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Lymphatic changes in cancer and drug delivery to the lymphatics in solid tumors

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**ABSTRACT**

Although many solid tumors use the lymphatic system to metastasize, there are few treatment options that directly target cancer present in the lymphatic system, and those that do are highly invasive, uncomfortable, and/or have limitations. This review gives a brief overview of lymphatic function and anatomy, discusses changes that befall the lymphatics in cancer and the mechanisms by which these changes occur, and presents limitations for drug delivery to the lymphatic system. We then go on to summarize relevant techniques and new research for targeting cancer populations in the lymphatics and enhancing drug delivery intralymphatically, including intralymphatic injections, isolated limb perfusion, passive nano drug delivery systems, and actively targeted nanomedicine.

**KEYWORDS:** lymphatic system; lymphangiogenesis; cancer; VEGF-C; drug delivery

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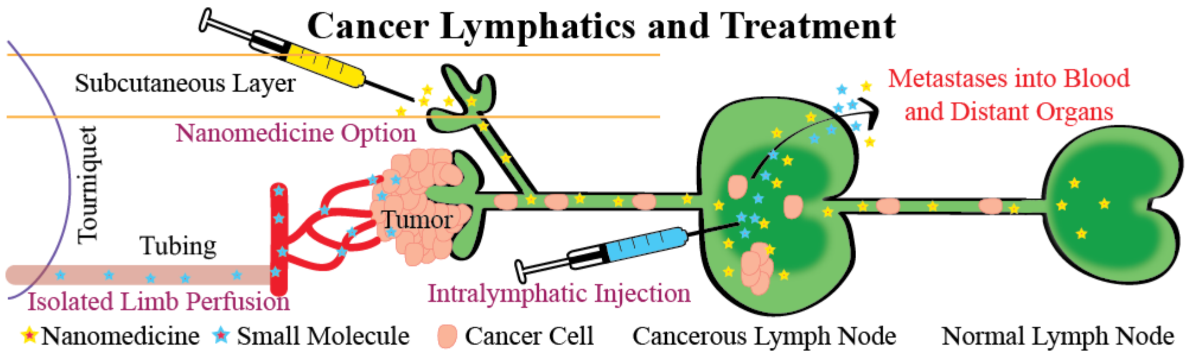

Cancer Lymphatics and Treatment
Subcutaneous Layer
Nanomedicine Option
Tourniquet
Tubing
Tumor
Isolated Limb Perfusion
Intralymphatic Injection
Metastases into Blood
and Distant Organs
Nanomedicine
Small Molecule
Cancer Cell
Cancerous Lymph Node
Normal Lymph Node

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## 1. INTRODUCTION

Before discussing drug delivery to the lymphatic system, it is important to understand the anatomy and physiology of the lymphatics. One of the main roles of the lymphatic system is the drainage of interstitial fluid from tissues back into circulation through the thoracic duct, preventing fluid build-up and edema [1–3]. Additionally, the lymphatic system is important immunologically, as one of its main functions is providing a networked system of vessels for the transport of immune cells throughout the body [1,4]. Antigens and antigen presenting cells enter into the lymphatic vasculature and travel to the nearby lymph nodes [4]. Lymphocytes within the lymph nodes that are exposed to antigen presenting cells trigger an immune response to the antigen [4]. The lymphatic system also plays an integral part in lipid metabolism [3,5,6]. As fats are consumed, enterocytes package lipids into lipoproteins. Lipoproteins are then transported by the lymphatic vessels [5].

The lymphatic system begins at the initial lymphatic capillaries where interstitial fluid enters [1]. After entering the lymphatics, interstitial fluid becomes lymph, the fluid that fills the lymphatic capillaries comprised of lipids, proteins, and white blood cells [4,6]. The cells that compose lymphatic capillaries are made of a monolayer of non-fenestrated endothelial cells that are typically collapsed until pressure in the interstitium increases [7,8]. As pressures rise within the initial lymphatic capillaries, the ends close and prevent the backwards flow of lymph [1]. Therefore, the anatomy of lymphatic capillaries contribute to their functionality, enabling them to act as valves [1,7]. As discussed later in section 3, many times, lymphatic drug delivery will occur through the lymphatic capillaries because the walls are quite thin, making this an easier place for substances to cross.

After the lymph has passed through the lymphatic capillaries, it flows to pre-collecting lymphatics, vessels that have one-way bicuspid valves and a smooth muscle layer capable of contracting to assist in propelling lymph forward [1]. Some areas in in the pre-collecting lymphatics are without a smooth muscle layer and will continue to absorb excess fluid [1,9]. From here, fluid flows into the collecting lymphatics, the walls of which are comprised of three layers – the intima consisting of endothelial cells, the media made of smooth muscle, and the adventitia, composed of collagen fibers [1]. These lymphatics have secondary valves which are typically bicuspid valves [10]. Lymphatic vessels are connected to the surrounding tissue by connective tissue fibers [2,11]. These fibers enable the lymphatics to remain open and prevent the lymphatic vessels from collapsing in on themselves, and the attachment of collagen fibers to elastin fibers allow the lymphatics to respond to movement [2,11]. After passing through the collecting lymphatics, lymph and its contents, including any drugs that have been successfully delivered to the lymphatic system, are drained either into the right subclavian vein or into the thoracic duct and back into blood circulation where the jugular and subclavian veins meet [1].

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Abbreviations:
vascular endothelial growth factor (VEGF)-C
vascular endothelial growth factor receptor (VEGFR)
nicotinamide adenine dinucleotide phosphate oxidase 3 (NOS3)
fatty acid synthase (FAS)
platelet derived growth factor (PDGF)
hypoxia inducible factor (HIF)

insulin-like growth factor binding protein 1 (IGFBP1)
insulin growth factor (IGF)
Kangai 1 (KAI1)
COOH-terminal interacting tetraspanin (KITENIN)
chemokine receptor (CCR)
chemokine ligand (CCL)
protein kinase B (AKT)
extracellular-signal regulated kinase (ERK)
Wingless-type mouse mammary tumor virus (MMTV)
MMTV integration site family (WNT)
semaphorin 7a (SEMA7a)
focal adhesion kinase (FAK)
interleukin- (IL-)
prospero homeobox protein 1 (Prox-1)
lymphatic vessel endothelial receptor 1 (LYVE-1)
lysophosphatidic acid (LPA)
nuclear factor (NF)
cyclooxygenase- (COX-)
sine oculis homeobox homolog 1 (SIX1)
transforming growth factor-β (TGF-β)
glycogen synthase kinase 3 β (GSK3β)
transforming growth factor β induced protein (TGFβIp)
Kirsten rat sarcoma viral oncogene homolog (Kras)
retinoblastoma gene (Rb)
inhibitor of differentiation (ID-)
sex determining region Y-box 18 (SOX-18)
protease-activated receptor 2 (PAR2)
sphingosine-1-phosphate (S1P)
sphingosine kinase-1 (SK1)
human epidermal growth factor receptor 2 (HER2)
Fms related tyrosine kinase 4 (FLT4)
tumor necrosis factor α (TNF-α)
forkhead box (FOX)
tropomyosin-related kinase B (trkB)
cellular inhibitor of apoptosis 2 (cIAP2)
nuclear receptor corepressor 1 (NCoR)
thyroid hormone receptor β1 (TRβ1)
E26 transformation-specific (ETS) domain-containing protein Elk-3 (ELK3)
soluble vascular endothelial growth factor receptor (sVEGFR)
cluster of differentiation (CD)
serine/threonine/tyrosine kinase 1 (NOK, also called STYK1)
sulfatase 2 (Sulf2)
decoy receptor 3 (DcR3)
phosphoinositide-3-kinase (PI3K)
inducible nitric oxide synthase (iNOS)
tumor associated macrophages (TAMs)
tyrosine kinase with Ig and EGF homology domains-2 (TIE-2)
TIE-2-expressing monocytes (TEMs)
chitinase 3-like protein 1 (CHI3L1)
macrophage colony-stimulating factor (M-CSF)
type 2 T helper cell (Th2)
Janus kinase (JAK)
signal transducer and activator of transcription 3 (STAT3)

Lymphoid organs include the bone marrow, thymus, spleen, appendix, tonsils, Peyer's patches, and lymph nodes [1]. The bone marrow and thymus are primary lymphoid organs and produce lymphocytes. The other lymphoid organs are secondary organs [1]. Lymphocyte maturation and immune response initiation occur in the secondary lymphatic organs [1]. As this review will be focusing mainly on the lymphatic vasculature and lymph nodes, we will only briefly discuss the anatomy of healthy lymph nodes. Secondary lymphatics are discussed in depth in another lymphatic review [1].

Drug delivery to lymph nodes is of interest in cancer treatment, as many solid tumors metastasize through the lymphatic system. Lymph nodes, like the lymphatic vessels, have an outer layer of smooth muscle, and thus contract as well [1]. As lymph flows through the lymph nodes, there is higher resistance compared to the rest of the lymphatics [1,12]. Upon entering the lymph node, lymph flows into the subcapsular sinus, around the lymphoid compartment, and is distributed into the nodal sinuses [1]. After reaching the sinuses, macrophages filter the fluid [1]. Most of the lymph is then pushed through the efferent vessels back into the lymphatic circulation, while some lymph enters the lymphoid compartment which is comprised of a highly impermeable membrane that prevents large molecules from entering the bloodstream [1,13]. The conduit system is full of specialized channels that allows lymphocytes, lymph, and small molecules to enter the lymph node [1,13]. High endothelial venules inside the lymphoid compartment permit the lymph within the lymphatic system to enter the bloodstream, and lymphocytes from the bloodstream to enter the lymph node [1,13,14]. Lymphatic anatomy, structure, and function is discussed more in depth in other reviews and papers referenced herein [1–4,7,8,11–14]. A schematic of lymphatic vessel anatomy can be found in Figure 1.

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phosphorylated-STAT3 (p-STAT-3)
ATP-dependent chromatin remodeler SMARCA4 (BRG1)
switch/sucrose non-fermentable (SWISNF)
human immunodeficiency virus (HIV)
polyglutamic acid (PGA)
polyethylene glycol (PEG)
poly(lactide-co-glycolide) (PLGA)
poly(lactic acid) (PLA)
methoxy poly(ethylene glycol)5,000-block-poly(ε-caprolactone)10,000 (mPEG-PCL)
carboxy poly(ethylene glycol)5,000-block-poly(ε-caprolactone)10,000 (cPEG-PCL)
polystyrene nanoparticles (PS)
PLGA-PMA:PLA-PEG (PP)
isolated limb perfusion (ILP)
Toll-like receptor (TLR)
scanning electron microscopy (SEM)
methyl poly(ethylene glycol)-distearoylphosphatidyl-ethanolamine (mPEG-DSPE)
poly-(ethylene glycol) phosphorethanolamine (PEG-PE)
polyethylenimine-stearic acid conjugate (PSA)
tyrosinase-related protein 2 (Trp2)
polyamidoamin dendrimers conjugated to alkali blue (PANAM-AB)
paclitaxel loaded onto PANAM-AB dendrimers (PTX-P-AB)
lymphatics-homing peptide (LyP-1)
polycaprolactone-polyethylenimine (PCL-PEI)
immunoglobulin G1 (IgG1)

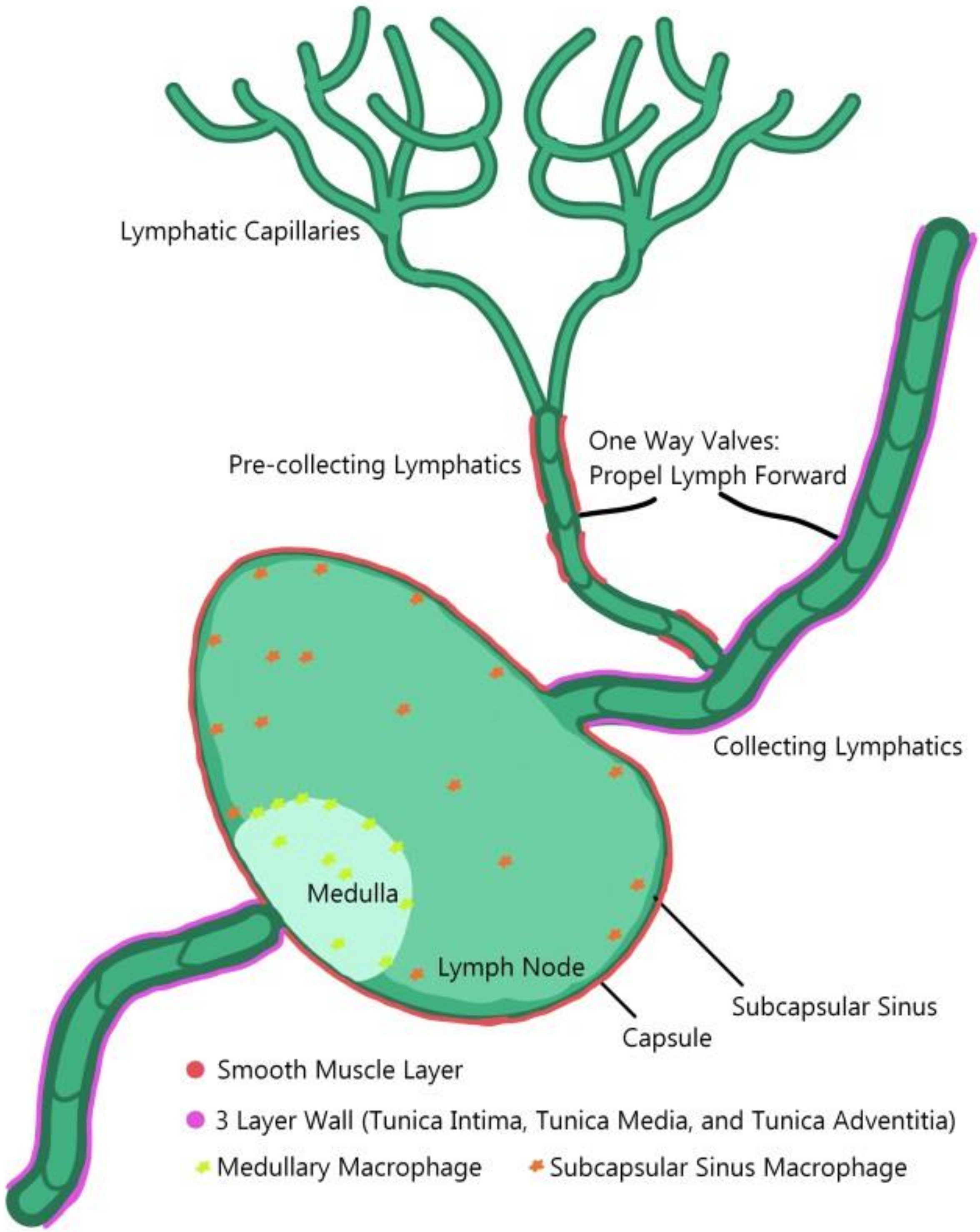


Figure 1. A brief schematic of lymphatic vessel anatomy, showing lymphatic capillaries, pre-collecting lymphatics with an intermittent smooth muscle layer, collecting lymphatics with a 3-layer cell wall, and location of lymph node macrophages. Lymph nodes are connected to collecting lymphatics and have a smooth muscle layer.

