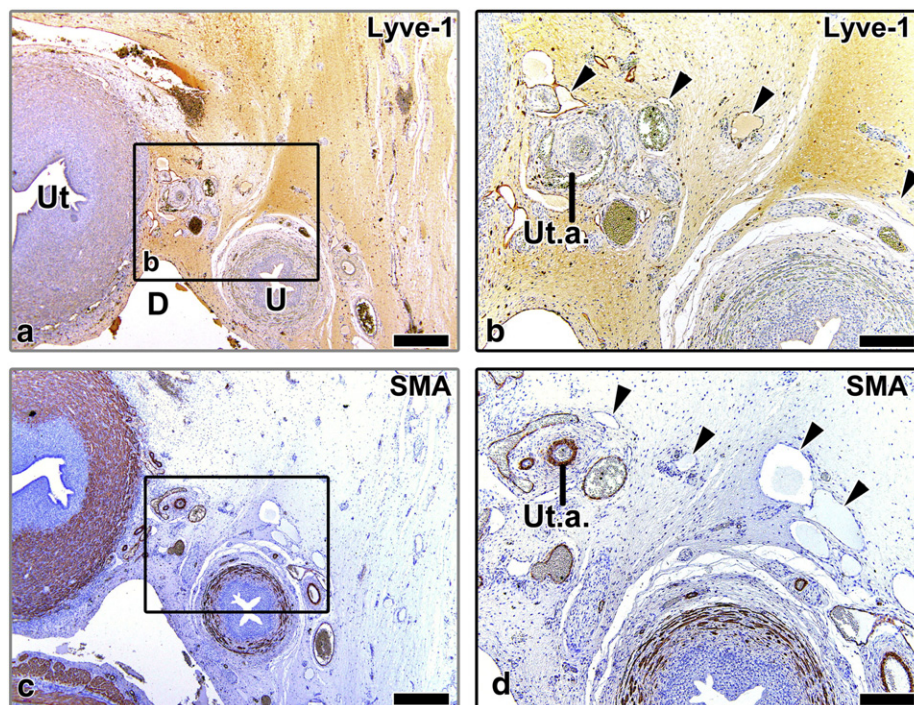


Fig. 4. Transverse LYVE-1 stained (a, b) and SMA stained (c, d) adjacent sections of female fetus aged 14 weeks at the level where the ureters (U) are dorsolateral to the uterus (Ut). Panel b is a detail window from overview panel a. The black arrowheads in panel b show lymph vessels around the uterine artery (Ut.a.) crossing the ureter superiorly. Panel d is a detail window from overview panel c. The black arrowheads show that lymph vessels do not contain smooth muscle fibers. D: pouch of Douglas. Scale bar overview 500 μ m; detail 200 μ m.

in a minority of the cadavers. A dorsal pathway was surprisingly not visualized by Ercoli and colleagues.

Removal of the lateral parametrium is uniformly performed during RHL in early-stage cervical cancer, but the extent of resection of the ventral, caudal and dorsal parametrium is still questioned. Several nerve sparing techniques have been developed differing mainly in the resection of the USL and VUL [19,20]. Höckel developed the concept of total mesometrial resection (TMMR), where the uterus is removed based on an embryologically defined ontogenetic unit. The deep layers of the VUL are not removed in a TMMR, but the USL are widely extirpated while preserving the IHP [21–23]. Höckel did not show that one of the two lymphatic drainage pathways from the cervix uteri runs in the USL. To the best of our knowledge, we are the first to immunohistochemically confirm the presence of lymph vessels draining the cervix uteri in the USL. Apart from that, we showed that this pathway has connections with lymph vessels draining the ureter, revealing the course of lymphatics throughout two distinct morphogenetic units.

Specifically, resection of the deep layer of the VUL is on debate. While the Okabayashi technique is ultra-radical in removing the ventral and caudal parametrium, the Wertheim–Meigs and TMMR variant are less radical at this point. Surgical damage of autonomic nerves running in the ventrocaudal parametrium/VUL in close relationship to the pelvic organs has proven to play a major role in post-operative dysfunctions [24,25]. In all studied fetuses, we could not detect lymph vessels draining the cervix uteri in the deep layers of the VUL. The lymphatic pathway draining the upper vagina runs in the paracolpal tissue and theoretically one can state that this should be removed as well. However, the appearance of this pathway was very small and diminished during embryonic development. In daily clinical practice, this pathway should be automatically included in any RHL as the en-bloc specimen contains the upper vagina and the surrounding vaginal cuff. Hence, the more favorable recurrence and survival rates of the Okabayashi technique [7,8] cannot be explained by the presence of lymphatic pathways in the deep layers of the VUL. A possible explanation for the better surgical outcome using this technique could be the more extended pelvic lymphadenectomy in comparison to less radical procedures. Another hypothesis is that the connected lymph vessels of bladder and upper vagina cause retrograde metastasis. Occlusion of upper lymphatic drainage pathways resulting from advanced lymphatic metastasis could cause lymphatic flow regurgitation [26] and enhance retrograde lymphatic spread. Finally, direct tumor extension, as can be seen in the lateral parametrium, could also be present in this posterior layer of the VUL. The latter indicates a possible benefit of removing the deep layers of the VUL.

