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The Lymphatic System and Pancreatic Cancer

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Abstract

This review summarizes current knowledge of the biology, pathology and clinical understanding of lymphatic invasion and metastasis in pancreatic cancer. We discuss the clinical and biological consequences of lymphatic invasion and metastasis, including paraneoplastic effects on immune responses and consider the possible benefit of therapies to treat tumors that are localized to lymphatics. A review of current techniques and methods to study interactions between tumors and lymphatics is presented.

Keywords

Lymphatics; Lymph node; Pancreatic Cancer

Introduction

As the fourth leading cause of cancer-related deaths (a five-year survival rate of less than 7%), pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancers in the United States [1]. Worldwide, PDAC results in over 330,000 deaths annually [2]. Unlike the stable or decreasing trends of incidence and death rates for other cancers, the incidence of PDAC continues to rise, underscoring the critical need for new and effective therapies for this disease [1]. Unfortunately, most PDAC cases are diagnosed when the primary tumor has already spread to regional and distant locations [1] eliminating surgery as a curative treatment option. Cancer metastasis into and through the lymphatic vasculature and lymph nodes occurs frequently in PDAC patients [3–5] and is strongly correlated with poor prognosis [6–9]. Evaluating lymph node status has been proven to be a significant factor when determining therapy selection for cancer patients [10–12].

The lymphatic vasculature offers the most direct route from the primary tumor to the frequentlyinvaded draining lymph nodes during PDAC metastasis. The lymphatic system is responsible for maintenance of tissue fluid homeostasis, absorption of dietary fat, and leukocyte and antigen transport from tissues to lymph nodes for the initiation of immune responses [13–15]. Originating in nearly all vascularized tissues, blind-ended lymphatic

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capillaries, or initial lymphatics, are specialized for the uptake of interstitial fluids, macromolecules, and leukocytes. They are composed of a single layer of endothelial cells with discontinuous intercellular junctions and lack a basement membrane [16,17]. The endothelial membrane of the initial lymphatics is attached to the extracellular matrix (ECM) *via* anchoring filaments, which facilitate the opening of the lymphatic lumen during increased interstitial fluid pressure [18,19]. Upon entry into the lymphatic capillaries, lymph and its macromolecular and cellular contents are transported to larger pre-collecting lymphatic vessels and then to collecting vessels, composed of not just the endothelial layer but also smooth muscles to facilitate flow and bi-leaflet valves to prevent backflow [20–22]. The afferent collecting lymphatics enter the lymph nodes where the lymph is filtered, and upon exiting the lymph nodes through the efferent collecting vessels, the lymph passes through the major trunks of the lymphatic system, the thoracic duct and the right lymphatic trunk, and is then returned to the circulatory system [14,23].

The network of lymphatic vasculature and lymph nodes responsible for draining the pancreas is quite complex. In the normal pancreas, the lymphatic vessels are typically located near blood vessels and are often found in the interlobular spaces of the pancreas [24]. Classification of pancreatic nodes has not been uniformly standardized, although pancreatic lymph nodes are generally divided into regions based upon their location around the pancreas and the areas of drainage of the pancreas: head/neck, body/tail, left side, or right side (reviewed in [25,26]). Studies correlating primary tumor location and lymph node involvement following resection have helped to identify the regional patterns and probabilities of lymph node metastasis, but more analysis will need to done for consistent accurate prediction of lymph node involvement [27–30].

Although clinicians and researchers understand the importance of lymphatic invasion and lymph node involvement for pancreatic cancer patient prognosis and therapy selection, the biological processes that govern lymphatic invasion and metastasis remain under-studied. For example, there is currently disagreement within the field as to whether lymphatic vessel expansion at the primary tumor site and draining lymph node is necessary for lymph node metastasis. Also, it has not been conclusively determined whether metastasis to the lymph nodes is a sequential step in distant organ spread or a final destination for tumor cells to promote immunosuppression. The potential role of lymphatics supporting immune suppression has led to questions of how normal and tumor-associated lymphatic endothelia may contribute to immune modulation within the tumor microenvironment and invaded lymph nodes either through trafficking functions or direct interactions with immune cells. These and many more questions have yet to be fully explained: how does the lymphatic endothelium regulate the entry of tumor cells into vessels; how do tumor cells evade immune cell recognition within lymphatic vessels and lymph nodes; what are the therapeutic implications of targeting lymphangiogenesis or other lymphatic-directed functions in patients with PDAC? This review summarizes our current knowledge of the role of the lymphatic system in pancreatic cancer progression and metastasis and examines research techniques and clinical procedures used in this field of study. A better understanding of the processes of lymphatic invasion and lymph node metastasis in PDAC will significantly contribute to our overall understanding of this deadly disease and provide the groundwork for the development of novel efficacious therapies.

Pancreatic Tumor Resection and Lymphadenectomy

Surgical resection of pancreatic adenocarcinoma was first brought to clinical practice by Walther Kausch in Berlin in 1909 [31]. Beginning in the mid-1930s, American surgeon Allen O. Whipple further employed and modified the pancreat(ic)oduodenectomy (PD) procedure that would bear his name; he eventually condensed the surgery into a single operation, the first of which was successfully performed in 1940 [32]. In a traditional PD the head of the pancreas is removed along with the duodenum, gall bladder, and end of the common bile duct [27,33–42]. Several timely surgical advances facilitated increased success of the PD as performed by Whipple and his contemporaries including the first successful duodenectomy in a canine, the discovery that direct immediate restoration of biliary and pancreatic secretions into the gastrointestinal tract was not necessary for survival of patients, and the use of non-dissolvable silk suture rather than the more temporary catgut [32,43]. Additional scientific breakthroughs critical for decreased perioperative morbidity and mortality included the discovery and synthesis of vitamin K, the discovery of insulin, and the description of human blood types and subsequent establishment of blood banks [32,43]. Today, a broad range of similar pancreatic resection procedures are in use in modern surgical practices around the world. Differences in primary tumor placement within the pancreas—head/neck vs. body/tail—and tumor invasion into surrounding tissues and organs often necessitate customization of resection [44–55] beyond the traditional PD to such procedures as distal pancreatectomy with or without splenectomy [41,56], pancreaticogastrostomy [35], pylorus-preserving PD [37,38,40], pylorus-resecting PD [40], subtotal stomach-preserving PD, pancreatojejunostomy, duodenum-preserving head resection, wedge resection of inferior vena cava, and total [39] or regional [57] pancreatectomy [58,59].

As with surgical treatment of other malignancies, one of the most controversial aspects of modern pancreatic ductal adenocarcinoma resection has been the extent to which surrounding connective tissue and lymph nodes should be removed. Evidence suggests that metastasis to lymph nodes is an early event in pancreatic cancer progression, and presence of tumor cells in lymph nodes represents one of the most negative prognostic factors with respect to patient outcomes [8,27,29,37,60–63]. Conservative surgical views support the standard PD with loco-regional lymphadenectomy [27,31,34,36,38,42,56,59,64–74], while others, most notably numerous Japanese groups, advocate that a more radical PD with extensive removal of retroperitoneal soft tissue and extended lymphadenectomy [27,34,39,42,75–84] results in better patient outcomes. Collected studies in Table 1 [8,27,31,34,36,37,39,40,42,59,64–75,77–99] demonstrate the broad range of study designs and conclusions that have fueled this debate. A recent set of randomized, controlled clinical trials from several centers around the world and a mathematical model of outcomes prediction have concluded that extended lymphadenectomy does not improve survival over traditional, more conservative resection and that quality-of-life may be decreased with more radical surgery [67–70,72,74,81,83,87,88,95]. Leading international surgical groups have also applied their expertise to the ongoing conversation in this field. They have recently identified lymph node stations to be included in standard lymphadenectomy for head (5, 6, 8a, 12b1, 12b2, 12c, 13a, 3b, 14a, 14b, 17a, and 17b) and body/tail (10, 11, 18) pancreatic cancer resections [94] and have released recommendations suggesting discontinued use of

extended lymphadenectomy for treatment of PDAC [34,73,94]. The occasional case report continues to demonstrate the biological diversity of pancreatic malignancy and challenge these recommendations. In 2013 a Japanese group reported that extended lymphadenectomy in a welldifferentiated, chemotherapy-responsive PDAC with para-aortic lymph node metastases resulted in patient survival of over ten years [75]. Peparini, *et al.* 2015 [93], addressed para-aortic lymph node involvement (stations 16a2 and 16b1), stating that involvement of these lymph nodes may be due to direct invasion of the primary pancreatic tumor as opposed to dissemination and seeding of migratory cancer cells in the traditional definition of metastasis and that their removal may favorably impact R margin status. Clinical trials and expert consensus recommendations consistently recommend standard lymphadenectomy over more radical resection strategies, but, as each case of pancreatic cancer is truly a unique disease, circumstances in which extended lymph node removal is beneficial may be more clearly defined in the future.

Outcomes Prediction: Lymphatic-Specific Metrics

Outcomes prediction for pancreatic cancer patients has traditionally been based on stage classification according to the TNM (tumor, node, metastasis) system at diagnosis [100]. Pancreatic cancer is rarely diagnosed in a pre-metastatic state. Comprehensive examination of lymph node and lymphatic vessel involvement would provide clinicians with important information about the progression characteristics of an individual patient's tumor such as its pattern and route of spread, likelihood of local/distant recurrence, and potential immunomodulatory effects. Four outcomes predictive metrics specifically address lymph node/lymphatic vessel involvement: lymph node disease (LND), lymph node burden (LNB), lymph node ratio (LNR), and lymphatic vessel invasion (LVI). Each of these measures provides distinct information regarding disease pathology and may be useful in refining prognoses. LND is defined as the confirmed presence of metastatic tumor cells in at least one lymph node. The total number of positive lymph nodes confirmed at resection constitutes LNB. LNR is the ratio of the number of positive nodes to the total number of nodes examined [101]. LNR has been shown to be an effective tool to further stratify the TNM stage N1 patient population for outcomes prediction while decreasing likelihood of understaging and stage migration [102–104]. LVI may refer to lymphatic vessel invasion as determined by immunohistochemical staining of tissue sections or more broadly, to lymphovascular invasion, which may or may not distinguish between invaded hematogenous and lymphogenous vessels [63,105]. The relative prognostic value of each of these lymph node-/lymphatic vasculature-specific metrics is controversial. Prospective and retrospective clinical studies evaluating the utility of these criteria are collected in Table 2 [6,33,38,44,51,62,63,101–104,106–114]. Contradictory conclusions from these studies highlight the remaining need for additional work before use of these metrics is informative in the general clinical setting.