Over the past several years, ongoing research has begun to elucidate the effects the lymphatic system can have in various illnesses, such as cancer. Although the lymphatic system is a part of the pathology of several disease states, it is can be difficult to target with conventional medications administered systemically. This review seeks to briefly explore the role the lymphatic system plays in cancer, discuss new insights into the mechanisms for increased lymphangiogenesis in cancer, and highlight options, benefits, and challenges for drug delivery to the lymphatics.

## 2. THE ROLE OF LYMPHATICS IN CANCER

### 2.1. Lymphatics in cancer

Solid tumors metastasize via the lymphatics in approximately 80% of cases, first by spreading to the sentinel node, the lymph node immediately downstream of the tumor that receives lymph proximally from the area of the tumor [15]. From there, the cancer can spread to other regional lymph nodes, to more distant nodes, or to other organs, though the mechanism by which this occurs has not been fully elucidated [15]. As the cancer cells spread throughout the lymphatic system, they may metastasize into the blood stream, other tissues, or organs that are perfused by lymphatic vessels [16]. This highlights the need to have a reliable way to deliver drugs to cancer cell populations within the lymphatics. Although lymphatic vascular changes in solid tumors have been well described and studied, there is limited literature describing lymphatic changes in blood cancers. In this review, we will be focusing on lymphatic changes in solid tumors.

### 2.2. Factor, molecule, and enzymatic variations in cancer leading to lymphatic changes: a mechanistic discussion of recent studies

Lymphatic vasculature near tumors is more dense than in normal tissue, and lymphatic vessels can develop intratumorally [17]. This indicates that cancer stimulates lymphangiogenesis. It has been shown that in patients with breast cancer, disease free survival and overall survival are significantly lower in patients with a high lymphatic vessel density compared to patients with low lymphatic vessel density [17]. This finding may be applicable to other cancers as well, and increased lymphatic involvement is associated with poorer outcomes. There are several factors, signaling molecules, and upregulated enzymes found in the tumor microenvironment that lead to increased lymphangiogenesis, and this review will discuss new findings on several of these from works published in the last couple of years. A simple overview of many of the factors discussed in this paper are presented in Figure 2. A table with information about which factors were studied with each cancer is also included (Table 1).

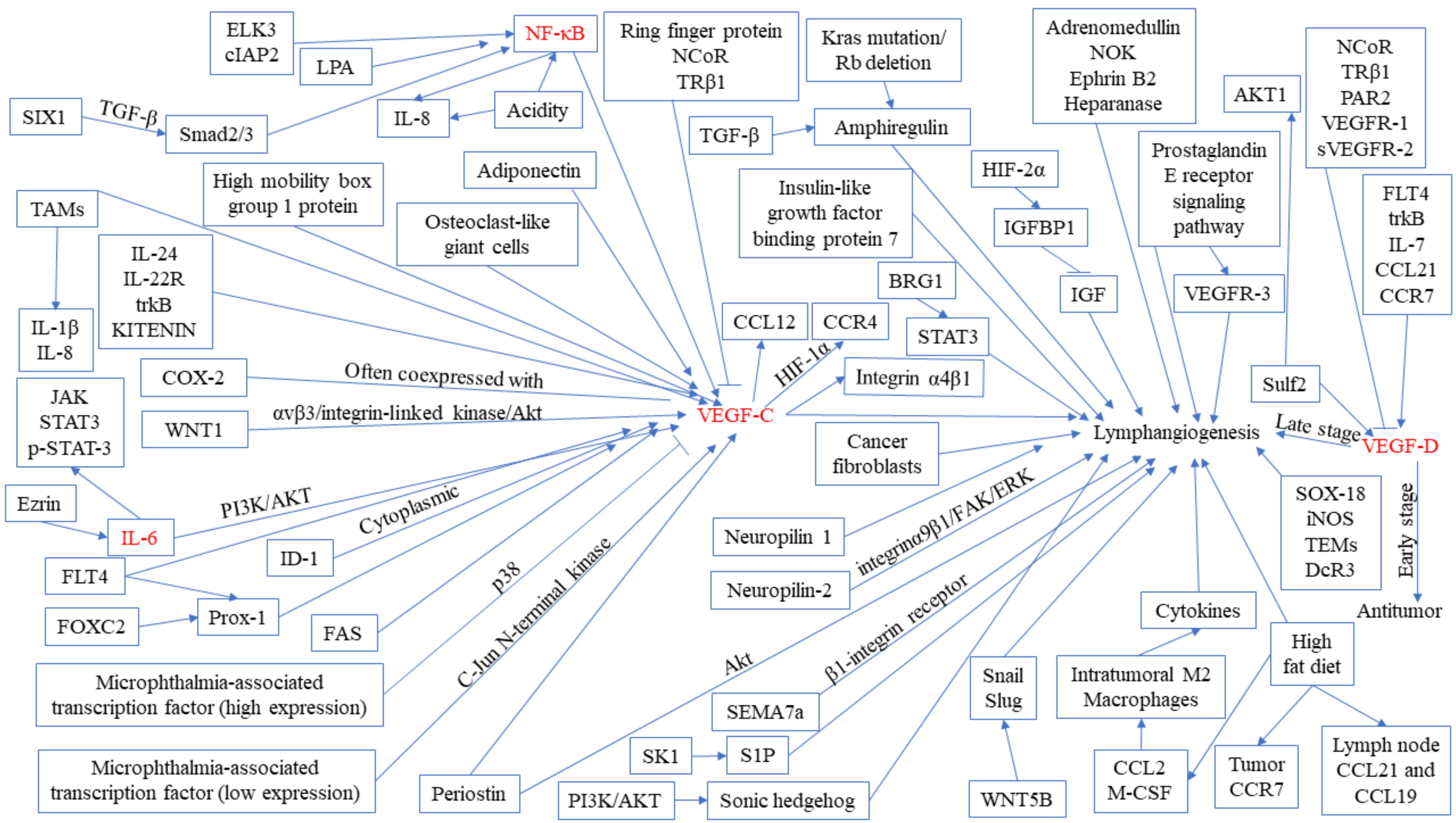


Figure 2. A simplified overview of the factors discussed in section 2.2 that contribute to lymphangiogenesis. Abbreviations can be found in the footnotes and in section 2.2. Items in red have central roles or are implicated across several discussed cancers.

| Cancer | Markers |
|---|---|
| **Gastric** | VEGF-C [18] |
| | IL-6 [19] |
| | Ring finger protein 180 [20] |
| | ID-1 [21] |
| | Sonic hedgehog [22] |
| | iNOS [23] |
| **Melanoma** | VEGF-C [24,25] |
| | FAS [24] |
| | Microphthalmia-associated transcription factor [25] |
| | c-Jun N-terminal kinase and p38/mitogen-activated protein kinase [25] |
| | Integrin α4β1 [26,27] |
| | FLT4 [28] |
| | High fat diet – CCL18, CCL21/CCR7 axis [29] |
| | SOX-18 [30] |
| **Cervical** | VEGF-C [31] |
| | SIX1 [31] |
| | TAMs [32] |
| | VEGFR-3 [33] |
| **Breast** | VEGF-C [34–37] |
| | IL-6 [34] |
| | Ezrin [34] |
| | COX-2 [35,38] |
| | Prostaglandin E2 and E receptor [35] |
| | NCoR and TRβ1 [36] |
| | TAMs [39,40] |
| | IL-24 [41] |
| | ELK3 [37] |
| | S1P [42] |
| | SEMA7a [43] |
| | DcR3 [44] |
| | Sulf2 [45] |
| | Mast cell density [46] |
| **Squamous Cell Carcinoma** | COX-2 [47,48] |
| | WNT5B [49] |
| | WNT-1 inducible signaling pathway protein-1 [50] |
| | Prox-1 [51] |
| | FOXC2 [51] |
| | VEGF-D [52] |
| | Neuropilin 1 receptor [53] |
| | IL-6 [54] |
| | VEGF-C [47,54–56] |
| | Periostin [55] |
| | ID-1 [57] |

| | |
|---|---|
| | A-smooth muscle actin protein [58] |
| | NF-κB [56] |
| | Notch1 [56] |
| | Cancer-associated fibroblasts [58] |
| **Gallbladder** | VEGF-C [59] |
| | Tropomyosin-related kinase B [59] |
| | TNF-α [60] |
| | cIAP2 [61] |
| **Colon** | VEGF-C [62,63] |
| | BRG1 [64] |
| | Integrin α4β1 [62] |
| | KITENIN [65] |
| | Smads [31] |
| | High mobility group box 1 protein [63] |
| | Neuropilin-2 [66] |
| **Lung** | VEGF-C [67] |
| | IL-7 [68] |
| | IL-1α [69] |
| | CCR7 [70] |
| | CCL21 [70] |
| | PDGF [71] |
| | TAMs [40] |
| | Insulin-like growth factor binding protein 7 [72] |
| | Prostaglandin $E_2$ [67] |
| | Adrenomedullin [73] |
| **HeLa Tumors/Ovarian** | Osteoclast-like giant cells [74] |
| | Podoplanin [75] |
| | NOK [76] |
| **Chondrosarcoma** | Adiponectin [77] |
| **Pancreatic** | Heparanase [78] |
| | Ephrin B2 [79] |
| | PAR2 [80] |
| | Angiopoietin-1 and angiopoietin-2 [81] |
| | Kras and Rb gene and Kras and INK4a [82] |
| **Neuroblastoma** | sVEGFR-2 [83] |
| **Prostate Cancer** | CD151 [84] |
| | Obesity [85] |
| | Caveolin1 [86] |
| **Hepatocellular Carcinoma** | HIF-1α [87] |
| | HIF-2α [87] |
| **Non-Specifically Studied** | LPA and receptors LPA1-3 [88] |

Table 1. A list of the markers discussed in section 2.2. The markers are arranged by the cancers in which they were studied. Abbreviations can be found in the text and within the footnotes.

Lymphatic changes in cancer may occur due to a variety of chemical changes and signals. Often, vascular endothelial growth factor (VEGF)-C expression is an important player in lymphatic vessel growth in cancer. VEGF-C expressed in tumor cells binds to and activates its receptor, vascular endothelial growth factor receptor (VEGFR)-3 found on lymphatic endothelial cells. After activation of VEGFR-3, Homeobox transcription factor HOXD10 is activated, and is involved in lymphatic endothelial cell migration and formation of cord-like structures, as it regulates the expression of VE-cadherin, claudin-5, and nicotinamide adenine dinucleotide phosphate oxidase 3 (NOS3) [89]. Examples of cancers that have upregulated VEGF-C include melanoma, squamous cell carcinomas, gastric, cervical, breast, gallbladder, colon, lung, ovarian, chondrosarcoma, pancreatic, neuroblastoma, and prostate cancers [18–20,24,25,28,32,34–36,41,47,50,51,54,55,59,62,63,71,90]. Many variables can increase VEGF-C expression in cancers. Additionally, other factors, molecules, and enzymatic variations that contribute to lymphatic changes in cancer are discussed in more detail below. Sections are divided based on types of cells involved in the lymphangiogenesis process.

### *2.2.1. Cancer Cell Involvement in Lymphangiogenesis*

Many times, cancer cells themselves are expressing or emitting factors and molecules that contribute to lymphangiogenesis. A major way cancers promote lymphangiogenesis is through the expression of VEGF-C. One factor that can contribute to VEGF-C upregulation is fatty acid synthase (FAS), a protein that catalyzes the production of fatty acids from acetyl-CoA and malonyl-CoA. FAS overexpression is correlated with a poor prognosis, and it may play a role in lymphangiogenesis, as melanoma mouse models treated with FAS inhibitors show a reduction in lymph node metastases due to decreased production of VEGF-C [24]. Mice given B16-F10 melanomas and treated with FAS inhibitors show a decrease in the volume of lymph node metastases by 39% and lower VEGF-C expression. Another factor, platelet derived growth factor (PDGF) is overexpressed in cancers [71]. In one study, about 65% of non-small cell lung cancer tissues had PDGF-BB or VEGF-C overexpession, and about 50% had overexpression of both factors simultaneously. Lymphatic microvessel density was significantly higher in cancers with PDGF-BB and VEGF-C overexpression, along with increased likelihood of lymph node metastasis and significantly shorter survival.

Other factors that can contribute to lymphangiogenesis include hypoxia inducible factors (HIFs). In hepatocellular carcinoma, HIF-1α expression is associated with increased lymphatic metastases [87]. HIF-2α silencing also leads to lymphangiogenesis, as it typically inhibits lymphangiogenesis by inducing insulin-like growth factor binding protein 1 (IGFBP1). Insulin growth factor (IGF) increases lymphangiogenesis, and IGFBP1 inhibits IGF. Interestingly, silencing of Kangai 1 (KAI1) COOH-terminal interacting tetraspanin (KITENIN) leads to decreased expression of VEGF-A, HIF-1α, and VEGF-C, but is significantly associated with lymph node metastases in colon cancer [65]. KITENIN positive tumors are not associated with higher lymphatic vessel density, indicating that perhaps it may only contribute to metastases via angiogenesis. HIFs also play a role in managing chemokine expression. Chemokines and chemokine receptors involved in lymphangiogenesis include chemokine receptor (CCR)4 and chemokine ligand (CCL)12 [27]. VEGF-C increases CCR4 expression on lymphatic endothelial cells, HIF-1α regulates its expression, and CCL12 acts as a chemoattractant for lymphatic endothelial cells by stimulating lymphangiogenesis via protein kinase B (AKT) and extracellular-signal regulated kinase (ERK) phosphorylation.