Lymphatic pathways in the parametrium have never been studied immunohistochemically. Based on a recent wave of discoveries of lymphatic endothelial markers [27], substantial progress in the understanding of the development and molecular mechanisms controlling the lymphatic system has been made during the last few years. Studying

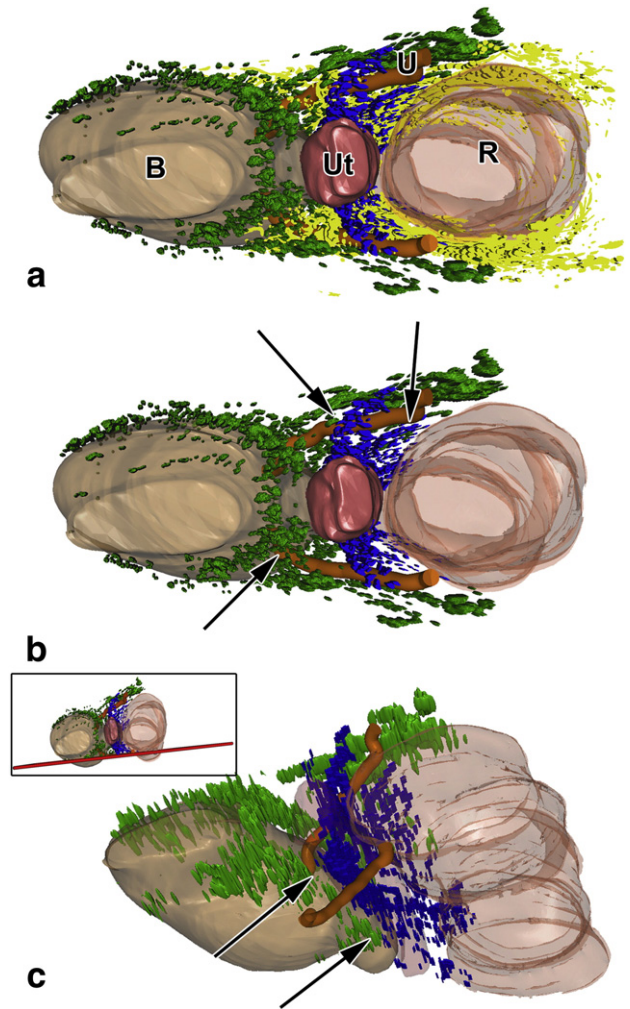


Fig. 8. A 3D reconstruction of a female fetus aged 14 weeks showing the lymph vessels from the bladder (green), lymph vessels from the cervix uteri (blue) in relationship to the autonomic nerves (yellow) and pelvic organs. Panels a and b show a superior view on the lymphatics from the bladder (B) and rectum (R), revealing a supra-ureteral pathway (upper left arrow) and a dorsal pathway (upper right arrow). The VUL contains only lymph vessels from the bladder (lower arrow). Panel c offers a lateral view (sliced from 3D model, as is shown in upper left window). There is no connection between lymphatics from the bladder and cervix uteri (Ut) in the VUL (upper arrow). Laterally from the ureter, lymph vessels from the bladder fuse with those from the supra-ureteral pathway (lower arrow).

the pelvic lymphatic system in embryos has its limitations though. Accurate knowledge of the normal development of the human lymphatic system is necessary to interpret the results. LYVE-1 is a well-

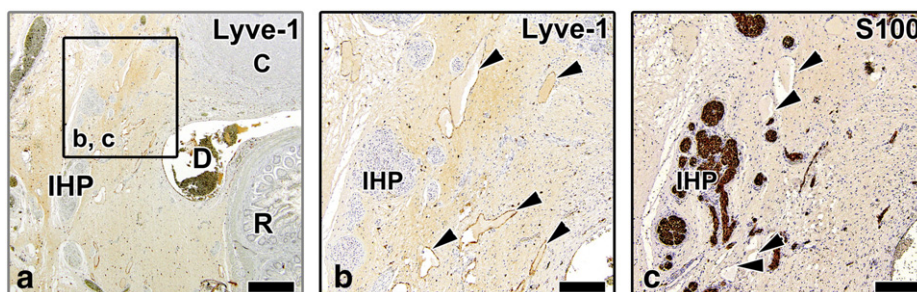


Fig. 5. Transverse LYVE-1 stained (a, b) and S100 stained (c) adjacent stained sections of a female fetus aged 15 weeks at the level where the inferior hypogastric plexus (IHP) is situated laterally to the cervix uteri (C) and rectum (R). Panel b is a detail window from overview panel a, showing the lymph vessels (black arrowheads) in the dorsal parametrium. The black arrowheads in panel c show that the dorsal pathway is situated medial to the IHP. Nerves are brown, D: pouch of Douglas, R: rectum. Scale bar overview 500 μ m; detail 200 μ m.