A major challenge in pancreatic cancer biology and treatment is the presence in lymph nodes of single or small clusters of tumor cells that are not detected by routine histopathological staining techniques [76,115–118]. Similar to insufficient surgical removal of primary lymph nodes [84,102–104,112,113], failure to detect occult micrometastatic deposits in nodes may result in patient misclassification, understaging, and improperly

informed outcomes prediction. Descriptions of “skip metastases” in which primary lymph nodes are negative but metastases are established in secondary nodes or distant organ sites [68,119,120] may be attributable, at least in part, to this phenomenon. Emerging immunohistochemical and molecular techniques such as epithelial cell adhesion molecule (EpCAM/BerEP4) [115,116], cytokeratin [76,117], and CA19-9 staining [117], and polymerase chain reaction for mutant K-Ras [118] have demonstrated efficacy in occult tumor cell detection in nodes, but their adaptation from the research laboratory to practical clinical application will require more extensive study.

Lymphangiogenesis

Signaling and Regulation

While clearly akin to angiogenesis in many regards, progress to define the process of lymphangiogenesis has revealed distinct molecular mechanisms that direct its inception, regulation, and roles in inflammatory disease and malignancy. Like angiogenesis, new lymphatic vessel growth can be directed by many growth factors and regulated by intra- and extracellular signaling mechanisms. Primary growth factors associated with lymphangiogenesis include vascular endothelial growth factor-A (VEGF-A), -C, and -D signaling *via* vascular endothelial growth factor receptor-2 (VEGFR-2) and -3 and neuropilin-2 (Nrp-2) [121–126], and angiopoietin-1 (Ang-1) and -2 signaling through receptor Tie-2 [127,128].

Recent evidence shows that in addition to the VEGFs and angiopoietins, several other chemical messengers are also capable of directly or indirectly inducing lymphangiogenesis *in vitro* and/or *in vivo* in experimental model systems. Such mediators include growth factors fibroblast growth factor-2 (FGF-2) [129], platelet-derived growth factor-BB (PDGF-BB) [130,131], nerve growth factor (NGF) [132], insulin-like growth factor-1 (IGF-1) and -2 [133], and hepatocyte growth factor (HGF) [134]; inflammatory cytokines interleukin-1 β (IL-1β) [135,136] and tumor necrosis factor- α (TNF-α) [135–137]; and other non-traditional signaling molecules lipid sphingosine-1-phosphate [138], cyclooxygenase 2 (Cox2), and EP3/4 [139]. A role for integrins in lymphangiogenesis is also emerging with evidence of binding of VEGF-A, -C, and -D to lymphatic endothelial-specific integrin-α9β1 [140,141]. While each of these factors does induce new lymphatic vessel growth, not all lymphangiogenesis is created equal; a study of corneal lymphangiogenesis in response to VEGF-A, VEGF-C, or FGF-2-loaded micropellets has revealed differences in both structure and function of lymphatic vessels and the proportion of blood to lymphatic vessels induced by these growth factors [142]. In the case of indirect stimulation of lymphangiogenesis, paracrine signals such as IL-1β [135,136] and TNF-α [135–137] can drive increased expression of the VEGFs, most notably VEGF-C. This likely occurs through activation of the NFκB promoter to induce VEGF-C expression [137]. Other indirect inducers of lymphangiogenesis include Cox2 and EP3/4, which may increase expression of VEGF-C and -D to modulate cell growth during inflammation [139], and NGF, which increased expression of VEGF-C, but not VEGF-A, in a mouse corneal model of lymphangiogenesis [132]. Some studies have attributed the secondary production of VEGFs, specifically VEGF-C, to an infiltrating macrophage population during periods of inflammation and malignancy

[135,138,143,144]. Huang, *et al.*, have also identified B cells and dendritic cells (DCs) as candidate immune cell populations that may secrete VEGF-A, -C, and -D to influence lymphatic vessel organization and growth [138]. B cells have also been shown to modulate lymphangiogenesis within lymph nodes in the context of tissue inflammation following experimental immunization [145]. In addition to their role in secretion of lymphangiogenic growth factors, Hall, *et al.*, have shown that tissue macrophages may directly contribute to new lymphatic vessel growth by transdifferentiation into a lymphatic endothelial progenitor-like phenotype and incorporation into growing vessels [146]. The roles of tumor-associated macrophages (TAMs) in lymphangiogenesis in the tumor microenvironment are discussed in more detail below.

Lymphangiogenesis is further controlled by regulation of growth factor and cytokine receptors on the lymphatic endothelial surface. Gene expression of VEGFR-1, -2, and -3 and Nrp-1, and -2 is regulated by transcription factors GATA-binding protein 2 (GATA2) and LIM domain only 2 (Lmo2) to influence both angiogenesis and lymphangiogenesis [147]. Another transcription factor, COUP transcription factor 2 (COUP-TFII), increases expression of Nrp-2 to augment VEGF-C signaling [148]. VEGFR-2 and -3 signaling is further modulated by Bone marrow kinase in X chromosome (BMX) following its upregulation upon VEGF-A stimulation of lymphatic endothelial cells (LECs) [149]. H-, N-, and K-Ras can also regulate VEGFR-3 signaling by inducing the up- or downregulation of that receptor [150]. Another mechanism of VEGFR-3 pathway signaling regulation in LECs is the IL-1β-dependent induction of miRNA-1236 [151] and the molecular scaffolding protein ASK-1 interacting protein-1 (AIP-1) [152]. The Slit2-Robo4 signaling axis has been shown to regulate surrogate lymphangiogenesis behaviors in lung LECs in culture by modulating VEGF-C/VEGFR-3 pathway signaling [153]. NFκB pathway signaling has been shown to further modulate inflammatory lymphangiogenesis by upregulating prospero-related homeobox-1 (Prox-1) and VEGFR-3 in a mouse model of peritonitis [138].

Tumor-associated Lymphangiogenesis

The discussion above has primarily focused on the regulation of inflammatory lymphangiogenesis typical of an injury or infection. A related, but in many ways physiologically distinct process, is that of tumor-associated lymphangiogenesis (TALA). Factors elaborated by tumor cells and other supporting cell types of the tumor microenvironment, such as cancer-associated fibroblasts (CAFs), TAMs, and DCs, interact with cognate receptors on the lymphatic endothelium both locally and in lymph nodes to influence lymphangiogenesis, lymph node metastasis, and tumor progression. Studies of human pancreatic cancer tissues have identified a role for TALA in lymph node metastasis and patient outcomes. Kurahara, *et al.* [154], found that high lymphatic vessel density (LVD) in PDAC head tumors predicted increased lymph node metastasis and decreased survival. They also showed increased LVD within metastatic lymph nodes [154]. Wang, *et al.*, found that increased peritumoral LVD in human pancreatic carcinoma tissues correlated with unfavorable tumor differentiation status, increased LVI, and more lymph node metastasis, while this was not the case for intratumoral LVD [53]. These data highlight the importance of peripancreatic lymphatics in the progression and metastasis of pancreatic cancer and their potential utility as both a predictor of patient outcomes and a possible therapeutic target.

As in inflammatory lymphangiogenesis, the VEGF-C/-D signaling pathways appear to play an important role in TALA, although the exact mechanisms of their activity remain somewhat less clear. Kurahara, *et al.,* found increased VEGF-C and -D expression in patient PDAC tumor margins compared to the tumor interior, and they reported that high VEGF-C and -D expression in tumor margins correlated with increased LVI (VEGF-C) and lymph node metastasis (VEGF-C and -D) and decreased five-year survival; expression levels of these proteins did not correlate with either hematogenous invasion or distant metastasis [48]. A similar study of patient samples also showed increased VEGF-C and -D immunostaining at pancreatic adenocarcinoma tumor margins that correlated with increased LVD, lymphatic and blood vessel invasion, lymph node metastasis, and overall survival [155]. Von Marschall, *et al.,* corroborated these findings with their evidence of increased VEGF-D and VEGFR-3 expression in human PDAC tissue and of increased LVI, presence of intra- and peritumoral lymphatics, and lymph node metastasis [156]. Deletion of VEGF-D in mice resulted in impaired peritumoral lymphangiogenesis and decreased lymph node metastasis while having no effect on lymphatic development or inflammatory lymphangiogenesis suggesting a tumor microenvironment-specific role for VEGF-D signaling [157]. In a Rip1Tag2 model of pancreatic β-cell carcinogenesis, Kopfstein, *et al.*, showed that VEGF-D expression in these tumors induced peritumoral lymphangiogenesis and lymph node and lung metastases [158]; a very similar study examining the role of VEGF-C in this context found increased lymphangiogenesis and lymph node metastasis but not distant metastases [159]. In an orthotopic PDAC model, treatment with anti-VEGF-C shRNA decreased tumoral LVD and inhibited tumor growth [160]. A role for microRNAs may also exist in the regulation of pancreatic TALA. Keklikoglou, *et al.,* recently described a mechanism of regulation of VEGF-C production in PDAC cells by miR-206. They showed that, in addition to regulating K-Ras and annexin-A2 gene expression, restoration of miR-206 expression blocked tumor-associated angiogenesis and lymphangiogenesis, and its overexpression in pancreatic cancer cell lines disrupted the cell cycle restricting proliferation, impaired migration and invasion *in vitro*,and delayed tumor xenograft growth *in vivo* [161]. While the role of hypoxia in lymphangiogenesis remains unclear, HIF-1α expression has been shown to correlate with VEGF-C expression in PDAC of the pancreatic head and may be responsible for increased lymphangiogenesis and LN metastasis [57]. Contrary to these studies, Sipos, *et al.*, examined the expression levels of lymphangiogenic factors, LVD, and effects on lymph node metastasis in human PDAC and orthotopic PDAC mouse models and found that VEGF-C and -D were not overexpressed in tumor tissues and that LVD within tumors was decreased while peritumoral LVD was increased. They found no correlation between LVD or expression of VEGF-C or -D and rate of lymph node metastasis or patient outcomes and concluded that PDAC metastasis is independent of lymphangiogenesis [162].