Integrins and their receptors play a major role in lymphangiogenesis. Lymphatic vessel expression of integrin α4β1 is increased by the presence of tumors and lymphangiogenic growth factors, such as VEGF-C [26]. Integrin α4β1 was recently found to be a member of the VEGF-C/VEGF receptor pathway [62]. Lv et al. examined the role of integrin α4 in colon cancer and its relationship with VEGF-C expression and lymphangiogenesis [62]. Expression of integrin α4, VEGF-C, and VEGFR-3 were increased in the colon cancer tissues, and lymphatic microvessel density was increased in cancerous versus non-cancerous tissues. There is a positive correlation between integrin α4 and VEGF-C, and integrin α4 and VEGF-C are significantly associated with poorer prognosis. Integrin α4β1 promotes survival and proliferation of lymphatic endothelial cells near the tumor, contributing to lymphatic metastases. Wingless-type mouse mammary tumor virus (MMTV) integration site family, member 5B (WNT)5B is upregulated in oral squamous cell carcinoma with high lymphatic metastatic potential, regulates Snail and Slug proteins through WNT signaling, and induces tube formation [49]. Snail and Slug are proteins involved in epithelial to mesenchymal transition [91]. This, in turn, induces lymphangiogenesis. WNT-1 inducible signaling pathway protein-1 regulates VEGF-C expression via the αvβ3/integrin-linked kinase/Akt pathway [50]. Semaphorin 7a (SEMA7a) is a glycophosphatidylinositol membrane anchored protein, and it likely causes increased lymphangiogenesis due to activation of the β1-integrin receptor [43]. Breast cancers with SEMA7a upregulation are associated with decreased overall survival and increased distant metastases. Additionally, neuropilin-2 activation in lymphatic endothelial cells increases lymphangiogenesis through activation of integrinα9β1/focal adhesion kinase/ERK (integrinα9β1/FAK/ERK) pathway signaling [66]. Periostin, a ligand for the αvβ3/integrins, has also demonstrated the propensity to induce lymphangiogenesis mediated through the AKT pathway and cause VEGF-C upregulation [55].

Interleukins also affect lymphangiogenesis. Interleukins of interest in breast cancer and lymphangiogenesis include interleukin (IL)-24 and IL-6. IL-24 is a cytokine that binds to IL-22R, and its expression is reduced in breast cancer cells [41]. Reduced expression is seen in populations with increased lymphatic metastases. Decreased expression of IL-24 and IL-22R in tumor samples have increased markers for lymphangiogenesis, including podoplanin, prospero homeobox protein (Prox)-1, and lymphatic vessel endothelial receptor (LYVE)-1, a lymphatic vessel marker. Introducing IL-24 to cells reduced VEGF-C and VEGF-D expression. IL-8 is also important. Lysophosphatidic acid (LPA) and its receptors (LPA1-3) can be overexpressed in cancer, and they may contribute to lymphangiogenesis, as LPA increases IL-8 expression by activating the nuclear factor (NF)-κB pathway in endothelial cells [88]. *In vitro*, it was determined that LPA-induced lymphangiogenesis and IL-8 production are a result of the LPA2 receptor in lymphatic endothelial cells. IL-8 production may also be enhanced in acidic microenvironments [92]. Acidosis in the cancer microenvironment increases lymphatic endothelial cell growth, and there are increased mRNA levels of IL-8 in lymphatic endothelial cells in acidic environments. Acidity also increases NF-κB activation, and inhibition of NF-κB decreases IL-8 expression. IL-7 also increases lymphangiogenesis by increasing VEGF-D and inducing the c-Fos/c-Jun pathway [68].

In efforts to understand what increases VEGF-C expression in melanoma, Puujalka et al. examined 22 human melanoma cell lines and determined that there is a negative correlation between mRNA expression of VEGF-C and microphthalmia-associated transcription factor [25]. Patients with high VEGF-C expression and low microphthalmia-associated transcription factor expression had a significant increase in metastasis development. C-J N-terminal kinase and p38/mitogen-activated protein kinase are implicated in this inverse relationship. Increased c-Jun N-terminal kinase signaling is seen in VEGF-C low and microphthalmia-associated transcription factor high melanoma. This melanoma is highly proliferative with low lymphangiogenesis. However, when p38 signaling is predominant in the tumors, the opposite is seen, with a VEGF-C high and microphthalmia-associated transcription factor low melanoma that is metastatic and has increased lymphangiogenesis.

Cyclooxygenase- (COX-)2 has been implicated in promoting lymphangiogenesis and contributing to lymph node metastases [48]. Often, COX-2 and VEGF-C are co-expressed, and co-expression is significantly correlated with lymphangiogenesis, metastases within the lymphatic system, and density of the lymphatic vessels located proximally to the tumor [47]. Co-expression of VEGF-C and COX-2 acts as a negative prognostic factor in oral tongue cancer [47]. COX-2 affects metastases in post-partum breast cancer, as it contributes to lymphangiogenesis in the mammary gland [38]. Another study in breast cancer shows that COX-2 can stimulate cancer cell invasion and produces VEGF-C, leading to increased lymphangiogenesis from prostaglandin $E_2$ activation of prostaglandin E 1 and 4 receptors [35]. Insulin-like growth factor binding protein 7, prostaglandin $E_2$ and prostaglandin E receptor signaling pathways contribute to lymphangiogenesis. IGFBP7 is associated with increased metastases and a higher lymphatic vessel density [72]. Prostaglandin $E_2$ and prostaglandin E receptor signaling pathways are involved in lymphangiogenesis, as prostaglandin E receptor signaling increases the expression of VEGF-C and VEGFR-3 [67]. Celecoxib, a COX-2 inhibitor, decreases both VEGFR-3 and podoplanin expression [67].

Smads also play a role in lymphangiogenesis. In a cervical cancer model, sine oculis homeobox homolog 1 (SIX1) promotes lymphangiogenesis by enhancing transforming growth factor-β (TGF-β) activation of Smad2/3, thereby increasing VEGF-C expressing tumor cells [31]. Smads direct signals from extracellular TGF-β receptors to the nucleus. In colon cancer, smad4 acts in the TGF-β signaling pathway and inhibits lymphangiogenesis, likely by decreasing VEGF-C secretion [90]. Inhibition of Smad3 phosphorylation also affects lymphangiogenesis [93]. Treatment with lithium inhibits glycogen synthase kinase 3 β (GSK3β) activation in colon cancer; this in turn inhibits Smad3 phosphorylation and reduces transforming growth factor β induced protein (TGFβIp) expression in colon cancer cells, decreasing lymphangiogenesis and lymphatic endothelial cell migration. Gore et al. found that lymphangiogenic genes and lymphatic endothelial cells are present in higher numbers in pancreatic ductal adenocarcinomas that have mutated Kirsten rat sarcoma viral oncogene homolog (Kras) and deleted retinoblastoma gene (Rb) and in models with mutated Kras and deleted INK4a [82]. Pancreatic tumors with a Kras mutation and Rb deletion secrete amphiregulin, a prolymphangiogenic factor. It was noted that TGF-β1 increases amphiregulin secretion in these pancreatic cells, though this does not hold true for pancreatic cancer cells without Smad4 [82].

Other factors that have demonstrated a propensity to increase lymphatic metastases include inhibitor of differentiation- (ID)-1, NF-κB, Notch1, sex determining region Y-box 18 (SOX-18), ephrin B2, heparin sulfate proteoglycans, and protease-activated receptor 2 (PAR2). ID-1 is a protein that assists in cellular activities throughout the cell life [94]. ID-1 also plays a role in tumor growth, as ID-1 silencing decreases lymphangiogenesis and VEGF-C expression [57]. Further, in tissue samples with well-differentiated cancer cells, ID-1 is primarily expressed in the nucleus; however, in samples with poorly differentiated cancer cells, the ID-1 expression mainly occurs within the cytoplasm [21]. This data clearly indicates that nuclear expression inhibits lymphangiogenesis and angiogenesis, and the opposite effects are seen with cytoplasmic expression, with increased lymphangiogenesis and angiogenesis. In esophageal squamous cell carcinoma, NF-κB and weak expression of Notch1 are present in tumors that develop lymph node involvement [56]. NF-κB and Notch1 expression are significantly inversely correlated with each other, and this expression is significantly associated with VEGF-C expression, metastases within the lymphatic system, and lymphangiogenesis [56]. SOX-18 expression may be important in lymphatic metastases, as suppression of SOX-18 decreases the rate of metastases and reduces lymphatic vessel density in melanoma [30]. Although SOX-18 is necessary for lymphangiogenesis in embryos, it also appears to be important in tumor-associated lymphangiogenesis, as it is re-expressed in lymphatic vessels after tumor-induced lymphangiogenesis. Ephrin B2, a membrane anchored protein, binds to the receptor, ephrin B4, to stimulate angiogenesis and lymphangiogenesis [95]. This process occurs normally, as well as in some disease states. Administration of an anti-ephrin B2 antibody decreases pancreatic tumor growth and VEGF-induced vascularization in xenograft mice [79]. Heparan sulfate proteoglycans are part of the extracellular matrix, and they have increased expression in several cancer types [78]. Increased expression is positively correlated with stage, grade, and metastases. Heparanase increases invasiveness of tumors, and heparanase elevation significantly increases lymphangiogenesis proximally to the tumor in the tumor microenvironment. PAR2 is a G protein-coupled receptor that is highly expressed in pancreatic cancer cells [80]. *In vitro* tube formation assays show that although PAR2 does not inhibit the tube forming of lymphatic endothelial cells, it inhibits cancer cell induced tube formation. Although its presence increases tumor growth, it limits lymphatic metastases and tumor-induced lymphangiogenesis.

Cobec et al. showed that podoplanin is important in promoting ovarian cancer metastasis [75]. Podoplanin expression in ovarian carcinoma tumor cells was compared to peritumoral and intratumoral lymphatic density. Density of proliferating lymphatics intratumorally positively related to proliferating tumor vessels peritumorally and number of mature vessels intratumorally. Peritumoral microvessel density of proliferating lymphatics correlate with peritumoral mature vessels. Additionally, proliferating tumor cells at the invasive front have higher expression of podoplanin, indicating that podoplanin assists in tumor progression and metastasis to the lymphatic system. Further, hypermethylation of ring finger protein 180 DNA promoter is significantly associated with metastases to the lymph nodes, and there is a negative correlation between ring finger protein 180 expression and lymph node metastases [20]. Ring finger protein decreases podoplanin expression and lymphangiogenesis in mice, as well as hepatocyte growth factor, matrix metalloproteinase 2 and 14, and VEGF-C and VEGF-D.

One signaling molecule that plays a role in lymphangiogenesis is sphingosine-1-phosphate (S1P). S1P is a sphingolipid produced by sphingosine kinase-1 (SK1) [42]. S1P is produced intracellularly, and it affects cells via autocrine and paracrine mechanisms [42,96]. SK1 is overexpressed in several tumor types, and in 3-D collagen matrices, S1P causes endothelial cell sprouting, indicating overexpression of SK1 can contribute to lymphangiogenesis [42]. In patients with breast cancer, S1P levels in tumor tissues are significantly higher in patients with high white blood cell counts, patients without human epidermal growth factor receptor 2 (HER2) overexpression, and in patients who had cancer tissues with high phospho-SK1 expression [97]. Significantly higher levels of S1P are seen in tumor tissues of patients with lymph node metastases [97].

Fms related tyrosine kinase 4 (FLT4) is a tyrosine kinase receptor for VEGF-C and VEGF-D that has been studied in melanoma, and it is important for lymphangiogenesis. When VEGF-C secreted by tumors binds to this receptor, lymphatic vessels can form, and tumor metastases may be initiated [28]. After administration of an FLT4 antagonist in a melanoma model, there was decreased tumor size and metastases in the lungs, fewer proliferative lymphatic vessels in the lungs in the intratumoral region, and a decrease in proliferating melanoma cells compared to untreated mice. In tissue with melanoma, there were higher amounts of FLT4 and tumor necrosis factor α (TNF-α), and lymphatic markers, including FLT4, VEGF-C, and Prox-1, had marked reductions in mice treated with an FLT4 inhibitor. TNF-α is associated with inflammation in cancer, and is involved in lymphatic metastasis [60]. Its expression is positively correlated with VEGF-D expression in gallbladder cancer patients, and VEGF-D can lead to an increase in lymphangiogenesis and lymph node metastases. Prox-1 and forkhead box (FOX)C2 expression affect lymphangiogenesis and angiogenesis [51]. Prox-1 expression is significantly correlated to stage of the tumor, lymphatic vessel density, metastases to the lymph nodes, and a poorer prognosis. *In vitro*, Prox-1 regulates growth and proliferation, invasiveness, and lymphangiogenesis. The effects of Prox-1 on lymphangiogenesis are regulated through VEGF-C. Higher immunoreactivity of FOXC2 is associated with an increase in microvessel density and worse prognosis. FOXC2 causes an increase in Prox-1 expression, and it can cause angiogenesis by increasing VEGF-A.

Farahani et al. examined the role of neuropilin 1 receptor in dysplastic epithelium and cutaneous squamous cell carcinoma [53]. Their findings show neuropilin 1 receptor is expressed in differentiated cells in the skin epithelium and squamous cell carcinoma, and upregulation is significant in oral squamous cell carcinoma and oral epithelial dysplasia. In non-cancerous tissue, the expression is similar to normal skin epidermis, though it is upregulated in dysplastic tongue epithelium and oral squamous cell carcinoma in both basal and proliferating epithelia. This is interesting, as this phenomenon not observed in cutaneous squamous cell carcinoma. In a xenograft with HSC3, a human oral squamous cell carcinoma from a cervical lymph node with high neuropilin 1 receptor levels, there was extensive lymphangiogenesis in the tumor.

Recent studies have examined the effects of tropomyosin-related kinase B (trkB) signaling and cellular inhibitor of apoptosis 2 (cIAP2) expression in lymphatic metastases of gallbladder cancer. TrkB increases gallbladder cancer invasion, expression at the invasive front is correlated with more advanced tumors, and survival decreases with increasing expression [59]. Signaling from activated trkB increases invasion, resultant from the induction of epithelial mesenchymal transition and activation of matrix metalloproteinases-2 and -9. Inhibition of trkB decreases VEGF-C and VEGF-D, tumor growth, lymphangiogenesis, and tumorigenesis. Additionally, cIAP2 expression was recently correlated with negative patient outcomes in gallbladder cancer [61]. It acts as a lymphangiogenesis factor for cancer cells and promotes lymph node metastases by activating the NF-κB pathway [61].

Interestingly, tumor cell type and hormones can be associated with VEGF-C tumor expression. For example, Hatano et al. showed HeLa tumors that contain osteoclast-like giant cells form larger tumors in xenograft mouse models, and lymphangiogenesis and macrophage infiltration in these tumors occur more rapidly compared to tumors without osteoclast-like giant cells [74]. Quantitative PCR shows VEGF-C mRNA expression is increased in tumors with these cells; therefore, one of the ways osteoclast-like giant cells within tumors stimulate lymphangiogenesis is by increasing VEGF-C in the microenvironment. Additionally, adiponectin, a hormone produced by differentiated adipocytes, has been studied in chondrosarcoma. Some research has indicated that high levels of adiponectin contribute to tumor stage, increased angiogenesis, and adds to a pro-metastatic tumor environment [77]. Increased adiponectin is associated with higher VEGF-C expression and tumor stage in patients. Huang et al. investigated the process by which lymphangiogenesis is promoted by adiponectin in human chondrosarcoma cells and found that the increase in VEGF-C expression in response to adiponectin is facilitated via the calmodulin-dependent protein kinase II, AMP activated protein kinase, and p38 signaling pathway.