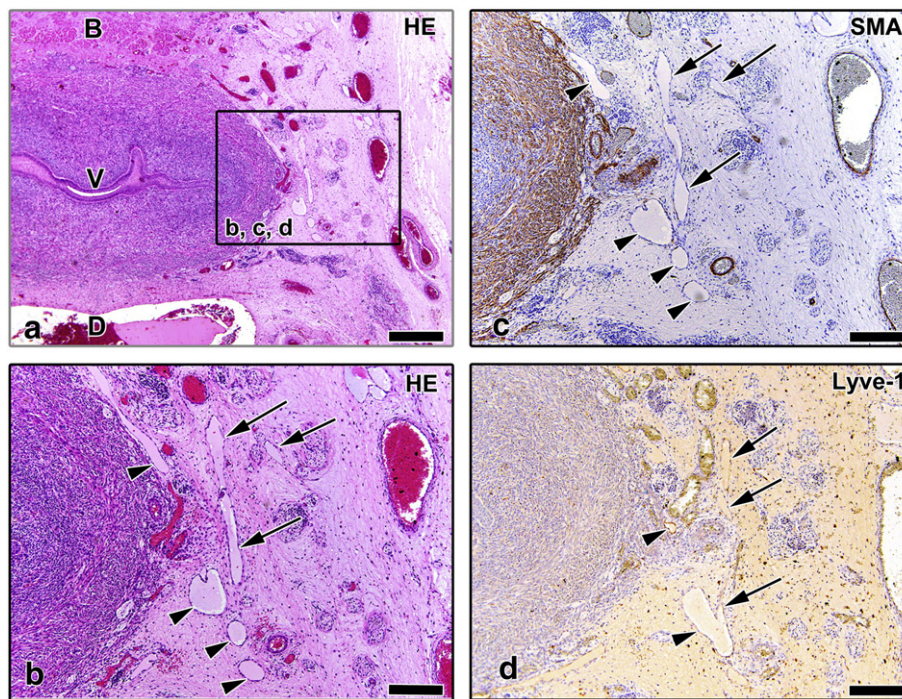


Fig. 9. Transverse HE stained (a, b), SMA stained (c) and LYVE-1 (d) stained adjacent sections of a female fetus aged 15 weeks at the level of the upper vagina (V). At the level of the upper vagina, lymph vessels from the bladder depicted with the black arrows (b, c, d) flow into the lymph vessels from the upper vagina which are pointed out by the black arrowheads (b, c, d). B: bladder, D: pouch of Douglas. Scale bar overview 500 μ m; detail 200 μ m.

known marker to identify lymphatic endothelial cells (LECs). In early embryonic stages veins might be LYVE-1 positive as well, given the fact that the lymphatic system develops out of the venous system [28]. However, in the 5th week LYVE-1 positivity shifts to solely LECs [29]. LECs remain LYVE-1 positive during development [27], but the expression might be diminished as the capillary lymphatic plexus matures to collecting lymphatic vessels [30]. Furthermore, LYVE-1 can be also expressed in tissue macrophages [31], as we showed in our fetuses as well. Both limitations in the use of LYVE-1 to recognize the different developmental phases in the formation to a mature lymphatic network emphasize the importance to conduct a similar immunohistochemical study on adult pelvis. Although we consistently found a uniform distribution pattern of lymphatic drainage pathways from the cervix uteri towards the pelvic plexuses in all fetuses aged 14 to 24 weeks, significant changes could occur in later developmental stages or even under pathological circumstances. Moreover, it is highly important to examine the presence of the horseshoe-shaped fascia in adult pelvis.

In conclusion, we have identified a supra-ureteral pathway and a dorsal pathway as major lymphatic drainage pathways of the cervix uteri in fetal pelvis. The superficial and deep layers of the VUL did not contain lymph vessels draining from the cervix uteri. Thus, the favorable survival outcomes of the Okabayashi technique cannot be explained by radical removal of lymphatic pathways in the ventrocaudal parametrium. Based on our findings, we might postulate that a V1C2L3D3 resection would be sufficient in early-stage cervical cancer. Currently we stick to the traditional Okabayashi technique in larger tumors, because in these cases direct tumor extension into the deep ventral parametrium (V2, V3) might be clinically important. Nevertheless, 3D exploration of the human fetal anatomy requires accurate verification in human adult tissue. Forthcoming immunohistochemical studies should be focused on evaluating the presence of lymphatics in the deep ventral parametrium (V2, V3) in human adult tissue. The infra-ureteral pathway, defined by Ercoli and colleagues but not confirmed in our study, must be analyzed as well. Moreover, studies should be focused on elucidating the anatomical ground of Okabayashi's technique's favorable results. Once

our results have been verified in human adult tissue, appropriate modification of parametrectomy during RHL would be clinically justified.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ygyno.2013.10.030>.

Conflict of interest

We declare no conflicts of interest.

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