In vitro experiments have examined the effects of tumor-secreted VEGF-C on LEC surrogate lymphangiogenesis behaviors. Supernatant from a high VEGF-C-secreting cell line, MiaPaCa-2, increased LEC migration, and MiaPaCa-2 co-culture with LECs increased LEC tubulogenesis [163]. These effects may be dependent on KAI-1 regulation as overexpression of that gene in MiaPaCa-2 resulted in decreased VEGF-C secretion, lymphangiogenesis, and lymph node metastasis [164]. Re-expression of tumor suppressor p16 in a MiaPaCa-2 orthotopic model had no effect on levels of VEGF-C or -D, but

nevertheless resulted in decreased lymphangiogenesis, LVD, and lymph node metastasis suggesting an alternate mechanism of regulation [165].

Overall, many studies examining the relationships among VEGF-C/-D expression and lymphatic-related phenotypes have found that high VEGF-C/-D levels correlate with increased lymphangiogenesis, lymphatic vessel invasion, and lymph node metastasis (or their surrogate *in vitro* counterpart behaviors). Whether a direct pathway can be drawn from tumor-associated lymphangiogenesis, to tumor cell invasion into lymphatic vessels, to tumor cell trafficking to lymph nodes, to establishment of lymph node metastases, to tumor cell exit of the lymph node by blood or lymphatic vessels and seeding of metastases at distant sites, to direct effects on patient outcomes is still unclear. Some of the studies we have discussed have supported portions of this pathway from lymphangiogenesis to distant metastases, but other data suggest that disease progression does not necessarily follow this linear sequence—*i.e.* the concept that lymphangiogenesis may not be required for lymphatic vessel invasion due to entry into pre-existing lymphatics, or the possibility of trafficking of tumor cells to lymph nodes through blood vessels, or the results from Sipos, *et al.* [162], showing that lymph node metastasis and patient prognosis are *not* linked to VEGF-C/-D levels. Also complicating this discussion is the fact that tumor cells may themselves respond to VEGF-C/-D signals in an autocrine manner further influencing their metastatic behaviors. Additional studies to systematically dissect each of the biological components of this proposed metastatic pathway are needed to concretely define their connections and contributions to disease progression.

Traditional neural signaling molecules also act to influence lymphatic vessel biology in the tumor microenvironment. Suppression of neural cell adhesion molecule (NCAM) induced VEGF-C and -D expression resulting in increased lymphangiogenesis and lymph node metastasis in the Rip1Tag2 mouse model [166], while the presence of NCAM expression in pancreatic cancer tissues from patients correlated with better prognosis [167]. In another example derived from the Rip1Tag2 model, Slit2 induced Robo1 in LECs to increase lymphangiogenesis and lymph node metastasis [168]. As previously mentioned, signaling of Slit2 through another receptor, Robo4, may also influence lymphangiogenesis behaviors such as growth, migration, and tubulogenesis, by modulating VEGF-C/VEGFR-3 signaling [153]. Nrp-2, a classical semaphorin receptor and VEGF pathway co-receptor, has also been shown to be a key regulator of TALA. It is expressed on intra- and peritumoral lymphatic vessels and lymph nodes; blocking its function *in vivo* decreased TALA, impaired tumor-associated LV function, and reduced lymph node and distant metastases [169]. These effects may be the result of impaired lymphatic sprouting [170]. Nrp-1 and -2 are also expressed on pancreatic tumor cells themselves [171,172]. In a model of colorectal cancer, TALA was stimulated by upregulation of Nrp-2 in LECs, and LVD correlated with the level of Nrp-2 expression; this Nrp-2 induction was mediated by integrin-α9β1 signaling in a VEGF-C/VEGFR-3 pathway-independent manner [173].

Other signaling pathways have also been implicated in regulation of TALA. In a mouse model of pancreatic β-cell carcinoma, both Ang-1 and -2 induced peritumoral lymphangiogenesis, but this new lymphatic vessel growth did not result in increased metastasis to either local lymph nodes or distant sites [174]. Ang-2 expression in orthotopic

PDAC xenografts resulted in increased LVD and lymphatic metastasis, and high levels of Ang-2 in patient serum samples correlated with lymph node metastasis and decreased survival. In MiaPaCa-2 cells, Ang-2 altered message levels of cytoskeletal and motility pathway molecules as well as decreasing expression of tumor suppressor genes [175]. The transforming growth factor- β (TGF-β) pathway may also be involved in TALA as expression of endoglin on intra- and peritumoral blood and lymphatic vessels in PDAC correlated with poor patient prognosis [176].

PDAC Invasion of Lymphatic Vessels and Metastasis to the Lymph Nodes

Background

Lymphatic vessel invasion and subsequent metastasis to the lymph nodes are early and significant events frequently observed during pancreatic cancer progression [4,5]. Although lymphatic invasion and metastasis to the lymph nodes does not directly contribute to PDAC morbidity in patients, these pathologies are important indicators of the metastatic potential of this disease. In the clinical setting, lymph node status is used to assess disease progression, to select appropriate therapies, and to predict survival [11,12]. Nearly all studies concur that lymph node status correlates with poor prognosis for pancreatic cancer patients [6,8–10]. Studies also agree that invasion of lymph nodes by PDAC occurs most frequently through the lymphatic vasculature rather than through direct/contiguous extension of the primary tumor to the lymph node [109,177,178]. However, the prognostic value of mode of lymph node invasion is arguable: some studies report poorer overall survival in patients with lymphatic vessel-directed metastasis as compared to direct invasion [178], while other reports show no survival difference between the two modes of lymph node invasion [109,177]. Although lymph node invasion by PDAC occurs most frequently through the lymphatic vasculature, the LVD at the tumor site has not been conclusively correlated with either lymph node metastasis or prognosis due to conflicting study results [53,156,162,179]. This is also true for studies examining the expression of pro-lymphangiogenic factors such as VEGF-C and -D [162,180,181] (and in pancreatic endocrine tumors [182]). The lack of standardized protocols for quantifying LVD in patients makes comparative analysis among collected data sets difficult. Some studies enumerate only intratumoral lymphatics in whole tumor sections, while others examine tumor margins for peritumoral lymphatics, and still others examine the sum of lymphatic vessels in both regions. In the continued absence of a standardized method, LVD has limited value as a metric for assessing pancreatic cancer progression.

PDAC tumors are often hypovascular with only sporadic blood and lymphatic vessels found among the tumor cells [183]. These intratumoral lymphatic vessels are typically collapsed and nonfunctional due to direct compression by the tumor cells and the high internal pressure of the PDAC tumor microenvironment [180,184,185]. However, even in the absence of functioning intratumoral lymphatic vessels, tumor cells are still capable of disseminating to lymph nodes, although identification of reliable sentinel lymph nodes remains challenging [27]. The lymphatic vessels located at the tumor margins are frequently described as enlarged with open lumens capable of being filled with tumor cells [180,184], and drainage studies show that these peritumoral lymphatic vessels are, in fact, functional [185]. Sipos

and colleagues demonstrated that even in the absence of elevated LVD values and active lymphangiogenesis, PDAC patients still frequently presented with lymph node metastases [162]. This suggests that PDAC cells are capable of invading the pre-existing lymphatic vasculature, especially enlarged vessels at tumor margins.

Mechanisms/Players

Mechanisms regulating lymphatic invasion are not completely understood, but are gaining increasing research interest. Most of our knowledge of vascular invasion has come from studies of the blood vasculature that are now being extended to studies of lymphatic vessel properties and function. Initially, invasion of lymphatic vessels by tumor cells was considered to be a passive process with increased interstitial fluid pressure driving tumor cells into draining lymphatic vessels [186]. Although increased interstitial pressure may contribute to tumor cell invasion, the concept of lymphatic-mediated tumor metastasis as a process that utilizes a “path of least resistance” is greatly oversimplified, and proteomics studies have identified distinctions between primary pancreatic tumors and their corresponding lymph node lesions [187]. Comparisons of pancreas tumors with and without lymph node metastases revealed differences in protein expression intrinsic to these two pathological tumor presentations [188]. In an effort to better understand the potential drivers of lymphatic metastasis, results of studies of leukocyte intravasation into lymphatic vessels are now being examined for commonalities to tumor cell intravasation. Three key molecular players of invasion have emerged as likely candidates in the regulation of tumor-lymphatic interactions and metastasis: chemokine signaling, paired binding of adhesion protein partners, and alterations in lymphatic vessel barrier integrity.

a) Chemokines—Chemokines secreted by lymphatic endothelial cells contribute to inflammation and initiation of immune responses in part by regulating the chemotaxis of antigen presenting cells to the lymph nodes. These same molecules are also being studied for similar roles in tumor metastasis to lymph nodes. Two widely researched candidate chemokines are CCL21 and CXCL12 and their respective G-protein coupled receptors (GPCRs), CCR7 and CXCR4.

During normal immune responses, lymphatic endothelial cells secrete CCL21 to increase migration of $CCR7^+$ DCs toward the vessel and then to guide DCs to the lymph nodes [189,190]. Tumor cells, including those of pancreatic cancer, overexpress CCR7 and are capable of responding to CCL21 cues to facilitate their dissemination to the lymph nodes [191–195]. Guo, *et al.,* noted a correlation between CCR7 expression in tumor cells and frequency of lymph node metastasis in pancreatic cancer patients [196]. Sperveslage, *et al.*, confirmed these results and also demonstrated that lymphatic vessels of PDAC patients had significantly higher expression of CCL21 compared to lymphatic vessels of the normal pancreas. Expression of CCL21 in lymphatic vessels correlated with increased lymphatic invasion and lymph node metastasis in these patients, as did overexpression of CCR7 in pancreatic tumor cells *in vivo* [197].