Other key factors that increase lymphangiogenesis by altering VEGF-C expression include nuclear receptor corepressor 1 (NCoR) and the thyroid hormone receptor β1 (TRβ1), E26 transformation-specific (ETS) domain-containing protein Elk-3 (ELK3), and high mobility group box 1 protein. Ncor and TRβ1 decrease the invasiveness of tumors [36]. Martinez-Iglesias et al. studied the means by which this occurs and found that NCoR and TRβ1 decrease VEGF-C and VEGF-D expression in breast cancer cells, decreasing lymphangiogenesis and lymph node metastases in a tumor xenograft mouse model. They are negatively associated with lymphangiogenic genes and LYVE-1 in breast cancer tumors. ELK3 suppression inhibits triple negative breast cancer metastasis. Oh et al. examined the mechanism behind this with a triple negative breast cancer cell line, MDA-MB-231, with suppressed ELK3 [37]. Peritumoral lymphatic vessels did not develop in the ELK3 suppressed tumors due to decreased NF-κB signaling, which leads to lower VEGF-C expression. High mobility group box 1 protein is a chromatin protein in the nucleus that supports transcription. Li et al. examined colon cancer samples taken from patients, and presence of this protein significantly increased with VEGF-C expression in cancer tissues [63]. Due to increased VEGF-C expression, lymphatic vessel density and lymph node metastases are increased with high mobility group box 1 protein expression. *In vitro* studies show high mobility group box 1 protein upregulates VEGF-C mRNA expression and VEGF-C protein. This upregulation is dose dependent and is facilitated through NF-κB.

VEGFRs are also important players in lymphangiogenesis in cancers. Soluble vascular endothelial growth factor receptor (sVEGFR)-2 is a lymphangiogenesis inhibitor. In advanced neuroblastoma, sVEGFR-2 can be downregulated, and the degree of downregulation is correlated to disease progression [83]. In metastatic neuroblastoma, there is downregulation of the lymphangiogenesis inhibitors VEGFR-1 and sVEGFR-2. In cervical cancer, VEGFR-3 expression is significantly associated with peritumoral lymphatic vessel density and lymph node metastases, which is correlated to a poorer prognosis [33].

Factors and proteins important for normal cellular function that contribute to lymphangiogenesis in cancer include caveolin-1, CD151, and adrenomedullin. Caveolae are necessary for standard cellular activities, such as signal processing, lipid transport, gene regulation, and pathway activation [98]. Formation of caveolae require caveolin-1 and polymerase I and transcript release factor/cavin-1 [86]. Caveolin-1 is has increased expression in prostate cancer tissue compared to normal tissue, and overexpression contributes to tumor aggressiveness. When polymerase I and transcription release factor are absent from prostate cancer cells, there is a significant increase in tumor progression and metastasis because both angiogenesis and lymphangiogenesis are increased. A protein, cluster of differentiation (CD)151, is located on cell surfaces. In both tumor cells and normal cells, it regulates cellular migration [99]. In cancer, it enhances invasiveness, and higher expression levels are associated with an increased lymphatic vessel density [84]. Additionally, studies in a mouse model show that there is significantly higher CD151 expression and lymphatic vessel density in tumors that metastasize compared to tumors without metastases. Prognosis is poorer with increasing CD151 expression. Adrenomedullin is another factor that causes lymphatic endothelial cell proliferation [73]. Overexpression in tumors is associated with increased lymphangiogenesis, proliferation of lymphatic endothelial cells, and enlarged lymphatic vessels [73]. Karpinich et al. demonstrated that adrenomedullin secreted by tumors is important in tumor and lymph node lymphangiogenesis.

Protein kinase expression can also increase lymphangiogenesis in some cancers. NOK (serine/threonine/tyrosine kinase 1 STYK1) is a tyrosine protein kinase involved in tumorigenesis and tumor metastasis [76]. Liu et al. showed that increased expression of NOK helps the occurrence of angiogenesis and lymphangiogenesis. HeLa cells with and without NOK expression were implanted into nude mice. Blood vessels in HeLa tumors with NOK expression were higher, and there is an increase in lymphatic vessels intratumorally and peritumorally compared to HeLa tumors without NOK expression.

Other pathways, factors, and ligands that contribute to lymphangiogenesis include activation of the sonic hedgehog pathway, the nitric oxide pathway, sulfatase 2 (Sulf-2), and decoy receptor 3 (DcR3). Activation of the sonic hedgehog pathway signaling occurs in several tumor types, and in gastric cancer, expression of sonic hedgehog is associated with lymphatic metastases and higher lymphatic vessel density [22]. It is thought that the phosphoinositide-3-kinase (PI3K)/AKT pathway may play a role in sonic hedgehog pathway activation, as inhibition of PI3K/AKT decreases the activity of matrix metalloproteinase 9 and blocks lymphangiogenesis and epithelial-mesenchyme transition. A recent study has demonstrated that the nitric oxide pathway may play a direct role in lymphangiogenesis in gastric cancers as well [23]. Inducible nitric oxide synthase (iNOS) expression is associated with lymphangiogenesis. Expression of iNOS in non-cancerous tissues is lower than expression in gastric carcinoma, and its expression is positively correlated with cancer presence within the lymphatics and increased lymphatic vessel density throughout and around the tumor. Sulf2 increases lymphangiogenesis in in breast cancer because of its regulatory role in VEGF-D expression [45]. Sulf2 significantly increases formation of lymphatic endothelial cells. Additionally, AKT1 is upregulated by Sulf2, and thus it increases lymphangiogenesis and lymphatic metastatic potential though VEGF-D and AKT1. DcR3, a Fas ligand that protects endothelial cells from apoptosis, is overexpressed in some cancers [44]. One study found that in 92% of breast cancer tissues, DcR3 is overexpressed in both the tumor tissue and the vascular endothelial cells. Additionally, lymphatic microvessel density increases as DcR3 overexpression increases.

*2.2.2. Macrophage and Mast Cell Involvement in Lymphangiogenesis*

Interestingly, macrophages play a role in lymphangiogenesis. Tumor associated macrophages (TAMs) are implicated in the upregulation of lymphangiogenesis. Initially in cervical cancer, it was shown that TAMs may be responsible for increasing lymphangiogenesis and lymphatic metastases, as mRNA levels of IL-1β, IL-8, VEGF-C, and VEGF-A are increased when macrophages are co-cultured with cervical cancer cells [32]. This was further studied in breast cancer [39]. Tyrosine kinase with Ig and EGF homology domains-2 (TIE-2) is an angiopoietin receptor. TIE-2-expressing monocytes (TEMs) are immunosuppressive cells, and TIE-2 and VEGFR pathways promote TEM-related immunosuppression. In breast cancer, TEMs have been shown to express LYVE-1, podoplanin, VEGFR-3, and Prox-1, and contribute to lymphangiogenesis. Fagiani et al. determined the effects and roles of angiopoietin-1 and -2 in tumor angiogenesis and lymphangiogenesis [81]. Angiopoietin-1 acts as a stabilizing factor, decreasing vascular permeability, and angiopoietin-2 is responsible for angiogenic sprouting. Individual expression of angiopoietin-1 or -2 caused peri-insular lymphatic vessels to form without changes in blood vessel density. When angiopoietin-1 or -2 are expressed, there is an increase in peritumoral lymphangiogenesis without metastases to lymph nodes or organs. Angipoietin-1 expressing tumors do not increase blood vessel density; however, angiopoietin-2 expressing tumors show a decrease in blood vessel functionality and a presence of highly permeable endothelia with hemorrhagic tumors.

Additionally, TAMs can assist in promoting tumor growth. This was initially demonstrated in lung adenocarcinoma where researchers show that TAMs have an M2-polarized subtype. Zhang et al. demonstrated that the presence of TAMs is correlated to worse outcomes due to lymphangiogenesis and lymphatic metastases [40]. A more recent study in breast cancer shows that a mechanism by which M2 macrophages promote metastasis is by producing chitinase 3-like protein 1 (CHI3L1) [100]. Further, tumors with higher metastatic potential overexpress IL-1α, and IL-1α overexpression promotes the activation of lymphangiogenesis through crosstalk with macrophages [69].

Other immune cells that may be related to lymphangiogenesis are mast cells. Mast cell density within the tumor microenvironment is a sign of tumor progression [46]. With increasing tumor size and volume, there is a positive correlation in mast cell density in lymph nodes with metastases. Positive correlations are seen between mast cell density, lymphatic vessel growth and density, and cancer presence in within lymphatic vasculature. Sometimes, apoptosing cancer cells can contribute to lymphangiogenesis via macrophages [101]. During tumor cell death, S1P is released and TAMs exposed to S1P produce lipocalin 2, promoting lymphangiogenesis, enabling the survival of caner by providing an avenue for metastases [101].

Diet may also affect lymphangiogenesis in cancer models. A study with a B16F10 allograft model shows a high fat diet contributes to melanoma growth, metastases, and lymphangiogenesis [29]. Mice had increased lipid vacuoles in the tumor and M2 macrophages. Lymph node CCL19 and CCL21 levels were increased, and CCR7 in tumors increased. Intratumoral M2 macrophages increase with increasing CCL2 and macrophage colony-stimulating factor (M-CSF) in response to a high fat diet. This allows for crosstalk between tumor cells and M2 macrophages, potentiating additional cytokines and lymphangiogenic promoters. Mature adipocytes therefore activate the CCL19, CCL21/CCR7 axis which is shown to increase lymph node metastases by inducing ERK 1/2 and AKT phosphorylation, enhancing VEGF-D expression [70]. Although obesity is associated with an increased risk of acquiring cancer, in prostate cancer, lymphangiogenesis may be inhibited in obese patients. In one study, Lyve-1 expression was negatively correlated with body weight and epididymal fat, indicating obesity may inhibit metastases via the lymphatics in prostate cancer [85]. The mechanism behind this is unclear, though Moreira et al. determined that the mechanism is independent of leptin or insulin.

VEGF-D has demonstrated conflicting roles in various cancers. Honkanen et al. hypothesized that VEGF-D may have different effects based on tumor stage, and they studied this phenomenon in squamous cell carcinoma. In early stage skin tumors, VEGF-D decreased type 2 T helper cell (Th2) response and increased m1/Th1 and Th17 polarization [52]. This decreases inflammation, promotes an environment that is anti-tumor, and allows for some tumor regression. However, in later stages of cancer, VEGF-D enables lymphangiogenesis and increases risk of metastases [52].

*2.2.3. T Cell Involvement in Lymphangiogenesis*

IL-6, a pro-inflammatory cytokine secreted by T cells and macrophages, is implicated in cancer progression and lymphangiogenesis [102]. IL-6 signaling plays a role in lymphatic metastasis development in oral squamous cell carcinoma [54]. Increased IL-6 and VEGF-C mRNA expression in patients are significantly correlated with lymph node metastases. IL-6 regulates VEGF-C mRNA levels through the PI3K/AKT pathway. In a xenograft mouse model, an anti-IL-6 receptor antibody showed decreased VEGF-C mRNA expression and lymphangiogenesis related to the tumor. Treatment also inhibited tumor metastases to the lymph nodes, indicating that IL-6 may play a role in increasing lymphatic metastases. Additionally, IL-6 and its receptors are expressed in many gastric cancer cell lines [19]. Exogenous IL-6 increases proliferation and invasion, VEGF-C production, and lymphangiogenesis in human dermal lymphatic endothelial cells. IL-6 administration causes an increase in Janus kinase (JAK), signal transducer and activator of transcription 3 (STAT3), phosphorylated-STAT3 (p-STAT-3) and VEGF-C protein levels. This indicates that IL-6 is responsible for lymphangiogenesis, tumor invasion, and tumor growth by acting on the JAK-STAT3-VEGF-C pathway [19]. ATP-dependent chromatin remodeler SMARCA4 (BRG1) is part of the switch/sucrose non-fermentable (SWISNF) chromatin-remodeling complex, and affects lymphangiogenesis through binding to STAT3 and modulating its activation [64]. Tumors with lower BRG1 expression have increased lymphatic vessel density, and its expression level is inversely associated with lymphatic metastases. Cancers with ezrin expression are associated with poorer outcomes, and in breast cancer cell lines, ezrin demonstrates the ability to regulate lymphangiogenesis by modifying STAT3, VEGF-A, VEGF-C, and IL-6 expression [34].

*2.2.4. Fibroblast Involvement in Lymphangiogenesis*

Cancer-associated fibroblasts promote cancer progression and have been studied in oral squamous cell carcinoma patient samples [58]. Samples were evaluated for lymphatic vessel density, microvessel density, and α-smooth muscle actin. In nearly 70% of tumors, there is positive expression of α-smooth muscle actin protein. The presence of this protein is significantly correlated to tumors with increased severity, lymphatic metastases, and lymphatic vessel density. In a nude mouse model, cancer-associated fibroblasts increase tumor cell invasion and significantly increase angiogenesis and lymphangiogenesis associated with the tumor compared to mice injected with oral squamous cell carcinoma cells alone. This study is fascinating, as it highlights the importance of other cancer-related cells in lymphangiogenesis.

### 2.3. Preparing for metastasis in the lymphatic system

Before cancers reach the sentinel lymph node, the tumors start a process called "preparing the soil." Hypervascularization of lymph nodes occurs before metastasis begins, and both lymphatic vessel lumen diameter and lymph nodes increase in size [15,103,104]. Additionally, it has been shown that the sinusoidal lymphatic endothelium grows before cancer reaches the lymph nodes, which may assist in cancer stem cell survival within the lymph nodes [15,104,105]. Tumors have increased interstitial pressure, high enough to passively push the cancer cells through the lymphatic vessels and into the sentinel lymph node [15]. Upon colonizing the lymph node, lymph flow may be blocked due to congestion at lymphatic sinuses caused by tumor metastasis. The decreased lymph flow allows tumor cells the chance to proliferate within the lymph node and lymphatic vessels [15]. Once cancer cells are in the sentinel node, they can continue to release factors that promote remodeling in the lymphatics downstream, increasing lymphatic vessel and lymph node size [16]. After metastasizing into lymph nodes, cancer cells may be able to enter the blood stream in the nodal sinuses, providing an additional avenue for metastasis [16]. Targeting and delivering drugs to cancer cell populations in the lymph nodes is critical to prevent further dissemination of the malignancy through the lymphatic system and to distant organs.

## 3. LYMPHATIC DRUG DELIVERY ESSENTIALS

### 3.1. Importance of drug delivery to lymphatics

In patients with cancer, lymphatic metastasis is associated with poorer prognosis [106]. Depending on cancer type, lymph node metastases from solid tumors outside the lymphatics begin in stage I to stage III, disseminating from the primary tumor. Along with treatment for the cancer, removal of affected lymph nodes is recommended to attempt to stop further cancer spread throughout the lymphatics or into other tissues and organs [107]. Solid tumors likely prefer to metastasize through the lymphatics compared to blood vessels because the lymphatic vasculature has wider vessel lumens, a decreased pressure gradient, a slower fluid flow, and increased endothelial permeability [15,16,108]. Additionally, throughout cancer progression, lymphatic vessels located proximally to the tumor undergo extensive remodeling that favor and enable cancer metastasis through this route, as discussed in the previous section [16].

Lymphatic involvement is an important indicator of disease progression and outcome, and there is poorer prognosis for patients with cancer after their cancer has metastasized to the lymphatic system. For example, in melanoma, an increased number of lymph nodes positive for metastases correlates with decreasing five year survival rates [106]. One staging system used in cancer is called the TNM system, where T is for the extent of the tumor, N is classification of lymph node involvement, and M is related to metastases [109]. If there is no cancer present in any lymph nodes, the N classification is N0. N1 indicates that one lymph node has cancer. Subclassifications include N1a to denote micrometastases and N1b for macrometastases. N2a indicates that there are 2 to 3 lymph nodes with micrometastases, and N2b is used when there are 2 to 3 lymph nodes involved and at least one has a macrometastasis. N2c is used to describe cancer without lymph node involvement when satellite or in transit metastases are observed. When the cancer has metastasized to four or more lymph nodes, matted lymph nodes are present, or satellite metastases or in transit metastases with involvement of at least 1 lymph node is seen, the cancer is staged at N3. The poorer prognosis related to lymphatic metastases may be due to difficulty treating the cancer that is located within the lymph nodes and lymphatic vasculature.

Cancer is not the only disease state in which the lymphatics play a role. Several other disease states, including Milroy's disease, human immunodeficiency virus (HIV), and psoriasis, involve the lymphatic system [110–112]. Targeting drugs directly to the lymphatics may be able to improve outcomes in diseases that either compromise the lymphatics or are harbored within the lymphatic system; however, getting free drug molecules into the lymphatics is challenging. In theory, targeting drugs to the lymphatic system can directly treat problems related to the lymphatics, something that would be extremely beneficial to those with diseases in the lymphatics or an aberrant lymphatic system; in practice, direct drug delivery to the lymphatic system is quite difficult, based on the nature of common medications. The limitations that prevent drugs from entering the lymphatic system are discussed in the following sections.

### 3.2. Lymphatic delivery limitations and challenges

According to Bayer, more than 90% of all drugs available on the market are small molecules [113]. Chemotherapeutic regimens typically use small molecule drugs that are administered systemically, such as via the intravenous or oral route [107,114]. These drugs are unable to reach adequate concentrations in the lymphatic system to treat cancer cells that are in transit or that have metastasized to lymph nodes [115–117]. Chen et al. showed that in patients with breast cancer, carboplatin administered intravenously has very low nodal accumulation [115]. Supersaxo et al. shows that small molecules administered subcutaneously have minimal uptake into the lymphatics [116]. Another study by Wilson et al. shows intravenously administered small molecules demonstrate very little accumulation in lymph nodes [117]. Because many solid tumors use the lymphatic system as the primary means for metastasis, this is a major downfall of standard therapies that are currently in place [107,114]. Additionally, since drugs are unable to reach therapeutic concentrations in the lymphatic system, the lymphatics can act as a reservoir for cancer cells [118].

Several factors influence lymphatic uptake. Limitations to lymphatic uptake include route of administration, molecular weight, size, charge, and hydrophobicity, and will be discussed in the following sections.

#### *3.2.1. Route of Administration*

Subcutaneous, intramuscular, or intradermal administration shows greater lymphatic uptake compared to orally or intravenously administered drugs [115,117,119]. Systemically administered small molecules are unable to be taken up lymphatically in clinically significant concentrations [114,115,117,119]. Peyer's patches are areas of lymphatic tissues in the intestines that may come into contact with orally administered substances. These tissue nodules assist in monitoring the bacterial flora in the intestines, and play an immunological role, mounting a response should any pathogenic bacteria overgrowth occur [120]. Uptake of drugs through the Peyer's patches is more difficult compared to uptake through initial lymphatics, as Peyer's patches have a basal lamina and tight junctions [120]. Administration of drugs intravenously also bypasses the lymphatics, leaving regional lymph nodes with low drug concentrations [114,115]. Once in the blood stream, drugs will reach target organs and enact their effects without coming into contact with the lymphatic system in clinically relevant concentrations [115,117].

Using a subcutaneous delivery method, instead of orally or intravenously administered drugs, ought to show increased uptake into the lymphatics because injection into the subcutaneous layer increases interstitial pressure, which in turn should cause the lymphatics to open from their collapsed state and drain the excess fluid from the area [107,114]. Additionally, the lymphatic anatomy is advantageous to drainage as well. The lymphatic capillaries have larger gaps between endothelial cells compared to blood vessels and lack a basement membrane to assist in drainage in the presence of interstitial pressure [1,7]. This will contribute to the removal of injected material from the site of administration. A study by Chen et al. demonstrated that carboplatin administered subcutaneously compared to intravenous administration increased the lymphatic uptake in breast cancer patients [115]. Additionally, Wilson et al. demonstrated a slight advantage to administering free small molecules subcutaneously versus intravenously [117].

After drugs are administered subcutaneously, they can enter the lymphatic system through three main mechanisms – transcellular uptake by binding to external receptors on lymphatic vessels; phagocytosis or by attachment onto dendritic cells; and passive paracellular uptake [107]. Transport by dendritic cells is more likely to occur when materials are large (>70 kDa or >50 nm), have too much of a negative charge or positive charge (<-47 mV or >56 mV demonstrated by Lunov et al.), or are hydrophobic, as they are restricted to the injection site and may have problems moving though interstitial water channels [107,121–123]. When substances are taken up by dendritic cells, they may not be able to exert their pharmacological effect against their target within the lymphatics [124]. Similar to subcutaneously administered medications, substances given intradermally can drain through lymphatic capillaries located in the dermal space and reach the lymph nodes [125,126]. Intramuscular administration of drugs has also been shown to facilitate greater lymphatic uptake compared to intravenous administration, though not as much as subcutaneous administration [107,119,127].

Another method that can be used for lymphatic drug delivery is intralymphatic injections. Intralymphatic injections are not commonly done, as they can cause lymph node damage. Repeated doses to lymph nodes cause nodal structure damage and formation of scars [128]. In a study by Randomski et al, intralymphatic ports were given to patients for dendritic cell infusions (Figure 3). Ports lasted for an average of 7.5 weeks. Although this study shows that it is feasible to use intralymphatic ports for direct drug delivery, there are some downsides. For example, the ports must be replaced often. Additionally, in a cancer model, this may not be the best option for treatment as the lymphatic system has one-way flow and should there be any in transit cancer cells before reaching the lymph node, the cell population would not be treated. Drugs must be administered upstream of metastases in the lymphatics or benefits may not be seen. Further, this study looked at the delivery of dendritic cells. Most drugs are small molecules and would likely perfuse out of the lymph node and nearby lymphatic vasculature before making much of an impact due to their size and molecular weight limitations [113].

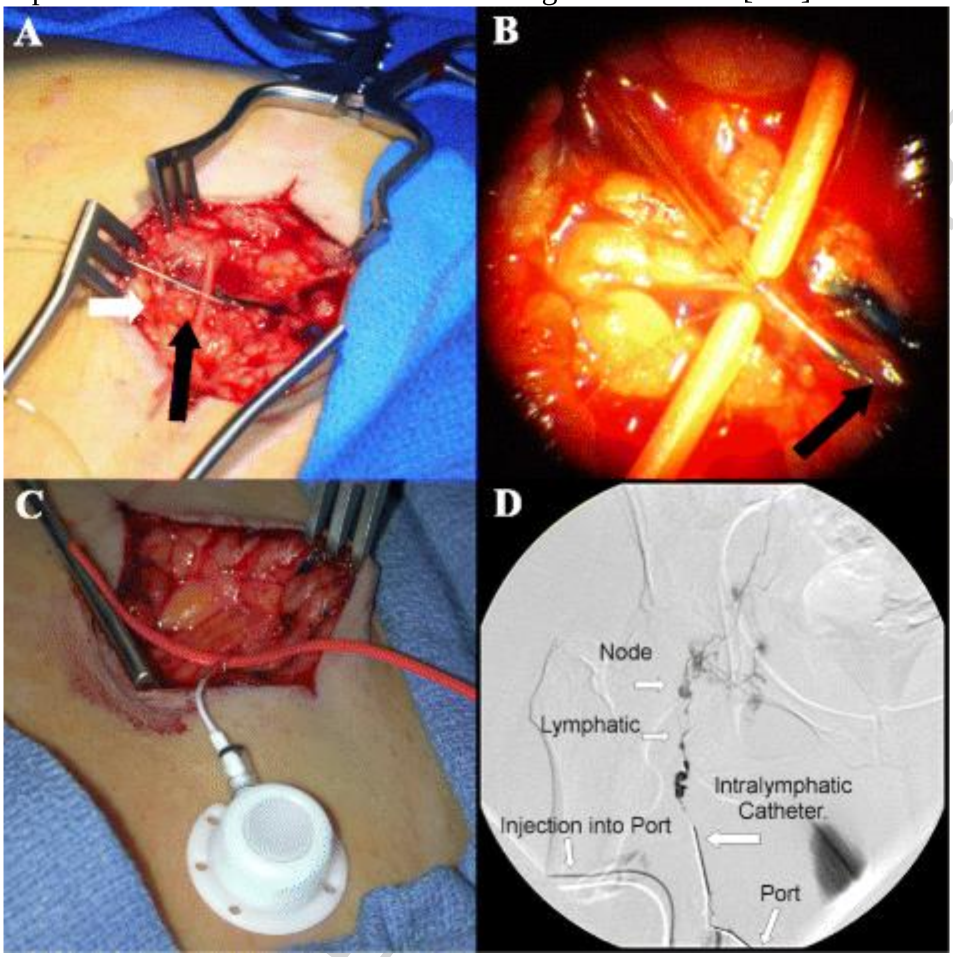


Figure 3. The procedure for intralymphatic port placement. A) Image of femoral vessels. The white arrow is showing the femoral lymphatic vessel surrounded with a vessel loop. The black arrow shows the cannula to be placed in the lymphatic vessel. B) Image through operative microscope. Black arrow is showing the cannula as it enters the lymphatic vessel. C) Intralymphatic port after lymphatic cannulation, before placement in the subcutaneous pocket. D) Lymphangiogram of subcutaneous right femoral lymphatic port. Image demonstrates patency of port. Contrast material is entering the lymphatics through the port and is seen in inguinal lymph nodes. Figure obtained through open access distributed under the terms of Creative Commons Attribution 4.0 International License (license link http://creativecommons.org/licenses/by/4.0/). Reprinted from Journal for ImmunoTherapy of Cancer, 4:24, Michal Radomski, Herbert J. Zeh, Howard D. Edington, James F. Pingpank, Lisa H. Butterfield, Theresa L. Whiteside, Eva Wieckowski, David L. Bartlett, and Pawel Kalinski, Prolonged intralymphatic delivery of dendritic cells through implantable lymphatic ports in patients with advanced cancer, 2016 [128].

Isolated limb perfusion (ILP) is another method for permeating the lymphatics with therapeutic agents. ILP is a treatment strategy for cancer that is located solely on a limb. The limb is isolated through clamping and cannulating the major artery and vein to the limb and using a tourniquet to compress the other vessels [129]. Treatment is given directly to the affected limb. During the procedure, intracompartmental pressure is increased from 13 mmHg up to a maximum of 90 mmHg and measured carefully (Figure 4) [130]. This technique is useful for tumors, such as melanoma, that have in transit metastases in the lymphatic vessels. Oloffson el al. showed that of patients treated with ILP with in transit melanoma metastases, 65% had a complete response and 20% had a partial response [129]. Perhaps one reason why ILP is beneficial in this setting is the increase of pressure in the limb causes the initial lymphatics to open and forces the small molecule chemotherapeutics into lymphatic vessels in the affected area. A shortcoming of this treatment is it is only useful for cancers that are located on the limb, and would not be useful for tumors, metastases, or micrometastases in other areas.


Figure 4. Probe tip and machine used in isolated limb perfusion to monitor compartmental pressure. Reprinted from European Journal of Surgical Oncology, Volume 22, Issue 2, Peter Hohenberger,Lothar H. Finke,Peter M. Schlag, Intracompartmental pressure during hyperthermic isolated limb perfusion for melanoma and sarcoma, pages 147-151, 1996, with permission from Elsevier [130].

### *3.2.2. Molecular Weight of Administered Substances*

Particles with a molecular weight greater than 16,000 Daltons have been shown to preferentially accumulate into the lymphatics [116]. In one study, mitomycin C, a small molecule, administered intramuscularly was not detected in the lymphatics 30 minutes after administration

[119]. However, when it was conjugated to dextran, molecular weight increased and accumulation in the lymph nodes was seen through approximately 48 hours. As dextran molecular weight increased, accumulation in the lymph nodes increased. Another study by Bagby et al. examined lymphatic uptake of particles with varying molecular weights of hyaluron conjugated to a near infrared dye [131]. Hyaluron molecular weights studied were 6.4 kDa, 35 kDa, 74 kDa, 132 kDa, 357 kDa, and 697 kDa. The 74 kDa hyaluron conjugate had the highest lymphatic uptake in the axillary, popliteal, and iliac nodes; larger molecular weights had less lymphatic uptake, indicating that there is both a lower limit and upper limit to how heavy particles can be for optimal lymphatic uptake. Molecular weight changes had a significant impact on the lymphatic uptake of the hyaluron and dye conjugate. Smaller molecular weight substances can be removed quickly by the blood stream. Substances with larger molecular weights accumulate at the site of injection and are cleared through the lymphatics, as larger gaps in the endothelial wall and lack of a basement membrane allow for easier clearance through lymphatic vessels than blood vessels.

### *3.2.3. Size of Administered Substances*

Ideally, substances should be sized between 10-80 nm for lymphatic uptake [114]. One study shows that nearly 75% of 40 nm liposomes are cleared from the subcutaneous injection site after administration; however, liposomes sized at 400 nm have less than 20% clearance from the injection site [132]. Particles smaller than 10 nm are more likely to be cleared from the injection site by blood vessels because, due to their small size, they can readily fit through the tight junctions in blood vessels [114]. Additionally, since the flow is greater in the blood vasculature, there is preferential uptake for particles less than 10 nm compared to the lymphatics [108]. This 10 nm size does not appear to be a strict cut off, as some proteins and dendrimers smaller than 10 nm can be taken up lymphatically, though small dendrimers are quicker to diffuse out of the lymphatics and into the interstitium [108,133]. Particles larger than 80 nm drain slowly from the site of injection. In some cases, particles may accumulate at the injection site because their size prevents them from entering either blood vessels or lymphatic vessels. These particles must be sequestered and cleared by dendritic cells [114,134–136]. Another study that demonstrates size is an important factor in lymphatic uptake is by Abellan-Pose et al. DiD labeled polyglutamic acid (PGA)-polyethylene glycol (PEG) nanocapsules with a mean size of 100 nm showed significantly higher fluorescence, and therefore accumulation, in livers, hearts, lungs, and kidneys of mice compared to those injected with 200 nm nanocapsules at 24 hours post administration [137]. Accumulation was also noted to be higher in mesenteric, axillary, mediastinal, and cervical lymph nodes in mice treated with 100 nm nanocapsules compared to mice treated with 200 nm nanocapsules.