The expression of CCL21 in lymphatic endothelial cells is regulated by numerous inflammatory cytokines including TNF-α and IL-1β and is also influenced by increases in

transmural flow [198], both of which are often present in tumor microenvironments. *In vitro* co-culture work has demonstrated that CCR7-expressing tumor cells have increased chemotaxis toward CCL21-expressing lymphatic endothelial cells [199–201]. This chemotactic axis is used by tumor cells specifically for invasion into lymphatic vessels; tumor cell chemoattraction to blood endothelial cells does not use this mechanism [200,202]. Blocking CCR7 or CCL21 expression and/or function inhibits lymphatic vessel invasion and metastasis to the lymph nodes *in vitro* and *in vivo* [201,203,204]. This chemokine signaling axis appears to be regulated by and to work in concert with VEGF-C to synergistically promote lymphatic invasion of $CCR7^+$ and $VEGFR\text{-}3^+$ tumor cells [200].

Another chemokine axis that influences lymphatic metastasis is the CXCL12-CXCR4 axis. It has been widely documented that CXCR4-expressing tumor cells, including PDAC cells, home to organs with high CXCL12 expression, such as the lungs, bone marrow, and lymph nodes [54,195,205,206]. In PDAC patient tissues, high expression of CXCR4 was found in tumors, while lymph nodes expressed high levels of CXCL12 [54,207]. This expression pattern positively correlated with increased LVD values in the pancreas, lymph node metastasis frequency, and poor disease prognosis. Tumor-associated, but not normal uninflamed, LECs secrete ample amounts of CXCL12 in the tumor microenvironment and attract $CXCR4^+$ tumor cells to lymphatic vessels and lymph nodes [208,209]. Blocking the CXCR4-CXCL12 signaling axis has resulted in impaired lymph node metastasis in numerous tumor models [210–212]. An *in vitro* breast cancer model demonstrated that CXCL12-treated LECs permitted greater transendothelial migration by breast cancer cells, and this permissiveness could be reversed by blocking CXCR4 in the LECs [213]. An *in vivo* model of melanoma demonstrated that stem-like, dual positive $CD133^+/CXCR4^+$ tumor cells were strongly associated with CXCL12-producing LECs and that these cells were resistant to chemotherapy [208]. Combinational treatment with a CXCR4 antagonist relieved this resistance and increased the efficacy of chemotherapy thereby reducing tumor growth and metastasis. This study suggested that CXCL12 secretion from lymphatic vessels supported a pro-metastatic and pro-survival niche for tumor cells. Further studies are required to elucidate whether or not these types of mechanisms are employed in PDAC and/or its tumor microenvironment.

b) Adhesion Proteins—Physical interactions between tumor cells and lymphatic endothelial cells may be another crucial regulator of tumor cell intravasation. Adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular adhesion molecule 1 (VCAM-1) are typically used by DCs to gain entry into inflamed lymphatic vessels during migration toward lymph nodes [198,214]. Mounting evidence indicates that these same leukocyte adhesion molecules may also be important for controlling tumor cell entry into lymphatic vessels [215–217]. In a non-inflamed state, the lymphatic endothelium does not express or only very weakly expresses these adhesion molecules [214,218]. Inflammatory conditions—such as those found during infection or tumor development—or a wound healing response quickly increase the expression of these molecules on the lymphatic endothelium [198,214]. Increased transmural flow, also characteristic of an inflamed microenvironment, upregulates ICAM-1 and Eselectin expression on an *in vitro* lymphatic endothelium resulting in increased DC binding [198]. A

recent report shows that binding and transendothelial migration of breast cancer cells is also influenced by *in vitro* fluid flow, although the mechanisms governing these behaviors have not been elucidated [219]. When placed in co-culture with tumor cells, LECs display marked upregulation of adhesion molecules. Kawai, *et al.* (2008 and 2009), have demonstrated that invasive breast cancer cells, which express the αLβ2 ligand for ICAM-1, are capable of inducing the expression of E-selectin and ICAM-1 on lymphatic endothelial cells. They also demonstrated that blocking ICAM-1 impaired the ability of these tumor cells to bind to a lymphatic endothelium [217,220]. Studies of the ability of adhesion proteins on lymphatic vessels to regulate tumor cell entry should be expanded to pancreatic cancer cell lines to determine if PDAC tumor cells can use similar mechanisms to bind and gain access to the lymphatic vasculature.

c) Lymphatic Vessel Barrier Integrity—The intrinsic cellular and molecular organizational characteristics of lymphatic vessels facilitate entry of immune cells and fluids from a collecting tissue bed—properties that may also allow these vessels to support tumor cell metastasis. The initial lymphatic capillaries within tissues are composed of only a single layer of endothelial cells with loose junctions between neighboring cells [15,221]. Unlike the tightly-formed, continuously-arranged junctions between neighboring endothelial cells of the blood vasculature [222], the junctional proteins—vascular endothelial cadherin (VE-cadherin), platelet/endothelial cell adhesion molecule-1 (PECAM-1), claudins, occludins, *etc*. —of initial lymphatic vessels are discontinuously arranged, creating gaps between overlapping lymphatic endothelial cells [16]. These discontinuous junctions along with preformed openings in the basement membrane [17] enable uptake of macromolecules, fluids, and cells by the initial lymphatic capillaries. As lymph and cells are transported up the lymphatic vasculature to the collecting lymphatic vessels, the discontinuous intercellular junctions become more constant and successive to prevent leakage prior to arrival at the lymph nodes [16].

Data suggest that tumor cells are capable of modulating the barrier integrity of the lymphatic endothelium to further facilitate lymphatic vessel invasion [223]. Lipoxygenase secretion by breast cancer cells has been shown to disrupt VE-cadherin junctions and induce endothelial cell repulsion, resulting in breaches in the lymphatic endothelium. Tumor-secreted VEGF-C also facilitates invasion by creating leaky lymphatic vasculature. VEGF-C induces the internalization of VE-cadherin, which, in turn, promotes tumor cell transendothelial migration [224,225]. In a pancreatic tumor model, inhibiting Ang-2 signaling with a soluble Tie-2 receptor decreased lymphatic-directed metastasis to the lymph nodes [175]. This result may be explained by studies demonstrating that Ang-2 disrupts the barrier integrity of the lymphatic endothelium and increases lymphatic permeability through phosphorylation of VE-cadherin resulting in button-junction formation in the initial lymphatic capillaries [226].

Lymphatic Vasculature and the PDAC Microenvironment

The PDAC microenvironment is arguably one of the most complex of any tumor microenvironment, replete with CAFs, immunosuppressive leukocytes, tumor-associated blood/lymphatic endothelial networks, nerves, and a considerably dense ECM compartment

(Figure 1). Each of these components facilitates PDAC progression and dissemination and has the capacity to influence the normal lymphatic vasculature within the pancreas.

Cancer-Associated Fibroblasts

One of the most striking features of PDAC is the robust desmoplastic reaction seen within the primary tumor. Due to their abundance in the PDAC microenvironment, CAFs exert a strong influence over other microenvironmental cell types including the lymphatic endothelium [227]. One of the main protein regulators of desmoplasia in PDAC is sonic hedgehog (Shh) [228,229]. Bailey, *et al.* (2009), noted that Shh signaling in the CAFs of PDAC tumors led to the creation of a pro-angiogenic and prolymphangiogenic stromal compartment. When Shh signaling was inhibited in the CAFs, LVD decreased and lymph node metastasis was reduced. Data such as these suggest that CAFs primarily influence the lymphatic endothelium *via* secretion of various effector proteins. It has been demonstrated that CAFs of various tumor types, including PDAC, secrete a wide range of pro-lymphangiogenic factors such as VEGF-C, VEGF-D [230,231], VEGF-A [232], epidermal growth factor (EGF) [233], PDGF, and FGF [183]. CAFs also secrete chemokines, including CXCL12, which has been shown to correlate with increased tumor aggressiveness, LVD values, and lymph node metastases in PDAC patient tissues [54,234]. In addition to their direct action on lymphatic endothelia, many of these same secreted factors as well as proinflammatory cytokines allow CAFs to indirectly support lymphangiogenesis and lymphatic vessel invasion through the recruitment of pro-lymphangiogenic immune cells such as TAMs and DCs [235,236]. Lastly, CAFs secrete matrix metalloproteinases (MMPs) and other proteases that remodel the ECM of tumors [237]. This remodeling promotes tumor invasion of stroma and tumor vasculature and releases sequestered growth factors and cytokines from the ECM for tumor growth, angiogenesis, and lymphangiogenesis. A recent study by Shi, *et al.* highlights an additional protease-related mechanism by which CAFs may influence pancreatic cancer progression and lymphatic metastasis. Specific pancreatic stromal compartment deletion of protease-activated receptor-2 (PAR-2), a GPCR highly expressed in PDAC, resulted in decreased primary tumor size (due to anti-angiogenesis effects) but increased LVD and lymph node metastases [238].

Immune Cells and Immune Regulation

One of the main functions of lymphatic vessels is to transport leukocytes to lymph nodes for immune response initiation, uniquely positioning LECs to modulate immune responses in ways that may support tumor progression. As immune cell trafficking conduits, LECs are responsible for the transport of both antigens and antigen presenting cells (APCs), such as DCs, to the lymph nodes for immune response optimization [190]. By regulating the expression and secretion of various chemokines in response to inflammation, injury, or tumor development, LECs can alter the recruitment of immune cells to the lymph nodes, and, as a result, influence the ensuing immune response (reviewed in [239,240]). Partially due to lymphatic-directed recruitment, tumor-draining lymph nodes demonstrate a more immunosuppressive environment as compared to normal lymph nodes with an increased presence of regulatory T cells (T_{regs}), myeloid-derived suppressor cells (MDSCs), immature and tolerogenic DCs, and immunosuppressive cytokines [241–244]. These immunosuppressive cells and cytokines accumulate in the lymph as a result of increased

lymphatic drainage from the tumor site [245]. Within the lymph nodes TGF-β, a major driver of immune suppression, supports the differentiation and activation of T_{regs} as well as promoting tolerogenic and immature phenotypes of DCs [246]. As T_{regs} differentiate and accumulate, they secrete more TGF-β to further drive immune suppression. IL-10 is another factor that supports the accumulation of immunosuppressive cells in the lymph nodes by promoting T_{reg} activity [247] and tolerogenic DC function [247,248]. Indoleamine 2,3-dioxygenase increases the generation of T_{regs} in the lymph nodes [249,250], while concurrently inhibiting effector T cell activity [251]. Other factors implicated in the accumulation of immunosuppressive cells in lymph nodes include IL-4, VEGF-A, and prostaglandin E_2 [252].