### *3.2.4. Charge of Administered Substances*

Particle charge has been shown to play a role in lymphatic uptake of substances. In one study by Hawley et al, poly(lactide-co-glycolide) (PLGA) nanoparticles with and without a poly(lactic acid) (PLA) and PEG coating had charges of -36 mV and -15 mV, respectively [138]. Nanoparticles with the -15 mV charge were detected in larger quantities in the lymphatics compared to nanoparticles with a -36 mV charge [138].

Another study by Doddapaneni et al. loaded nanoparticles made from different ratios of methoxy poly(ethylene glycol)$_{5,000}$-*block*-poly(ε-caprolactone)$_{10,000}$ (mPEG-PCL) and carboxy poly(ethylene glycol)$_{5,000}$-*block*-poly(ε-caprolactone)$_{10,000}$ (cPEG-PCL) [124]. mPEG-PCL nanoparticles alone had a charge of -6 mV, cPEG-PCL nanoparticles alone had a charge of -36 mV, and a 60:40 ratio of mPEG-PCL:cPEG:PCL had a charge of -19 mV. Nanoparticles were then loaded with drugs to treat NRAS mutant melanoma and administered proximal to the inguinal lymph node to determine efficacy against lymph node metastases. The mPEG-PCL nanoparticles were efficacious the inguinal lymph node near the site of administration, demonstrating that particles with a -6 mV charge can enter the lymphatics. The cPEG-PCL nanoparticle had no effect on lymph node metastases, indicating that at -36 mV, the nanoparticle was likely taken up by macrophages or sequestered by other dendritic cells due to its highly negatively charged nature. The -19 mV nanoparticle was efficacious against nodal metastases both at the inguinal lymph node and the axillary lymph node, indicating that nanoparticles with this charge can repel and distribute throughout the lymphatic system without being too highly charged, avoiding immediate phagocytosis by dendritic cells at the site of administration. Cationic substances can also be used for lymphatic delivery, but are associated with increased toxicity to nonphagocytic cells [139,140].

### *3.2.5. Hydrophobicity of Administered Substances*

Hydrophobicity of administered substances also impacts lymphatic drainage from the site of injection and lymph node retention. It has been shown that increasing hydrophobicity causes an increase in phagocytosis, and opsonins are attracted to hydrophobic surfaces [121,122]. Therefore, increasing the surface hydrophilicity will increase the amount of free substance that is available for lymphatic uptake that does not get seized by phagocytic cells [141]. Hawley et al. demonstrated this with PLGA nanoparticles with and without a PEG + PLA coating [138]. Nanoparticles made from only PLGA are highly hydrophobic and had limited uptake into the lymphatic system. PLGA nanoparticles given a PEG + PLA coating had increased drainage from the site of injection, increased detection in the lymph nodes, and increased lymphatic retention times. A study by Rao et al. also examined the effects of hydrophobicity of compounds and the relationship to lymphatic uptake [142]. This study compared lymphatic uptake of hydrophobic polystyrene nanoparticles (PS) to similarly sized nanoparticles made from PLGA-PMA:PLA-PEG (PP) with increased hydrophilicity. The nanoparticles were injected into the dorsal surface of the rat footpad. The cumulative lymph node AUC over 48 hours post injection was significantly higher for the PP nanoparticles compared to the PS nanoparticles of similar sizes. This study also indicates that increased hydrophilicity contributes to increased lymphatic uptake.

## **3.3. Passive and active lymphatic targeting delivery systems**

### *3.3.1. Passive Nanodelivery Systems*

Given the limitations of substances for optimal lymphatic uptake, the platforms that are commonly used to deliver drugs to the lymphatics include liposomes, micelles, and nanoparticles.

Liposomes are drug delivery vehicles that can be sized in the nanometer range and are composed of one or more phospholipid bilayers [143]. Hydrophilic drugs can be dissolved and loaded in the center of liposomes, and hydrophobic drugs can be loaded within the bilayer. Oussoren et al. evaluated subcutaneously administered liposomal uptake into the lymphatics on the basis of size, lipids used, and dose [144]. Liposomes less than about 150 nm entered the lymphatics, neutral liposomes had limited lymphatic uptake, and increasing doses did not have an effect on absorption. Oussoren's work demonstrates passive uptake into the lymphatics.

Micelles are made with amphiphilic molecules that have both a hydrophilic and hydrophobic portion [143]. They are made of a single layer, and hydrophobic drugs can be carried in the core of micelles. Chida et al. used epirubicin-loaded micelles made of poly(ethylene glycol)-b-poly(beta-benzyl L aspartate) to target breast cancer metastases in the axillary lymph nodes [145]. Epirubicin polymeric micelles had pH-triggered drug release and inhibited tumor growth and metastasis to the axillary lymph nodes. Micelles accumulated in the primary tumor and axial lymph node, and epirubicin was released proximally to the tumor due to the acidic microenvironment.

Another polymeric micelle composed of methyl poly(ethylene glycol)-distearoylphosphatidyl-ethanolamine (mPEG-DSPE) loaded with doxorubicin showed increased uptake of doxorubicin into A375 cells (51.2% free doxorubicin compared to 88.7% micelle) [146]. Micelles administered subcutaneously were taken up lymphatically and showed accumulation in draining lymph nodes. Doxorubicin can cause tissue damage; however, doxorubicin-loaded micelles had less tissue damage compared to doxorubicin alone. Weights of draining lymph nodes were lower in the micelle-treated group than the saline and doxorubicin treated groups, and micelles were able to eradicate tumor cells within the lymph nodes.

Zeng et al. created hybrid particles to target the lymph nodes with a cancer vaccine, keeping in mind that particles between 10 and 100 nm and a neutral or negative charge is preferred for lymphatic uptake with subcutaneous or intradermal injection [147]. Polymeric hybrid micelles were made with poly-(ethylene glycol) phosphorethanolamine (PEG-PE) and polyethylenimine-stearic acid conjugate (PSA). Melanoma antigen peptide tyrosinase related protein 2 (Trp2) and TLR-9 agonist were loaded into micelles with a < 30 nm size. Micelles made with equal parts PEG-PE and PSA were able to target lymph nodes near the site of injection and dendritic cells, and demonstrated an antitumor effect.

Polymeric nanoparticles are made out of block co-polymers with hydrophobic and hydrophilic properties, and hydrophobic drugs can be loaded into the hydrophobic nanoparticle core [143]. A study by Doddapeneni et al. shows that drug loaded PEG-PCL nanoparticles are able to passively target the lymphatic metastases after subcutaneous administration proximal to the site of the tumor [124]. Another nanoparticle prepared for lymphatic uptake is composed of methoxypoly(ethylene glycol)-b-poly(D L -lactic acid) (mPEG-PLA) and mixed poly(D L -lactic-co-glycolic acid) (PLGA/mPEG-PLA) [148]. These particles were used for delivery of a small molecule, resiquimod, that acts as an agonist for TLR7, an immunotherapy that is used for skin cancer topically, but has systemic toxicity if administered via any other route. These nanoparticles were taken up by dendritic cells and macrophages, activating an immune response against cancer. Though no toxicity was observed in immune cells after subcutaneous administration, there were cytotoxic effects against cancer.

Kishimoto et al. used nanoparticles composed of PLGA and PLA-PEG loaded with rapamycin [149]. Intravenous administration of this formulation with PEGylated uricase in mice and non-human primates inhibited antidrug antibody formation. In mice with uricase deficiency, uric acid levels were comparable to normalized mice. These nanoparticulates accumulated in the spleen, the lymphoid organ responsible for inducing tolerance in organisms after administration of the nanoparticle and antigen. When this formulation was administered subcutaneously with adalimumab (Humira) in TNF-α transgenic mice, there was inhibition of antidrug antibody formation. This drug administration strategy may be useful in administering biological anticancer agents, as often, antidrug antibodies can be blamed for treatment failure or adverse reactions associated with biologics.

An amphiphilic gold nanoparticle 5 nm in size coated with 1-octanethiol and 11-mercaptoundecanesulfonic acid was used to deliver a TLR7 ligand to tumor draining lymph nodes to act as an immunostimulant [150]. Nanoparticles were administered subcutaneously and caused local immune activation. There was a cytotoxic T-cell response that was stimulated against the tumor. The treatment inhibited growth of large tumors and increased survival time in groups treated with the nanoparticle compared to freely administered drug.

Several recent studies have utilized materials conjugated to polymers for lymphatic delivery. Mannose alginate nanoparticles have been used for dendritic cell targeting within the lymphatic system [151]. Using ovalbumin as a model antigen conjugated to mannose, Zhang et al. found these nanoparticles increased antigen uptake into bone marrow dendritic cells. It is important to the get the antigen to the lymph nodes so the adaptive immune response can be stimulated for cancer immunotherapy. When nanoparticles were labeled with Cy7, they demonstrated uptake in the lymph nodes after subcutaneous administration.

Research by Verbeke et al. used an injectable porous hydrogel to deliver BDC peptide in a mouse model of type 1 diabetes [152]. In this study, BDC was delivered either in PLGA microparticles or conjugated to alginate polymer. Pore-forming gels were loaded with GM-CSF gold nanoparticles and peptide loaded PLGA microparticles, and T cells in draining lymph nodes were examined. Antigen-specific $CD4^+$ T cells increased significantly by day 5. Though this study occurs in a noncancerous model, it indicates that platforms like this can be used to affect the presence of immune cells in draining lymph nodes, perhaps assisting in cancer vaccination.

A study that examined polymer hydrogel nanodelivery was conducted by De Koker et al [153]. Mesoporous silica particles were infiltrated with poly(methyacrylic acid) and disulfide crosslinked. PEGylation increased lymphatic drainage, demonstrated by Alexa Fluor 488-cadaverine labeling. To determine if antigen presentation to lymphatic T cells occurred with this platform, SIINFEKL, the MHC1 epitope of the antigen ovalbumin was conjugated to nanoparticles. PEGylated poly(methylacrylic acid) nanoparticles successfully delivered the peptide with increased antigen presentation compared to poly(methylacrylic acid) nanoparticles alone. PEGylation increased lymph node targeting. This type of hydrogel may be useful for cancer vaccine delivery directly to the lymphatic system.

Borrajo et al. targeted the lymphatic system with PGA-PEG nanocapsules [154]. Nanocapsules were 100 nm, and uptake into the lymphatic system via different dosing routes was measured. As determined by fluorescence, unsurprisingly, subcutaneous administration had greater uptake into the lymphatic system compared to nanocapsules administered via the intravenous route. In an orthotopic lung cancer model with lymph node metastases, docetaxel-loaded nanocapsules administered subcutaneously demonstrated better antitumor efficacy and less toxicity compared to standard docetaxel. Further, it nearly completely eradicated the cancer in mediastinal lymph nodes compared to regular docetaxel which demonstrated very little efficacy in lymph nodes.

Another study looked at lipid-based nanocapsules in a hydrogel for lymphatic targeting. Wauthoz et al. used a lauroyl derivative of gemcitabine and administered the formulation subcutaneously or intravenously to target the lymphatic system, decrease the toxicity associated with gemcitabine therapy, and fight against mediastinal metastases in a mouse model with orthotopic non-small cell lung cancer in immunodeficient mice [155]. The biodistribution study indicates that the nanocapsules were targeted to the lymph nodes, caused no significant myelosuppression compared to normal saline, and significantly prolonged survival compared to standard therapy and control. Nanocapsules administered intravenously had an elimination half-life of 19 hours; however, nanocapsules administered subcutaneously in the hydrogel had an elimination half-life of 32 hours. Biodistribution studies showed that nanocapsules delivered subcutaneously in the hydrogel had similar levels of accumulation in the local lymph nodes within the first 8 hours; however, there was much higher nodal accumulation at later time points through 336 hours compared to intravenously administered nanocapsules. Further, there was limited distribution of nanocapsules to other organs at any time point. Taken together with the half-life data, this indicates the nanocapsules administered in hydrogel subcutaneously have a controlled release profile and higher specificity for the lymphatics compared to intravenously administered nanocapsules. Nanocapsules as a drug delivery vehicle for lymphatic targeting have proven useful; however, size plays a role in how much reaches the lymphatics [137]. To study this effect, polyaminoacid nanocapsules were loaded with docetaxel [137]. 100 and 200 nm nanocapsules were compared. The polyaminoacid assisted with colloidal stability in biological fluids, and the 100 nm nanocapsules had adequate docetaxel loading with a sustained release profile. Biodistribution after nanocapsules were administered via subcutaneous injection show that 100 nm nanocapsules reach the lymphatics faster than 200 nm nanocapsules. Another nanocapsule strategy utilizes a polysaccharide shell. Polyglucosamine/squalene nanocapsules labeled with indium-111 were monitored for biodistribution profile after subcutaneous administration in rabbits [156]. These nanocapsules demonstrate slow clearance from the injection site and accumulate in draining lymph nodes, whereas free $^{111}InCl_3$ drained into systemic circulation. Nanocapsules formed a depot at the injection site and had slow lymphatic drainage and long lymphatic retention.

Carbon nanotubes have also been used for drug delivery to reach cancer in the lymphatic system. One study loaded gemcitabine in magnetic multiwalled carbon nanotubes and compared these to magnetic-activated carbon particles [157]. Magnetism was conferred by $Fe_3O_4$ on the outside of nanotubes, and nanotube bundles had a diameter of 40-60 nm. Subcutaneous administration of the nanotubes in the hind paw foot pad shrunk lymphatic metastases and inhibited lymph node metastases under magnetic field, and the nanotubes had increased efficacy compared to magnetic-activated carbon particles.

Several studies have looked at dendrimers as a means to increase drug uptake into the lymphatics. A study examined how much doxorubicin gets into the lymphatics after subcutaneous and intravenous administration when administered as the plain hydrochloride salt, in a PEGylated polylysine dendrimer (12 nm), in a PEGylated liposome (100 nm), and in different Pluronic micelles (5 nm) [158]. Results were determined in thoracic lymph duct cannulated rats. Micelles had poor stability *in vivo* and their pharmacokinetics were similar to those of doxorubicin hydrochloride. The dendrimer formulation increased doxorubicin in the thoracic lymph after intravenous and subcutaneous dosing. Liposomes were also better than plain solution, but much less effective than dendrimers. Draining lymph nodes of the injection site retained amounts of doxorubicin from the formulations in the following order: dendrimer > liposome > micelles and solution. Dextrans have proven to be quite useful in conjunction with nanodelivery systems. In a study with liposomes, dextrans inhibited macrophage uptake and increased the number of liposomes draining into the lymphatics [159]. Further, liposomes with dextran loaded with doxorubicin had less tissue damage compared to liposomal doxorubicin alone.

One factor that can affect the lymph node targeting capability of dendrimers is the length of the drug linker [160]. As dendrimers are injected subcutaneously, they can interact with the interstitium and draining into the lymphatics can be limited. To study linker length effects, methotrexate was conjugated to PEGylated dendrimers. Dendrimers with shorter linkers had higher drainage, likely due to increased PEG protection; however, dendrimers with shorter linkers have less retention in lymph nodes. Drainage of dendrimers with longer linkers can be increased with dextran coadministration. This allows for more retention in lymph nodes because of the presence of longer linkers, and increased drainage from the injection site due to the presence of dextran. PEGylation appears to be quite important for dendrimers that target the lymphatics. A study using polylysine dendrimers examined the effects of PEGylation on absorption and trafficking through the lymphatic system with various weights of PEG, as well as 4-benzene sulphonate [161]. Dendrimers were given either intravenously or subcutaneously. With increasing PEG length, there was decreased absorption into the blood and increasing amounts in the lymphatic system. The sulphonate dendrimers had difficulty draining from the subcutaneous tissue. Methotrexate-conjugated PEGylated polylysine dendrimers were then used to determine if there was increased efficacy against lymphatic metastases [162]. More dendrimers reached the lymphatics when administered subcutaneously rather than intravenously, and methotrexate conjugated dendrimers inhibited lymph node metastases effectively.

Polyamidoamin dendrimers conjugated to alkali blue (PANAM-AB) have been synthesized as a lymphatic tracer [163]. When administered subcutaneously, lymph nodes were stained blue after 10 minutes. Lymphatic absorption is rapid, and compared to methylene blue solution, water-in-oil microemulsion, and multiple microemulsion, PANAM-AB had increased lymph node AUC and longer lymphatic retention times. In another study, paclitaxel was loaded onto PANAM-AB dendrimers (PTX-P-AB) [164]. There was more absorption into the lymphatics compared to standard Taxol®, as well as increased AUC values in the lymphatics, longer retention in lymph nodes, and increased metastasis inhibition.

*3.3.2. Active Lymphatic Targeting Systems*

In section 3.3.1, nanodelivery using passive targeting was discussed. Nanocarriers with targeting ligands have also been developed. For example, a study by Kaur et al. used liposomes with surface modifications to increase absorption of zidovudine, a medication for HIV, into the lymphatic system [165]. Mannose was added as a targeting moiety to increase macrophage uptake into the lymph nodes and spleen. Their results conclude that mannose-coated liposomes had the most uptake lymphatically compared to plain liposomes and free drug.

One study by Wang et al. used polymeric micelles conjugated to tumor lymphatics-homing peptide (LyP-1) [166]. LyP-1 micelles were more targeted to tumor lymphatic vessels compared to polymeric micelles without the targeting agent, which tended to accumulate near blood vessels. Additionally, LyP-1 micelles had the most efficacy against tumors *in vitro*. In a study by Li et al, micelles composed of two amphiphilic diblock copolymers, polycaprolactone-polyethylenimine (PCL-PEI) and PCL-PEG, were loaded with Trp2 peptide and CpG oligodeoxynucleotide as an adjuvant [167]. Different ratios of cationic PCL-PEI were used, and it was determined that 10% w/w PCL-PEI had the best distribution into the lymph nodes and efficacy after subcutaneous administration. Further, this ratio demonstrated low toxicity against dendritic cells and efficacy in a B16F10 melanoma mouse model. Another study by Luo et al. used LyP-1 conjugated to PEG-PLGA nanoparticles [168]. The LyP-1 conjugated nanoparticles were compared to nanoparticles without LyP-1, and the LyP-1 nanoparticles had significantly higher distribution in metastatic lymph nodes compared to unconjugated nanoparticles.

Bahmani et al. used a polymeric nanoparticle drug delivery system comprised of PLGA-PEG and PLGA-PEG maleimide to deliver immune therapeutics to the lymphatic system to increase survival after cardiac allograft [169]. Nanoparticles carried anti-CD3 and were coated with MECA79 monoclonal antibody as a way to directly increase lymph node accumulation by targeting the lymphatic system, specifically. Nanoparticles were taken up by immune cells in the lymphatics, and mice with heart allografts treated with this therapy had longer survival rates and increased $T_{reg}$ immune cells in both grafted tissues and draining lymph nodes. Another study used MECA79 as a coating for microparticles loaded with tacrolimus [170]. It was given intravenously and, because MECA79 targets high endothelial venules in lymph nodes, there was accumulation in draining lymph nodes in animals with allografts.

A study by Thomas et al. used 30 nm polymeric nanoparticles stabilized with Pluronic F-127 to target intralymphatic dendritic cells [171]. Formulations were administered intradermally ipsilaterally to the tumor, and accumulation was seen in the tumor-draining lymph node. Nanoparticles with CpG or paclitaxel caused dendritic cells to mature *in vitro*. The same was seen *in vivo* in tumor draining lymph nodes. $CD4^+$ T cells were of a Th1 phenotype, and there was increased $CD8^+$ T cells in the tumor, slowing tumor growth. Additionally, the $CD8^+$:$CD4^+$ T cell ratio was significantly higher in tumor draining lymph nodes compared to control, indicating a lower risk for lymph node metastasis, making this a promising therapeutic strategy. In another study, Jeanbart et al. used nanoparticles conjugated to tumor-associated antigens or CpG as vaccines [172]. This study also showed that targeting the tumor draining lymph node with vaccines increased cytotoxic $CD8^+$ T cell responses locally and systemically compared to a nontargeting vaccine.

Schmid et al. used nanoparticles with antibodies that target and bind to $CD8^+$ T cells in lymphatic tissues, as well as blood and tumor tissues, in mice [173]. They used anti-PD-1 on the surface of PEG-PLGA nanoparticles to target PD-1 expressing cells, and delivered SD-208, an inhibitor of TGF-β signaling, to PD-1-expressing cells. This platform was also able to deliver a TLR7/8 agonist to the tumor. The CD8-targeting nanoparticles had some accumulation in the sentinel lymph node, and their concentrations in the lymph node increased over time, potentially indicating that the nanoparticles are passively accumulating in the draining lymphatics.

Potential shortcomings of nanodelivery systems include their relative novelty on the market, a time-consuming manufacturing process, difficult scale up procedures, and cost. According to C. Lee Ventola, there were only 60 approved drugs with nanomedicine components in the formulation as of 2017 [174]. A way to potentially overcome time-consuming manufacturing and assist in scale-up is the use of microfluidics, which can produce larger volumes of liquid nanomedicines more rapidly than traditional bench techniques and improve batch-to-batch consistency [175].

### *3.3.3. Microneedle Systems*

Microneedle intradermal delivery systems are used to deliver drugs or imaging agents to the lymphatic system by draining through the lymphatic capillaries in the dermal space. Harvey et al. used microneedles to deliver proteins into the lymphatics [125]. When the microneedles were loaded with dyes, they demonstrated clearance through the lymphatic system. Loading the microneedles with insulin increased the $C_{max}$ and resulted in a higher $T_{max}$ in swine. Using microneedles to deliver insulin dramatically changed the pharmacokinetic profile and allowed for uptake into the lymphatic system.

Another study by Yang et al. utilized microneedles and transferomes to increase lymphatic uptake of doxorubicin [126]. The platform included microneedles made of hyaluronic acid with the ability to dissolve. Transferomes were located on the needle tips and loaded with doxorubicin. When placed on rat skin, transferomes were released in the dermis as microneedles dissolved. Utilizing both transferomes and microneedles enhanced doxorubicin uptake into the lymphatics, as measured by fluorescence intensity.

A study by Aldrich et al. used a nanotopography device to increase etanercept uptake into the lymphatics [176]. Etanercept is a biologic used for rheumatoid arthritis that is typically administered subcutaneously and has poor lymphatic uptake. Nanotopography works by disrupting tight junctions between cells in the skin, thereby increasing the delivery of drugs to the lymphatic system. The nanotopography device used was a microneedle device with a polyether, ether, and ketone film (SOFUSA™ device) pictured in Figure 5. Compared to subcutaneous or intradermally administered drugs, nanotopography had increased efficacy, as well as higher uptake and retention in lymph nodes draining the area as determined by radiolabeled etanercept.

One microneedle vaccine strategy uses a hollow microneedle array for delivery of nanoparticles to the intradermal space. This system has been studied in rats and demonstrates a burst transit through lymph nodes that drain the site of application [177]. Further, there appears to be less systemic exposure compared to subcutaneous or intravenous administration. Niu et al. developed a vaccine with ovalbumin as the antigen and Toll-like receptor (TLR) agonists imiquimod and monophosphoryl Lipid A in PLGA nanoparticles to be administered through hollow microneedles. Because of the draining through lymph nodes, there was faster antibody affinity maturation, increased IgG2a, and more interferon-γ secreting lymphocytes, indicative of Th1 response. The microneedle-nanoparticle delivery system performed better than intramuscular injection or simply administering the antigen through microneedles without using nanoparticles.

Benefits of microneedle systems include controlled delivery of drugs, and delivery directly to the intradermal space where draining lymphatic capillaries can take up the drugs. Additionally, data in the above studies have consistently shown that microneedles allow for higher efficacy, lymphatic uptake, or lymphatic retention compared to controls. This form of drug delivery may be cost prohibitive, and scale-up can be difficult; however, newer, easier methods for microneedle fabrication are currently being researched as potential, cost-effective alternatives [178,179].

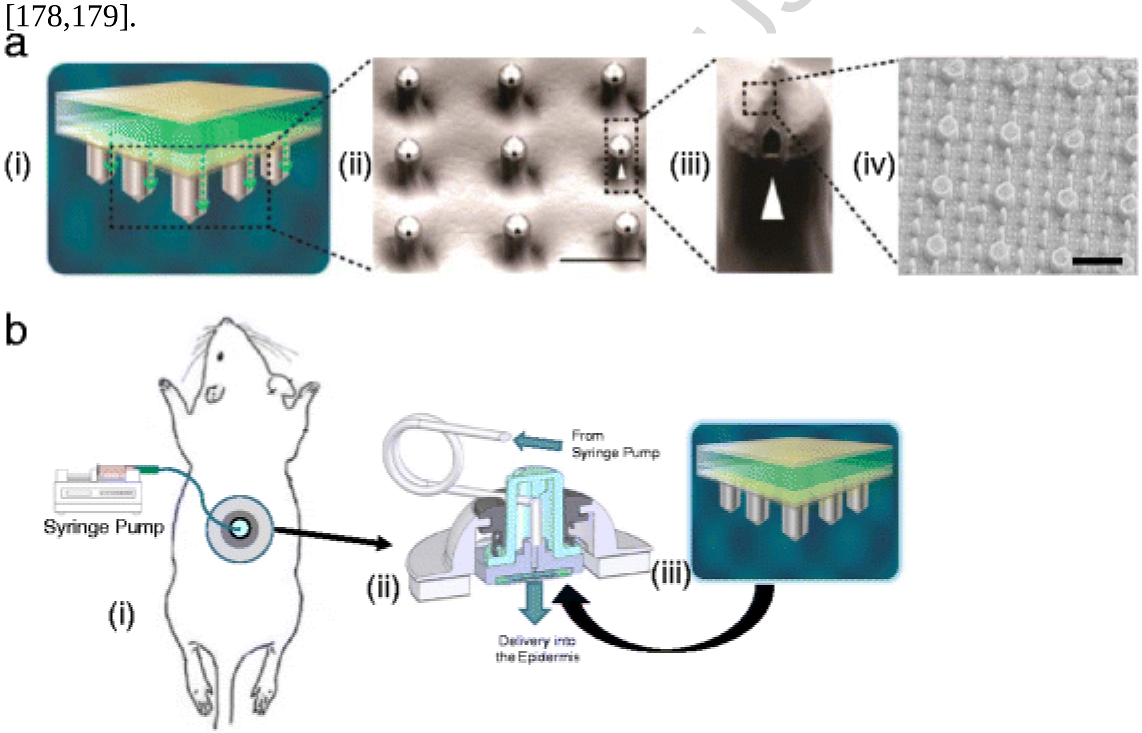


Figure 5. a) Image of the SOFUSA™ nanotopography device used to increase etanercept uptake into the lymphatics. (i) microfluidic block, with silicon microneedles on the bottom, attachment adhesive, microfluidic distributor, and attachment adhesive on top. Each array has 100 350 µm long and 110 µm wide microneedles with a 30 µm hole for drug delivery. (ii) Scanning electron microscopy (SEM) image of microneedles with nanotopographic film (scale bar 300 µm). (iii) SEM image of one microneedle. (iv) SEM image of nanostructures present on microneedles (scale bar 3 µm). b) (i) Device placement on back skin of rats. (ii) cross-section of nanotopography device. (iii) image of full microfluidic block. Figure obtained through open access distributed under the terms of Creative Commons Attribution 4.0 International License

(license link http://creativecommons.org/licenses/by/4.0/). Reprinted from Arthritis Research & Therapy, 19:116, Melissa B. Aldrich, Fred C. Velasquez, Sunkuk Kwon, Ali Azhdarinia, Kenneth Pinkston, Barrett R. Harvey, Wenyaw Chan, John C. Rasmussen, Russell F. Ross, Caroline E. Fife, and E. M. Sevick-Muraca, Lymphatic delivery of etanercept via nanotopography improves response to collagen-induced arthritis, 2017 [176].

*3.3.4. Other Methods of Drug Delivery to the Lymphatics*

Other research has been conducted using additional methods for targeting the lymphatic system. For example, monoclonal antibody aggregate uptake into the lymphatics has been studied. Subcutaneously administered protein aggregates were administered in mice [180]. Murine and human monoclonal immunoglobulin G1 (IgG1) were labeled with fluorescent dye, and aggregates were created. Biodistribution studies measured with *in vivo* fluorescence imaging demonstrated that aggregates less than 1 micron had increased uptake into lymph nodes one hour after injection compared to micron sized aggregates.

Gels have demonstrated the propensity to target and deliver drugs to the lymphatic system when formulated properly. For example, one study examined a polypeptide hydrogel for immunotherapy in melanoma [181]. The hydrogel consisted of injectable self-assembled PEGylated poly(L-valine) and was formulated as a 3D porous hydrogel that had the ability to recruit dendritic cells. Loaded in the hydrogel, tumor cell lysates as an antigen and a TLR3 agonist (polyinosinic:polycytidylic acid) were administered and demonstrated sustained release. Recruited dendritic cells were activated. The hydrogel allows for an increase in antigen time at the site of injection and increases the amount that reaches the lymph nodes. Subcutaneous administration causes a cytotoxic T-lymphocyte response and increases $CD8^+$ T cells in draining lymph nodes. This formulation demonstrated good efficacy against melanoma tumors *in vivo*.