In addition to cellular and cytokine transport, LECs also transport tissue antigens (and in the case of cancer, tumor antigens) from peripheral tissues to lymph nodes. Studies have demonstrated that LECs, particularly those in the lymph nodes, are capable of scavenging these tissue and tumor antigens and cross-presenting them on major histocompatibility complex-I (MHC-I) [253,254]. This can lead to immune tolerance through deletion of naive $CD8^+$ T cells as LECs lack co-stimulatory molecules needed to activate the T cells and instead express programmed death-ligand 1 (PD-L1), an inhibitory signal for T cells [255]. LECs can also present scavenged exogenous tissue/tumor antigens on MHC-II molecules and likely induce immune tolerance through interactions with the inhibitory lymphocyte activation gene-3 (LAG-3) protein on $CD8^+$ T cells [256]. These studies shed light on the phenomenon that when tumor cells are denied lymphatic vessel experience, such as through direct implantation into lymph nodes, tumor immunity is impaired through a robust $CD8^+$ T cell response [257]. LECs also modulate immune responses by inhibiting DC maturation [242]. Binding of DCs to the lymphatic endothelium *via* macrophage-1 antigen (Mac-1) and ICAM-1-mediated interactions during transendothelial migration can reduce the expression of co-stimulatory molecules on DCs needed for T cell activation. Studies such as these inspire new ideas regarding increased lymphangiogenesis at the tumor periphery and draining lymph nodes, suggesting that it may influence tumor progression in two ways: 1) increasing metastatic routes for dissemination and 2) immune suppression through increased antigen scavenging and decreased DC maturation leading to T cell inhibition and immune tolerance [258]. Further investigation is needed to substantiate the immunosuppressive properties of the lymphatic endothelium and its specific contribution to disease progression as a component of the tumor microenvironment.

A reciprocal concept in relation to the capacity of LECs to affect immunity is that of immune cells inducing effects on LECs. One such tumor infiltrating immune cell type, TAMs, can be found in many tumor microenvironments, including PDAC [259–262], and their presence often correlates with poor patient prognosis [263–266]. TAMs promote tumor lymphangiogenesis through two mechanisms: paracrine secretion of pro-lymphangiogenic factors and transdifferentiation into LEC-like progenitor cells. TAMs secrete high levels of VEGF-C and -D, which, in turn, increases LVD in and around tumors [259,260,267,268]. Indeed, TAM density has been shown to significantly correlate with increased LVD, lymphatic vessel invasion, and lymph node metastasis in many cancers [259,268–271]. Inhibition or depletion of TAMs from tumor microenvironments significantly reduced LVD values and decreased the incidence of lymph node metastases compared to tumors with

TAMs present [272–274]. However, depletion of TAMs did not completely inhibit lymph node metastasis as tumor cells were still able to invade pre-existing lymphatic vessels. These macrophages also secrete proteases such as MMP-2, MMP-9, and plasmin/urokinase plasminogen activator (uPA) that remodel the extracellular microenvironment and release sequestered growth factors for lymphangiogenesis [275,276]. The plasmin/uPA system is also important for the proteolytic maturation of VEGF-C and -D increasing their affinity for VEGFR-3 [277]. It has yet to be determined if TAMs secrete any of the other factors known to promote lymphangiogenesis. The second way TAMs contribute to lymphangiogenesis is by transdifferentiating into LEC-like progenitors both in inflammatory and tumor settings [146,273,278,279]. Transdifferentiated macrophages undergo genetic reprogramming [146] with increased expression of lymphatic markers Lyve-1, Prox-1, podoplanin, and VEGFR-3 [146,273,280,281]. Expression of LEC markers enables TAMs to physically incorporate into the newly developing lymphatic vasculature. The percentage of transdifferentiated TAMs within these newly formed lymphatic vessels is often less than 10% [273,280] suggesting the main mechanism by which TAMs promote tumorassociated lymphangiogenesis is through secretion of pro-lymphangiogenic factors.

Lymphatic Vessel-Nerve Interactions

In addition to lymph node metastasis, one of the most devastating pathologies of PDAC is perineural invasion. The peripancreatic region is densely innervated, housing large nerve plexuses with sensory, sympathetic, and parasympathetic nerves extending into the pancreas [82,282,283]. Incidence of perineural invasion in PDAC approaches 100% [44,82] and has been implicated in local and distant recurrence [75,82,284,285] after resection and neuropathic pain [286,287]. In an effort to decrease these effects, it has been suggested that nerve tissue be removed as part of radical pancreatic resection procedures [71,75,82,84,285,288]. The interactions between nerves and the lymphatic vasculature within and around the pancreas are poorly understood. Studies have shown that lymphatic vessels are, in fact, innervated [289,290] and have suggested that these connections may represent an additional route of metastatic dissemination of tumor cells from either network to the other [77,291]. It is well documented that vasculature and nerves can respond to the same molecular cues—termed neurovascular guidance molecules—for development and remodeling [132,168–170,292,293], and many of these molecules, such as Nrp-1 and -2, NGF, brain-derived neurotrophic factor (BDNF), FGF, IGF-2, Netrin-1, Semaphorin-3A, Ephrin receptor B4, Slit2, and Robo1 may be differentially expressed or have altered signaling functions in the pancreatic tumor microenvironment, implicating them in cancer progression [171,172,294–300]. As well, somatic alterations in axon guidance pathway genes are observed in a subset of pancreatic cancer genomes [301]. Remarkably, Chen, *et al.*, showed that in the absence of both perineural and lymphovascular invasion, the five-year survival rate for pancreatic adenocarcinoma patients was 71% [63]. These studies underscore the importance of both lymphatics and nerves in the pancreatic tumor microenvironment and highlight the need for further mechanistic work interrogating the specific contributions of these tissue networks to disease progression and metastasis.

Comparative Tools and Models to Study Lymphatic Biology and Tumor-Lymphatic Interactions

The discoveries of lymphatic endothelium markers such as lymphatic vessel hyaluronan receptor-1 (Lyve-1) [302], Prox-1 [303], VEGFR-3 [304], and podoplanin (PDPN) [305–307] have facilitated the development of new research methodologies and models with which to study lymphatic vessel biology under homeostasis and various disease pathologies as well as interactions between lymphatic endothelial cells, immune cells, and tumor cells *in vivo*. The mouse cornea and skin have emerged as two popular mammalian platforms for this type of work. Historically used for studies of angiogenesis, the murine corneal model system has proven equally informative for studies of lymphatic biology because of its unique characteristics. The normal healthy cornea harbors a single limbal lymphatic vessel ring at its periphery and is otherwise devoid of lymphatic vessels. Upon insult or injury, inflammatory lymphangiogenesis occurs resulting in extension of newly-synthesized lymphatic vessels from the limbal arcade toward the site of the stimulus. Corneal injury can be recapitulated experimentally by placement of sutures, mechanical debridement, or chemical burn. A refinement of this inflammatory model enabling more mechanistic dissection of lymphatic vessel behavior is the corneal micropocket assay in which a micropellet can be loaded with a protein or drug of interest and implanted into the cornea [132,308,309]. Further modifications of traditional acute inflammatory protocols can induce wound recovery [132,310] and recurrent inflammation [132,311]—two additional distinct physiological microenvironments with implications for wound healing, chronic inflammatory disease, and tumor microenvironment research. Anatomical sites commonly used in skin imaging studies include murine dorsal surface, foot pad, and pinna. Unlike the cornea, the skin is vascularized with a dense network of lymphatic capillaries under steady state conditions. This presents an ideal system for studies of lymphatic vessel homeostasis and remodeling, local inflammatory lymphangiogenesis, and endothelium-immune/tumor interactions.

Both cornea and skin have also been employed in real time live-imaging and intravital microscopy studies [17,312–314]. Early experiments of this type relied on injection and uptake of large fluorescent conjugate molecules such as FITC-dextran or explant immunostaining (Reviewed in [315]) to label vasculature and other tissue antigens, but recently several genetically engineered mouse models [316–321] have enabled more sophisticated lymphatic vessel-specific experimental designs. In these immunocompetent models, fluorescent protein expression is driven by lymphatic endothelium-specific promoters such as Prox-1, Lyve-1, or VEGFR-3 in either a constitutive or inducible manner. Inducible systems offer the advantages of titration and temporal control of fluorescence expression within the lymphatic endothelial compartment. Fluorescently labeled tumor or immune cells may be delivered to and tracked in either cornea or skin providing insight into intravasation/extravasation behavior, cell trafficking and fate, and spread to draining lymph nodes. Translation of these techniques to studies of the pancreatic lymphatic vasculature specifically would provide insight into organ-specific lymphatic vessel biology and pancreatic tumor microenvironment contributions to lymphatic remodeling and lymphatic-mediated metastasis. We suggest combination of several existing technologies to examine

these questions. First, crossing a spontaneous pancreatic ductal adenocarcinoma model containing a fluorescent reporter gene, such as the PKCY [322] or KPCT mouse [323], with one of the available lymphatic-specific reporter mice would facilitate visualization of cells of pancreatic origin and lymphatic vessels in two colors. Implantation of a pancreas window [324] in these animals could enable long term intravital microscopy studies of lymphatic vessel biology and tumor metastasis throughout the course of disease progression, from PanIN formation through advanced metastatic disease. Finally, use of the CLARITY technique as previously described for brain [325] in concert with multiphoton microscopy and immunofluorescent staining would allow deep tissue visualization and reconstruction of full lymphatic vascular networks as well as detection of other important microenvironmental structures (such as nerve and blood vascular networks) and signaling molecules both peri- and intratumorally.