Qiao et al. developed a nanovaccine with polyelectrolyte complexation of chitosan and heparin to encapsulate VP1 protein antigen. This antigen is found in the virus that causes hand foot and mouth disease. TNF-α or CpG were used as adjuvants [182]. The vaccine was prepared with flash nanocomplexation to both reduce size and size distribution of the particulates. After subcutaneous administration, distribution was noted in proximal and distal lymph nodes, and retention was noted within the lymphatic system. There was immune activation, and treatment demonstrated efficacy and protection against a lethal virus challenge.

A study by Muraoka et al. set out with the goal of developing a more effective vaccine for cancer [183]. A synthetic long peptide antigen was formulated in a nanogel composed of cholesteryl pullulan and injected subcutaneously in mice. The peptide drained to the local lymph nodes and was taken up my macrophages in the node medulla, demonstrated in Figure 6. Interestingly, the peptide was only taken up specifically by macrophages located in the medulla and not immune cells located elsewhere in the interstitium or lymph node. This formulation presented the antigen to $CD8^+$ T cells and was able to inhibit cancer growth.

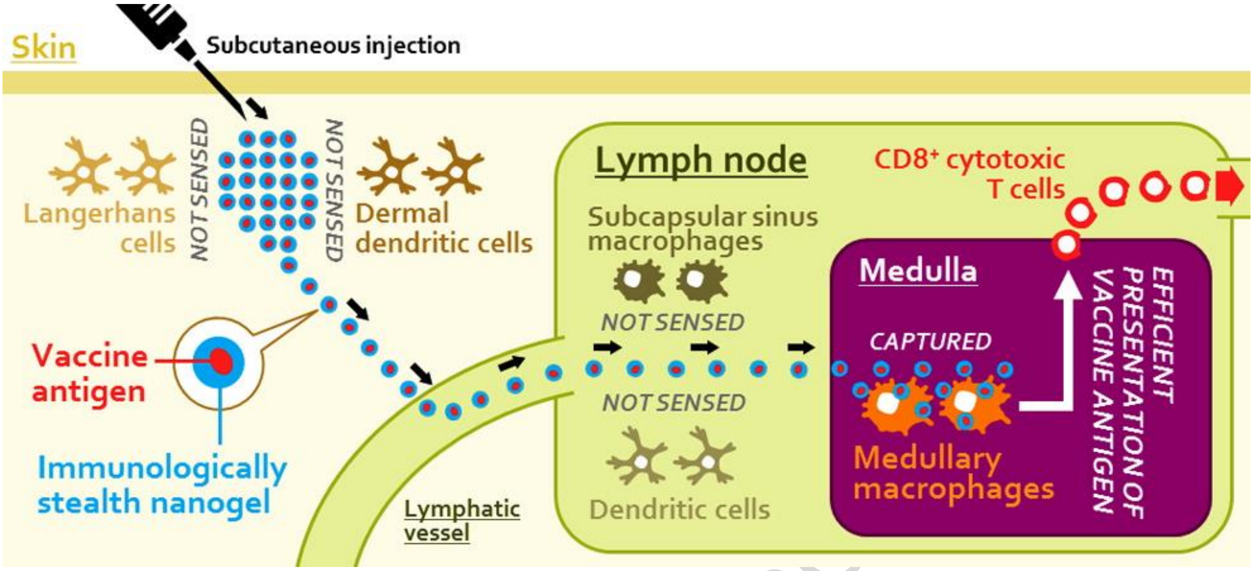

Figure 6. Illustration of stealth nanogel vaccine. Nanogel was administered subcutaneously in mice and is not sensed by immune cells in the skin or lymph node outside of the node medulla. In the node medulla, macrophages captured the vaccine and stimulated a $CD8^+$ T cell response. Reprinted with permission from D. Muraoka, N. Harada, T. Hayashi, Y. Tahara, F. Momose, S. Sawada, S. Mukai, K. Akiyoshi, H. Shiku, Nanogel-based immunologically stealth vaccine targets macrophages in the medulla of lymph node and induces potent antitumor immunity, ACS Nano. 8 (2014) 9209–9218. doi:10.1021/nn502975r. Copyright 2014 American Chemical Society. [183]

Microbubbles for theranostics have been developed. mRNA complexed to cationic liposomes were loaded into microbubbles for ultrasound-mediated drug delivery, with the goal of delivering mRNA to lymph nodes [184]. Subcutaneous injection in dogs showed microbubbles with and without the mRNA enter lymphatic vasculature and go to lymph nodes. These could be useful for imaging, diagnostics, and treatment.

Some research has examined lymphatic drug delivery through the oral route of administration. Though the lymphatic tissue in the intestines is thicker due to the presence of a basal lamina and is therefore less permeable than the initial lymphatics, an advantage to delivering drugs through this tissue is the potential of oral drug dosing. A study by Cao et al. attempted to formulate drugs to be taken up into the lymphatics through the intestines when given orally [185]. A liver-X receptor agonist, a treatment intended for atherosclerosis, has better uptake through the lymphatics when administered orally in formulations that contain a long chain lipid oleic acid in an emulsion. The long chain oleic acid assists in increasing lymphatic uptake of the drug because they are taken up by enterocytes, transformed into triglycerides, then converted to lymph lipoproteins. Additionally, this formulation allows for higher efficacy at lower doses.

## 4. CONCLUSION

The lymphatics play an integral role in several necessary physiologic processes, as well as numerous disease states, especially those with inflammation as a part of the pathology. In cancer, the lymphatic system is overgrown near the site of tumors, as cancer cells release factors, signaling molecules, and enzymes that promote lymphangiogenesis. These pro-lymphangiogenic molecules assist in the progression of cancer and metastasis of tumor cells to distant locations. As prognosis becomes poorer and overall survival decreases with lymphatic involvement, there is significant interest in delivering chemotherapeutic drugs to cancer within the lymphatic system. However, simply administering standard therapy options alone is not enough to treat cancer harbored in the lymphatics, as route of administration, weight, size, charge, and hydrophobicity of substances all affect lymphatic delivery, and standard, systemically administered treatments do not fit the necessary constraints for adequate lymphatic uptake. Using nanocarriers that meet the requirements for optimal lymphatic uptake as delivery options for chemotherapeutic agents may prove beneficial for treating lymphatic metastases and reaching cancer populations that otherwise would be undertreated with current therapies.

Although many treatments proposed within this review sound promising, few are used in practice. Studies with intralymphatic injections and isolated limb perfusion have been completed in humans as described in section 3.2.1; however, the many other methods of delivery are mainly studied in animals thus far. A major improvement that is needed in the field of lymphatic drug delivery is further development of the discussed methods. Developing formulations for translational and clinical use is necessary before understanding how beneficial these methods for lymphatic drug delivery will be in a patient population. Challenges to progress include expensive development and scale up for many of the methods discussed, as well as complicated or time-consuming manufacturing processes. These lymphatic delivery systems would not only have implications in cancer treatment, but they may be useful for delivery of drugs in other disease states that involve the lymphatics, such as HIV or psoriasis. The research discussed herein is promising; however, for the field to progress, there needs to be a greater push for taking steps to develop these platforms in a clinically relevant manner.

## CONFLICT OF INTEREST

The authors do not report any conflicts of interest for this article.

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| Cancer | Markers |
|---|---|
| **Gastric** | VEGF-C [18] |
| | IL-6 [19] |
| | Ring finger protein 180 [20] |
| | ID-1 [21] |
| | Sonic hedgehog [22] |
| | iNOS [23] |
| **Melanoma** | VEGF-C [24,25] |
| | FAS [24] |
| | Microphthalmia-associated transcription factor [25] |
| | c-Jun N-terminal kinase and p38/mitogen-activated protein kinase [25] |
| | Integrin α4β1 [26,27] |
| | FLT4 [28] |
| | High fat diet – CCL18, CCL21/CCR7 axis [29] |
| | SOX-18 [30] |
| **Cervical** | VEGF-C [31] |
| | SIX1 [31] |
| | TAMs [32] |
| | VEGFR-3 [33] |
| **Breast** | VEGF-C [34–37] |
| | IL-6 [34] |
| | Ezrin [34] |
| | COX-2 [35,38] |
| | Prostaglandin E2 and E receptor [35] |
| | NCoR and TRβ1 [36] |
| | TAMs [39,40] |
| | IL-24 [41] |
| | ELK3 [37] |
| | S1P [42] |
| | SEMA7a [43] |
| | DcR3 [44] |
| | Sulf2 [45] |
| | Mast cell density [46] |
| **Squamous Cell Carcinoma** | COX-2 [47,48] |
| | WNT5B [49] |
| | WNT-1 inducible signaling pathway protein-1 [50] |
| | Prox-1 [51] |
| | FOXC2 [51] |
| | VEGF-D [52] |
| | Neuropilin 1 receptor [53] |
| | IL-6 [54] |
| | VEGF-C [47,54–56] |
| | Periostin [55] |
| | ID-1 [57] |

| | |
|---|---|
| | A-smooth muscle actin protein [58] |
| | NF-κB [56] |
| | Notch1 [56] |
| | Cancer-associated fibroblasts [58] |
| **Gallbladder** | VEGF-C [59] |
| | Tropomyosin-related kinase B [59] |
| | TNF-α [60] |
| | cIAP2 [61] |
| **Colon** | VEGF-C [62,63] |
| | BRG1 [64] |
| | Integrin α4β1 [62] |
| | KITENIN [65] |
| | Smads [31] |
| | High mobility group box 1 protein [63] |
| | Neuropilin-2 [66] |
| **Lung** | VEGF-C [67] |
| | IL-7 [68] |
| | IL-1α [69] |
| | CCR7 [70] |
| | CCL21 [70] |
| | PDGF [71] |
| | TAMs [40] |
| | Insulin-like growth factor binding protein 7 [72] |
| | Prostaglandin $E_2$ [67] |
| | Adrenomedullin [73] |
| **HeLa Tumors/Ovarian** | Osteoclast-like giant cells [74] |
| | Podoplanin [75] |
| | NOK [76] |
| **Chondrosarcoma** | Adiponectin [77] |
| **Pancreatic** | Heparanase [78] |
| | Ephrin B2 [79] |
| | PAR2 [80] |
| | Angiopoietin-1 and angiopoietin-2 [81] |
| | Kras and Rb gene and Kras and INK4a [82] |
| **Neuroblastoma** | sVEGFR-2 [83] |
| **Prostate Cancer** | CD151 [84] |
| | Obesity [85] |
| | Caveolin1 [86] |
| **Hepatocellular Carcinoma** | HIF-1α [87] |
| | HIF-2α [87] |
| **Non-Specifically Studied** | LPA and receptors LPA1-3 [88] |

Figure 1. A brief schematic of lymphatic vessel anatomy, showing lymphatic capillaries, pre-collecting lymphatics with an intermittent smooth muscle layer, collecting lymphatics with a 3-layer cell wall, and location of lymph node macrophages. Lymph nodes are connected to collecting lymphatics and have a smooth muscle layer.

Figure 2. A simplified overview of the factors discussed in section 2.2 that contribute to lymphangiogenesis. Abbreviations can be found in the footnotes and in section 2.2. Items in red have central roles or are implicated across several discussed cancers.

Table 1. A list of the markers discussed in section 2.2. The markers are arranged by the cancers in which they were studied. Abbreviations can be found in the text and within the footnotes.

Figure 3. The procedure for intralymphatic port placement. A) Image of femoral vessels. The white arrow is showing the femoral lymphatic vessel surrounded with a vessel loop. The black arrow shows the cannula to be placed in the lymphatic vessel. B) Image through operative microscope. Black arrow is showing the cannula as it enters the lymphatic vessel. C) Intralymphatic port after lymphatic cannulation, before placement in the subcutaneous pocket. D) Lymphangiogram of subcutaneous right femoral lymphatic port. Image demonstrates patency of port. Contrast material is entering the lymphatics through the port and is seen in inguinal lymph nodes. Figure obtained through open access distributed under the terms of Creative Commons Attribution 4.0 International License (license link http://creativecommons.org/licenses/by/4.0/). Reprinted from Journal for ImmunoTherapy of Cancer, 4:24, Michal Radomski, Herbert J. Zeh, Howard D. Edington, James F. Pingpank, Lisa H. Butterfield, Theresa L. Whiteside, Eva Wieckowski, David L. Bartlett, and Pawel Kalinski, Prolonged intralymphatic delivery of dendritic cells through implantable lymphatic ports in patients with advanced cancer, 2016 [128].

Figure 4. Probe tip and machine used in isolated limb perfusion to monitor compartmental pressure. Reprinted from European Journal of Surgical Oncology, Volume 22, Issue 2, Peter Hohenberger,Lothar H. Finke,Peter M. Schlag, Intracompartmental pressure during hyperthermic isolated limb perfusion for melanoma and sarcoma, pages 147-151, 1996, with permission from Elsevier [130].

Figure 5. a) Image of the SOFUSA™ nanotopography device used to increase etanercept uptake into the lymphatics. (i) microfluidic block, with silicon microneedles on the bottom, attachment adhesive, microfluidic distributor, and attachment adhesive on top. Each array has 100 350 μm long and 110 μm wide microneedles with a 30 μm hole for drug delivery. (ii) Scanning electron microscopy (SEM) image of microneedles with nanotopographic film (scale bar 300 μm). (iii) SEM image of one microneedle. (iv) SEM image of nanostructures present on microneedles (scale bar 3 μm). b) (i) Device placement on back skin of rats. (ii) cross-section of nanotopography device. (iii) image of full microfluidic block. Figure obtained through open access distributed under the terms of Creative Commons Attribution 4.0 International License (license link http://creativecommons.org/licenses/by/4.0/). Reprinted from Arthritis Research & Therapy, 19:116, Melissa B. Aldrich, Fred C. Velasquez, Sunkuk Kwon, Ali Azhdarinia, Kenneth Pinkston, Barrett R. Harvey, Wenyaw Chan, John C. Rasmussen, Russell F. Ross,

Caroline E. Fife, and E. M. Sevick-Muraca, Lymphatic delivery of etanercept via nanotopography improves response to collagen-induced arthritis, 2017 [176].

Figure 6. Illustration of stealth nanogel vaccine. Nanogel was administered subcutaneously in mice and is not sensed by immune cells in the skin or lymph node outside of the node medulla. In the node medulla, macrophages captured the vaccine and stimulated a $CD8^+$ T cell response. Reprinted with permission from D. Muraoka, N. Harada, T. Hayashi, Y. Tahara, F. Momose, S. Sawada, S. Mukai, K. Akiyoshi, H. Shiku, Nanogel-based immunologically stealth vaccine targets macrophages in the medulla of lymph node and induces potent antitumor immunity, ACS Nano. 8 (2014) 9209–9218. doi:10.1021/nn502975r. Copyright 2014 American Chemical Society. [183]