Other research and pre-clinical imaging models have further studied lymphatic vessel- and lymph node-related pathologies in cancer. High resolution MRI has proven an effective non-invasive strategy for mapping involved mouse lymph nodes in pancreatic ductal adenocarcinoma [326]. Multiphoton laser scanning microscopy work in a model of melanoma showed that functional lymphatic vessels are not present within the tumor proper and that functional peri-tumoral lymphatic vessels are sufficient to mediate metastasis [185]. Other methods of lymph node and metastasis imaging (reviewed in [315]) have included injection of dyes or radiotracers such as Lymphoseek for lymphoscintigraphy, injection and uptake of cancer-specific radio-labeled antibodies and their accumulation in affected lymph nodes, injection of fluorescent antibody conjugates against the lymphatic endothelium in combination with fluorescent reporter-expressing pancreatic cancer cells, and use of combinatorial bioluminescence and fluorescence resonance energy transfer (BRET-FRET) nanoparticles for mapping lymphovascular and node networks [327]. Fluorescence lifetime imaging microscopy (FLIM)-FRET [328], optical coherence tomography [329], optical frequency domain imaging [330], photoacoustic tomography [331], higherorder harmonics generation [332], and Raman spectroscopy [333] imaging technologies offer other options for reconstructive deep tissue imaging and analysis of single cell signaling within an intact tumor microenvironment. Jeong and Jones, *et al.,* have also established a chronic lymph node window to facilitate long-term live imaging microscopy studies of lymph node biology, angiogenesis and lymphangiogenesis, and nodal deposition of metastatic tumor cells [334]. Application of CRISPR-Cas9 gene editing technology [335] may also prove useful in generating new pre-clinical models suitable for lymphatic vessel imaging in disease.

Clinical Imaging Techniques to Detect Pancreatic Cancer Lymph Node Metastasis

Despite research advances in comparative lymphatic vessel imaging, clinical imaging of pancreatic cancer patient lymphatic networks and lymph node status has remained challenging. Several groups have examined the utility of traditional clinical imaging platforms for detection of lymph node metastasis with limited success. Roche, *et al.*, have shown that examination of peripancreatic lymph nodes by CT cannot accurately predict presence of metastatic deposits [85]. Similarly, Imai, *et al.*, showed that CT, MRI, and FDG-

PET were not consistently accurate in predicting pre-operative para-aortic lymph node involvement in pancreatic cancer patients [86]. Conversely, another group had some success using endoscopic ultrasound (EUS) to differentiate benign and cancerous lymph nodes and to identify diseased pancreas; this technique has not been fully developed for widespread clinical use [336]. Cesmebasi, *et al.*, have recently reviewed other advances in clinical imaging techniques including EUS and lymphotropic nanoparticle-enhanced MRI, reporting that further refinement of these techniques may make them promising options to identify patterns of pancreatic cancer spread [25]. Other groups have focused efforts on imaging routes of pancreatic drainage in attempts to identify sentinel lymph nodes for pancreatic tumors arising in various locations within the pancreas. Injection of indocyanine green fluorescent dye into the pancreatic surface during PD surgery allowed visualization of pancreatic lymphatic vessels intraoperatively and resulted in identification of seven routes of lymphatic drainage highlighting the complexity of the pancreatic lymphatic vascular network [40]. In a similar study methylene blue dye was injected peri- and intratumorally during pancreatic cancer resection, but the authors concluded that detection of patterns of pancreatic lymphatic drainage and sentinel lymph node identification were not feasible with this protocol [39]. Another group injected activated carbon particles or regular insulin colloid at resection and examined their patterns of spread to surgicallyremoved lymph nodes by histology. They documented uptake in several groups of lymph nodes and recommended new radical resection guidelines based on their findings [30]. Development and testing of additional lymphatic imaging technologies and their adaptation to pancreatic adenocarcinoma patients may make pre-operative identification of lymph node metastases a reality in the future [337–339].

Therapy

Due to advanced stage at diagnosis and its complex microenvironmental organization, pancreatic ductal adenocarcinoma has proven to be very difficult to treat. Surgical removal of the tumor is the most effective option, but only approximately 15% of cases are considered resectable [340,341]. Of those cases in which resection is an option, incomplete removal of microscopic disease (R1 residual margin status) only slightly improves patient survival over those cases presenting with unresectable metastatic disease [342,343]. Non-surgical options for pancreatic cancer include radiation, chemotherapy, or a combination of both. Some approved chemotherapies for the treatment of pancreatic cancer are the use of FOLFIRINOX, gemcitabine, albumin-bound paclitaxel, cisplatin, and oxaliplatin (as well as others) [344,345]. However, these drugs have had limited success in prolonging patient survival. Development of targeted therapies that specialize in blocking crucial molecular pathways of the pancreatic tumor and its microenvironment is becoming an increasingly attractive therapeutic option.

Anti-angiogenic therapies were originally developed to starve tumors of important nutrients and oxygen and to reduce the number of potential routes for dissemination. However, clinical trials demonstrated that, when used alone, anti-angiogenic therapy was not sufficient to improve patient survival. Unexpectedly though, the results indicated that anti-angiogenic therapy significantly improved survival of patients with solid tumors when used in combination with conventional chemotherapies [346–348]. These findings led to the

evolution of the current vascular normalization theory: the use of anti-angiogenic therapy to block aberrant tumor angiogenesis and alleviate vessel dysfunction [349]. By restoring the balance between pro- and anti-angiogenic factors, anti-angiogenic therapies improved vessel organization, stabilized cell-to-cell junctions, increased pericyte coverage, and, consequently, reduced fluid leakage. All these factors, in turn, relieved blood flow irregularities resulting in improved delivery of chemotherapy to all parts of the tumor [350]. Unfortunately, in the setting of pancreatic cancer, anti-angiogenic therapies have had either no effect or only transient effects on improving patient survival even when used in combination with standard chemotherapies [351–355]. PDAC tumors are unusually hypovascular and desmoplastic negating the ability of even normalized vessels to deliver therapy [356]. The failure of anti-angiogenic therapy in PDAC may also be the result of tumor cells circumventing the VEGF-A/VEGFR-1 blockade through autocrine or paracrine secretion of alternative angiogenic factors, such as the prototypical lymphangiogenic factors which have overlapping angiogenic functions [355,357–359].

Targeting the tumor lymphatic vasculature as a treatment for cancer is beginning to gain interest among both basic and clinical research groups with the primary focus on anti-lymphangiogenic therapies. Lymphangiogenic growth factors are not critical for the maintenance of adult lymphatic vessels in homeostasis. This allows for extended treatment with anti-lymphangiogenic therapies in tumor settings without disruption of pre-existing vessels and with minimal drug-induced toxicities [360,361]. Numerous pre-clinical *in vivo* studies have demonstrated that blocking pro-lymphangiogenic factors VEGF-C and VEGF-D and their receptor VEGFR-3 significantly reduces tumor lymphangiogenesis and lymph node metastases in many tumor types including pancreatic [157], breast [362–365], melanoma [361], renal [366], lung [225,367], gastric [368,369], prostate [370], hepatocellular [371], and bladder [372]. Other protein targets of lymphangiogenesis that have shown promise in inhibiting lymphatic metastasis *in vivo* include the VEGFR-3 co-receptor Nrp-2 [169,173] and the angiopoietins Ang-1 and -2 [373,374]. Currently, two humanized neutralizing antibodies are in clinical trials for patients with solid tumors: VGX-100, which inhibits VEGF-C (NCT01514123) and IMC-3C5, which inhibits VEGFR-3 (NCT01288989).

The blockade of a single VEGF/VEGFR pathway will likely be insufficient to inhibit tumor lymphangiogenesis and lymph node metastasis due to the multiple compensatory and overlapping roles of the VEGF ligands and receptors [23,126,358,375]. Other growth mechanisms outside of VEGF/VEGFR signaling may also regulate lymphangiogenesis in the tumor setting, such as PDGF-BB/PDGFR [130] and FGF/FGFR [376]. Receptor tyrosine kinase inhibitors (RTKIs) often target multiple receptors allowing them to inhibit several signaling pathways simultaneously—including the VEGFR pathways. Both preclinical comparative studies and clinical trials have determined the safety and efficacy of numerous anti-angiogenic/-lymphangiogenic RTKIs for the treatment of cancer including foretinib [377], cediranib [378,379], and axitinib [380–382]. Some of these RTK inhibitors have also been approved for clinical use. Sorafenib, which inhibits VEGFR-1 and -3, PDGFR-β, FGFR-1, and Raf proteins, has been approved for renal cell (RCC) and hepatocellular carcinomas [383–385]; sunitinib, which inhibits VEGFR-1 and -3, and PDGFR-α and -β, has been approved to treat pancreatic neuroendocrine tumors, RCC, and gastrointestinal

stromal tumors [386–389]; and pazopanib, which inhibits VEGFR-1 and -3, PDGFR-α and -β, and FGFR, has been approved to treat RCC and soft tissue sarcoma [390–392] (RTKIs further reviewed in [13]). Vatalanib, which inhibits VEGFR-1, -2, and -3, and PDGFR-β, is currently in clinical trials for the treatment of various solid tumors including pancreatic, ovarian, and breast cancers. This RTKI has been shown to directly inhibit angiogenesis, lymphangiogenesis, and tumor growth in preclinical models of pancreatic cancer as well as other cancer models [393–397]. In a recent clinical trial, vatalanib resulted in a partial or stable response for some metastatic pancreatic cancer patients who had initially failed gemcitabine treatment [398]. Many of these lymphangiogenic receptor-targeting RTKIs hold promise for the treatment of early-diagnosed and resectable cancers [23]. Unfortunately, these are not typical characteristics of pancreatic cancer, and, consequently, many of these drugs have failed to significantly improve pancreatic cancer patient survival [381,399–401].

Lymphangiogenesis is not the only manner by which the lymphatic vasculature can promote tumor progression. As discussed previously, pre-existing lymphatic vessels can directly facilitate metastasis by transporting tumor cells to distant sites. Lymphatic endothelia may also contribute to immune suppression by altering DC and T cell responses. However, these functions are poorly understood and much more work needs to be done to determine if these functions can be specifically targeted in lymphatic vessels for the treatment of cancer.

Using Lymphatic Vessels to Deliver Therapies to Lymph Nodes

In pancreatic cancer, metastasis to lymph nodes and distant sites has often already occurred by the time of diagnosis. Anti-lymphangiogenic therapies may inhibit further tumor cell dissemination but will do little to reduce the growth of metastatic tumors that have already seeded at distant sites [225,379,402]. Successful treatment of tumor-invaded lymph nodes has been particularly difficult to achieve. Resection of invaded lymph nodes would intuitively seem to be a promising strategy; however, as discussed above, current clinical imaging technologies cannot reliably detect single cell or microscopic lymph node metastases [337,338], and excision of an excessive number of lymph nodes is controversial due to conflicting evidence regarding its survival benefits and concerns about post-operative quality-of-life [70,403]. Also, conventional intravenously-administered therapies display poor access to lymphatic vessels and lymph nodes resulting in sub-optimal drug concentrations within lymph nodes [404]. This enables tumor cells present within lymph nodes to evade treatment and potentiate future recurrence. Using the lymphatic vasculature as a delivery system for cancer therapies to the lymph nodes has gained increasing interest. For therapies to be effectively taken up by lymphatic vessels and not blood capillaries requires specific characteristics of drug formulations such as being of a particular size and molecular weight, lipophilicity and surface charge of the drug carrier, and concentrations of the drug and carrier (reviewed in [405,406]). A few anti-cancer drugs have been formulated to target the lymphatic system and have shown promise *in vivo*: a methyl poly(ethylene glycol)distearoylphosphatidylethanolamine micelle containing doxorubicin reduced the size of lymph node metastases in a melanoma model [407]; a PEGylation of interferon-α2 demonstrated anti-tumor efficacy in the lymph nodes of rats with breast cancer [408]; cisplatin conjugated to a copolymer block of poly(ethylene oxide)-block-poly(lysine) successfully treated lymph node metastases in a model of squamous cell carcinoma [409];

and gemcitabine loaded onto magnetic multiwalled carbon nanotubes (mMWNTs) resulted in better uptake of gemcitabine in the lymph nodes and regression of lymph node metastases in a subcutaneous model of pancreatic cancer [372]. The field of lymphatic-based drug delivery is still in its infancy and more studies are required to demonstrate efficacy and feasibility in patients.

Conclusions and Perspectives

Advances in surgical, radiation, and chemotherapeutic treatment regimens for pancreatic cancer have not greatly impacted overall survival rates for patients afflicted with this devastating disease. Early spread of tumor cells to lymph nodes and distant sites often precludes curative resection and facilitates cancer chemoresistance and immune evasion. While not the sole route of spread, the lymphatic system represents one of the major understudied players in the tumor microenvironment and in the process oftumor metastasis. The intrinsic functional and structural characteristics of the lymphatic system suggest roles in immune regulation (especially immune suppression), cell trafficking, and interactions with other tissue networks and cell types. The distortion of the physiological process of lymphangiogenesis in the tumor microenvironment and its consequent effects on the biology of the lymphatic system are complex and complicate targeting strategies. Clinical efforts to detect and use lymph node status to inform treatment decisions, guide surgical lymphadenectomy, and stratify patients into prognostic cohorts have improved but remain inconsistent across groups and are not yet standardized or broadly applied. Identification, refinement, and directed studies of lymphatic-specific metrics have highlighted the importance of these criteria as additional prognostic factors in this disease. Development of pre-clinical models of tumor-associated lymphangiogenesis and lymph node metastasis and new lymphatic-directed clinical therapeutics represent two significant areas of current research. Live imaging studies in genetically engineered models that recapitulate both molecular and behavioral signatures of specific cancer types will improve our understanding of the earliest events in tumor-associated lymphangiogenesis, tumor-lymphatic interactions, and lymph node invasion. Translation of these and other preclinical findings to clinically-relevant diagnostic criteria or therapeutic interventions remains an underexplored but promising strategy to ultimately improve PDAC patient quality-of-life and outcomes.

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Highlights

- We review the clinical relationship between pancreatic cancer metastasis to lymph nodes, clinical staging, and clinical outcome
- We review the clinical, pathological, molecular and biological features of lymphangiogenesis, lymphatic invasion and metastasis by pancreatic tumors
- We review the biological consequences of lymphatic invasion and metastasis including immunosuppression and tumor sequestration
- We review strategies to image and treat metastases in lymph nodes

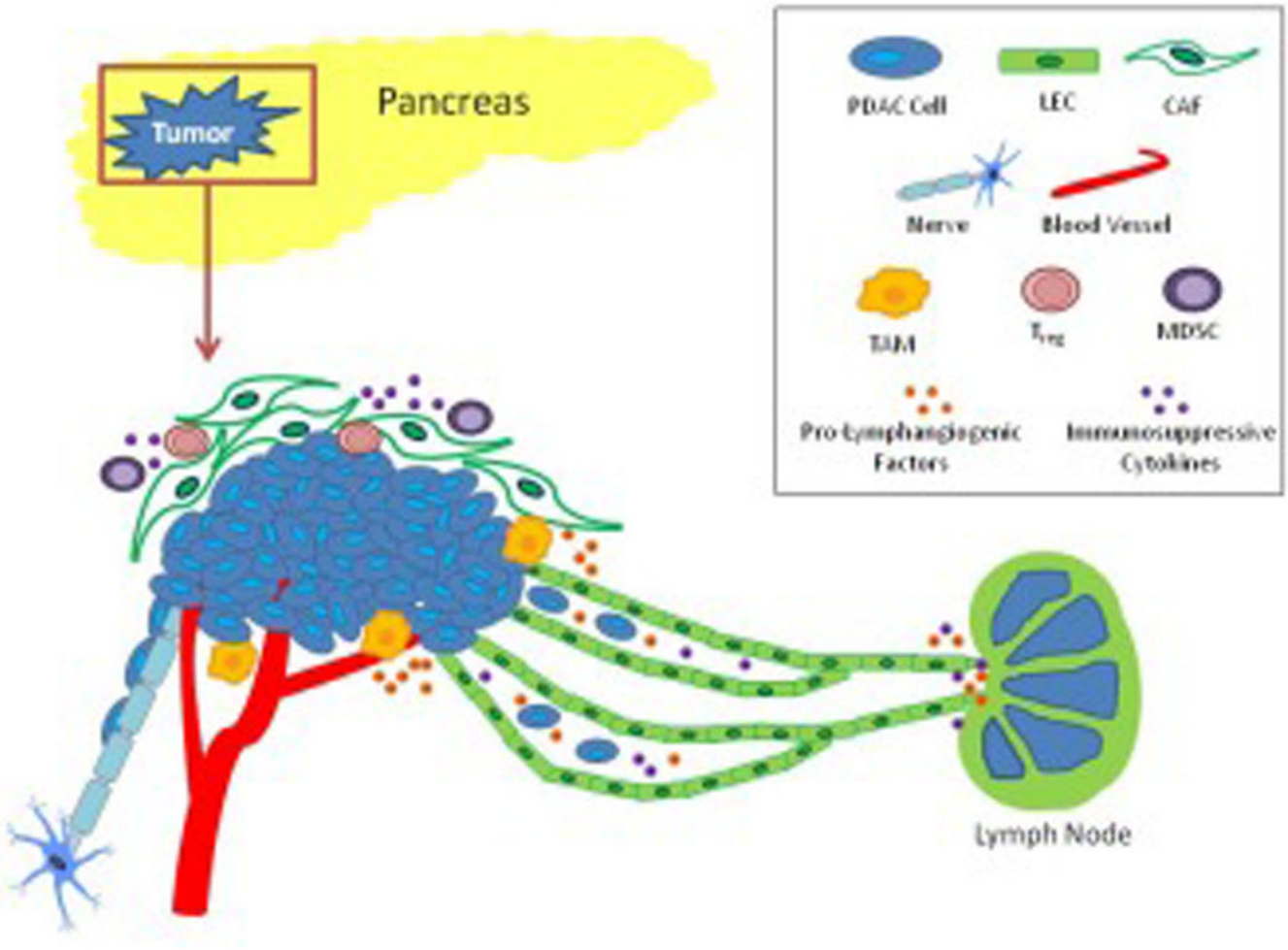

Figure 1. Pancreatic tumor microenvironment and lymph node metastasis

Cells of the tumor microenvironment are essential contributors to tumor growth, lymphatic invasion, and lymph node metastasis. CAFs and TAMs secrete pro-lymphangiogenic factors and proteases needed for lymphangiogenesis and metastasis. Lymphatic vessels act as conduits not only for tumor cell metastasis, but also for immunosuppressive cell and cytokine transport to lymph nodes. Nerves are also another route for pancreatic tumor metastasis and can communicate with lymphatic vessels to facilitate tumor metastasis from one network to the other.

Table 1

Standard (sLE) vs. Extended (eLE) Lymphadenectomy for Treatment of PDAC: Results and Recommendations of Collected Studies

Citation	Study Type	Results/Conclusions	Supported LE Type
Masui 2013	CR	In selected cases eLE can result in long-term patient survival: here well-differentiated, chemotherapy-responsive pancreatic cancer with PALN metastasis	Extended
Pedrazzoli 1998	P	Survival trends toward an increase in node positive patients with PD + eLE and retroperitoneal tissue removal over sLE; No increase in operative morbidity/mortality	Extended
Riall 2005	P	Survival trends toward an increase in pylorus-preserving PD with retroperitoneal LE and distal gastrectomy in PDAC patients, but this may be due to increased positive resection margins in standard resection group	Extended
Benassai 1999	R	Survival increased with eLE but may be due to patient selection; Additional prospective randomized trials are necessary	Extended
Ishikawa 1988	R	eLE and connective tissue removal is recommended for regional control of small (< 4 cm) tumors	Extended
Manabe 1989	R	Survival increased with radical pancreatectomy including soft tissue removal and eLE beyond suspected positive lymph nodes	Extended
Meriggi 2007	R	Extended peripancreatic and locoregional LE including removal of nerve and connective tissue reduces local recurrence in tumors < 4 cm in diameter	Extended
Nakao 1995	R	eLE including PALNs should be performed in patients with pancreas head cancer	Extended
Ohta 1993	R	eLE and removal of retroperitoneal connective tissue is recommended for curative resection in tumors macroscopically confined to pancreas	Extended
Fernández-Cruz 1999	Rv	Removal of primary tumor as well as eLE and removal of nerve plexus, soft tissue, and portions of nearby blood vessels are necessary to prevent tumor recurrence and spread	Extended
Samra 2008	Rv	Modified *en bloc* resection (including removal of lymphatics and neural tissue associated with superior mesenteric artery and retropancreatic tissue) may be best for PDAC of pancreas head	Modified radical/ Intermediate
Tol 2014	Exp	International Group on Pancreatic Surgery accepted Japanese Pancreas Society lymph node classification system, defined standard and extended LE procedures, and recommended standard LE for PDAC	Standard
Farnell 2005	P	Quality-of-life is decreased in patients following eLE with no survival benefit; No further trials should be performed comparing these two surgical methods	Standard
Gerdes 2005	P	Radical LE not recommended in cases of pylorus-preserving PD	Standard
Jang 2014	P	Prospective randomized clinical trial found no improvement in survival with eLE over sLE while eLE increased morbidity	Standard
Nimura 2012	P	No difference in 5-year or disease-free survival or number of involved lymph nodes; Local recurrence higher and quality-of-life lower with eLE; eLE not indicated based on trials	Standard
Pissas 1984	P	Describes pancreatic lymphatic drainage patterns and involved lymph nodes with discussion of appropriate surgical procedures to remove specific lymph node clusters; eLE may not be beneficial because of close proximity of pancreatic lymphatics and thoracic duct allowing early circulation of tumor cells	Standard
Yeo 2002	P	Extending pylorus-preserving PD with retroperitoneal LE and distal gastrectomy does not improve survival and increases morbidity in patients with periampullary carcinomas; eLE may show some benefit in PDAC with long-term follow-up	Standard

Citation	Study Type	Results/Conclusions	Supported LE Type
Henne-Bruns 1998	R	Retroperitoneal eLE does not improve survival over regional LE for pancreatic head tumors following R0 partial duodeno-pancreatectomy	Standard
Henne-Bruns 2000	R	Survival after partial PD does not improve with eLE and retroperitoneal tissue removal over regional LE	Standard
Hirata 1997	R	eLE does not always improve PDAC outcomes and may be responsible for increased post-operative mortality	Standard
Kanda 2011	R	A thorough but not radical degree of LE is recommended with differential dissections indicated for head vs. body/tail tumors	Standard
Pawlik 2005	R	eLE may only benefit 0.3% of patients; Too large of a population size would be necessary to sufficiently power a prospective trial making it infeasible	Standard
Shimada 2006	R	eLE does not improve outcomes in the presence of positive PALNs and is not recommended	Standard
Dasari 2015	Rv	Meta-analysis of randomized clinical trials; eLE does not improve survival over sLE but increases morbidity	Standard
Evans 2009	Rv	Survival is not improved with eLE over sLE; Recommend sLE during PDAC PD	Standard
Farnell 2008	Rv	Recommends standard PD without eLE based on survival and quality-of-life outcomes of four randomized clinical trials	Standard
Fujii 2013	Rv	Title somewhat misleading; Advocates LE with sufficient removal of LNs to provide accurate prognosis (to include lymph node ratio metric) but does not recommend extensive eLE	Standard
Iqbal 2009	Rv	Meta-analysis 1988–2005; eLE is associated with increased patient morbidity including delayed gastric emptying with no increase in survival	Standard
Ke 2014	Rv	eLE is associated with poor post-operative quality-of-life; sLE is recommended for patients with PDAC of pancreas head	Standard
Michalski 2007	Rv	Survival not improved and quality-of-life decreased with eLE; Not indicated except perhaps in the setting of additional randomized controlled trials	Standard
Pederzoli 1997	Rv	Current data does not show benefit of extensive LE; Additional prospective randomized trials are necessary	Standard
Pedrazzoli 2015	Rv	Lymph node stations 6, 8a, 8p, 12a, 12b, 12c, 13a, 13b, 14a, 14b, 14c, 14d, 16b1, 17a, and 17b should be removed as part of a sLE to accurately stage disease and decrease metastasis risk	Standard
Peparini 2015	Rv	Lymph node stations 16a2 and 16b1 (PALNs) should be included in sLE to minimize local invasion and improve resection margins	Standard
Schoellhammer 2015	Rv	eLE should not be implemented for PDAC patients due to lack of improvement in patient survival	Standard
Sergeant 2013	Rv	Sufficient evidence does not exist to indicate eLE over sLE for treatment of PDAC of pancreas head	Standard
Svoronos 2014	Rv	Five-year overal survival was not improved with eLE and eLE patients experienced a significant increase in post-operative diarrhea; sLE with PD should be used to treat PDAC of pancreas head	Standard
The studies below do not directly support one type of LE over another but provide pertinent results that should be considered in a discussion of this topic.			
Hirono 2012	P	Intraoperative physiological fluorescence imaging identified 7 lymphatic drainage pathways from pancreatic uncinate process; Removal of PALNs and skeletonization of superior mesenteric artery may both be beneficial	
Imai 2010	P	CT, MRI, and FDG-PET cannot accurately detect presence or absence of lymph node metastases; Intraoperative examination of frozen sections is recommended	
Kocher 2007	P	Sentinel pancreas lymph nodes were not identified intraoperatively; Prediction of positive lymph nodes to guide selective resection was not possible	

Citation	Study Type	Results/Conclusions	Supported LE Type
Nguyen 2003	P	There is no difference in quality-of-life metrics in standard vs. radical resection 2.2 years following surgery	
Roche 2003	P	Preoperative CT does not accurately predict positive lymph nodes especially in the case of micrometastasis and should not preclude curative resection or direct LE decisions	
Yeo 1999	P	Extending pylorus-preserving PD with retroperitoneal LE and distal gastrectomy does not increase morbidity and mortality over standard resection; More time and greater numbers of patients are needed to assess survival benefit	
Bittner 1989	R	Surgery for pancreatic cancer does not increase morbidity or mortality over other abdominal oncologic surgeries; Resection only benefits TNM stage I population	
Doi 2007	R	PALN metastasis correlated with increased mortality; Upon intraoperative confirmation of PALN metastasis alternative treatment strategies should be considered due to short survival duration even with eLE	

CR: Case report, Exp: Expert consensus statement, LE: Lymphadenectomy–(e) Extended, (s) Standard, P: Prospective study, PALN: Paraaortic lymph node, PD: Pancreat(ic)oduodenectomy, PDAC: Pancreatic ductal adenocarcinoma, R: Retrospective study, Rv: Review

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Table 2

Lymphatic-Specific Outcomes Metrics: Lymph Node Disease (LND), Lymph Node Burden (LNB), Lymph Node Ratio (LNR), Lymphatic Vessel Invasion (LVI)

		Analyzed/Significant Metrics*			
Citation	**Conclusions/Recommendations**	**LND**	**LNB**	**LNR**	**LVI**
Ausborn 2013	High LNR is associated with decreased overall survival of PDAC patients only when 53BP1 expression is low			■	
Berger 2004	LNR > 0.15, not number of LNs examined, predicts overall survival and disease-free survival in patients with PDAC		■	■	
Bhatti 2010	LNR is a better predictor of overall survival than LNB or LND in PDAC	■	■	■	
Chen 2010	Lymphovascular invasion (may be blood or lymphatic vessels) predicts 5-year survival in periampullary cancer and PDAC patients	■			■
House 2007	Presence of LND predicts survival in PDAC patients; In node positive disease, LNB and LNR are good predictors of survival	■	■	■	
John 2013	LNB and LNR can be used to predict cancer-specific survival in patients with PDAC of pancreas head regardless of resection margin status	■	■	■	■
Konstantinidis 2010	Regional lymph node metastasis and direct lymph node invasion equally negatively impact survival; LNR ≥ 0.2, LNB, and LVI are useful predictors of prognosis of PDAC patients		■	■	■
La Torre 2011	LND and LNR are both predictive of prognosis in PDAC patients; LNR maintains significance on multivariate analysis	■		■	
Murakami 2010	LND and LNB, not LNR, predict overall survival in PDAC patients; LNR also significant in univariate analysis	■	■	■	
Nakagohri 2006	LND and LVI are predictors of 3-year survival; LND is best predictor of all analyzed for PDAC patients	■			■
Pawlik 2007	LNR predicts prognosis and disease-specific survival in PDAC patients	■	■	■	
Riediger 2009	LNR, not LND or LNB, predicts overall survival in PDAC patients	■	■	■	
Robinson 2012	LND and LNR are good predictors of 5-year survival of PDAC patients; LNR better when > 0.15	■		■	■
Schwarz 2006	Number of lymph nodes examined and number of negative lymph nodes identified predict survival for exocrine pancreatic cancer patients		■		
Sergeant 2009	Extracapsular LNR predicts overall survival as does overall LNR; LNB predicts disease-free survival in PDAC patients	■	■	■	
Sierzega 2006	LND predicts prognosis in patients with PDAC of pancreas head; In	■		■	

Citation	Conclusions/Recommendations	Analyzed/Significant Metrics*			
		LND	LNB	LNR	LVI
	node positive disease, LNR > 0.2 predicts survival; Specifically, Group 8 and 12 lymph nodes were prognostic				
Slidell 2008	Number of positive LNs, presence of LND, and LNR are significantly associated with survival in PDAC patients				
Smith 2014	New predictive tool available for LNR and survival that incorporates specific patient metrics and includes population characteristics				
Tol 2015	LNR above 0.18 predicts 3-year survival in PDAC patients				
Yamamoto 2014	LNB and LNR are predictors of PDAC patient prognosis, not LVI; LNR is best predictor of all metrics examined				

* White: metric not analyzed; Light gray: metric analyzed but not statistically significant; Dark gray: statistically significant metric