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LYMPH NODE TRANSFER IN THE TREATMENT OF POSTMASTECTOMY LYMPHEDEMA

by

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To my soulmate, Tiina H.

ABSTRACT

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Lymph node transfer in the treatment of postmastectomy lymphedema

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Background: Lymphedema is a debilitating disorder with few treatment options. Clinical studies have shown that microvascular lymph node transfer may improve the lymphatic function of the affected limb. This study provides information about the clinical efficacy and safety of this procedure. Further, the biological background of this technique is clarified with an analysis of postoperative production of lymphatic growth factors and cytokines related to lymphangiogenesis.

Patients and Methods: The effect of lymph node transfer to recipient and donor sites was analyzed with lymphoscintigraphy, limb circumference measurements, and appearance of clinical symptoms. Axillary seroma samples were analyzed from four patient groups: Axillary lymph node removal (ALND), Microvascular breast reconstruction (BR), lymph node transfer (LN) and combined lymph node transfer and breast reconstruction (LN-BR).

Results: The postoperative lymphatic transport index was improved in 7/19 patients. Ten patients were able to reduce or discontinue compression therapy 6 - 24 months postoperatively. The donor lower limb lymphatic flow was slightly impaired (Ti >10) in 2 patients. No donor site lymphedema symptoms appeared during the 8 – 56-month follow-up.

A high concentration of the VEGF-C protein was detected in the seroma fluid of all flap transfer groups. The concentration of the anti-inflammatory and anti-fibrotic cytokine IL-10 was increased in the LN-BR group samples when compared to the ALND or BR group.

Conclusions: According to this preliminary study, the lymph node transfer seems to be beneficial for the lymphedema patients. However, a randomized study comparing the effect of BR and LN-BR is needed to evaluate the clinical efficacy of lymph node transfer. In addition, the effect of this surgery on the donor site needs to be studied further. The clinical effects of the lymph node transfer might be partly mediated by increased production of the lymphangiogenic growth factor (VEGF-C) as well as the anti-fibrotic cytokine (IL-10).

Key words: Lymphedema, lymphatic surgery, vascular endothelial growth factor, pro-inflammatory cytokines, anti-inflammatory cytokines, IL-10

TIIVISTELMÄ

Tiina Viitanen
Imusolmukesiirto rintasyöpähoitojen jälkeisen lymfedeeman hoidossa

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Tausta ja tavoitteet: Lymfedeema eli imunesteturvotus on etenevä imunestekierron sairaus, johon ei tunneta parantavaa hoitoa. Uusimpien tutkimusten mukaan mikrovaskulaarisella imusolmukesiirrolla voidaan parantaa lymfedeemaraajan imunestekiertoa. Tämän tutkimuksen tarkoituksena oli selvittää imusolmukesiirron tehoa ja turvallisuutta. Myös imusolmukesiirron biologista taustaa tutkittiin analysoimalla leikkauksen jälkeistä imutiekasvutekijä- ja sytokiinituotantoa.

Potilaat ja menetelmät: Imusolmukesiirron vaikutusta sekä lymfedeemaraajaan että kielekkeen ottokohtaan analysoitiin lymfoskintigrafian, raajan ympärysmittausten ja kliinisten oireiden avulla. Kasvutekijä- ja sytokiinimääritykset tehtiin leikkausalueen seroomanäytteistä neljällä eri potilasryhmällä, joille tehtiin joko kainaloevakuaatio, mikrovaskulaarinen rintarekonstruktio, imusolmukesiirto tai yhdistetty rintarekonstruktio ja imusolmukesiirto.

Tulokset: Imunestekierron nopeutuminen imusolmukesiirron jälkeen todettiin 7:llä potilaalla 19:stä. Kymmenen potilasta pystyi vähentämään tai lopettamaan yläraajan kompressiohoidon 6-24 kuukautta leikkauksen jälkeen. Alaraajassa, jolta puolelta kieleke otettiin, todettiin lievästi hidastunut imunestekierto 2:lla potilaalla. Yhdellekään potilaalle ei kuitenkaan seuranta-ajassa ole kehittynyt alaraajan turvotusoireita.

Kaikkien kielekesiirtoryhmien seroomanäytteissä todettiin korkea imutiekasvutekijä (VEGF-C) -pitoisuus. Anti-inflammatorisen ja anti-fibroottisen sytokiinin IL-10:n pitoisuus yhdistetyssä rintarekonstruktio- ja imusolmukesiirtoryhmässä oli korkeampi kuin kainaloevakuaatio- tai pelkässä rintarekonstruktioryhmässä.

Päätelmät: Osa lymfedeemapotilaista näyttäisi hyötyvän imusolmukesiirrosta. Jatkossa leikkauksen tehoa tulisi selvittää satunnaistetulla tutkimuksella, jossa verrataan pelkän rintarekonstruktion ja imusolmukesiirron vaikutusta imunestekiertoon. Myös leikkauksen vaikutusta kielekkeen ottoalueeseen on tutkittava lisää. Imutiekasvutekijä VEGF-C:n sekä antifibroottisen sytokiinin IL-10:n lisääntynyt eritys selittävät osittain imusolmukesiirron hyödyllisiä vaikutuksia.

Avainsanat: Lymfedeema, imunestekiertoa korjaava kirurgia, imutiekasvutekijät, proinflammatoriset sytokiinit, anti-inflammatoriset sytokiinit, IL-10

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ABBREVIATIONS

ALND	Axillary lymph node dissection
BMI	Body mass index
BR	Breast reconstruction
CDT	Complete decongestive therapy
DIEP	Deep inferior epigastric perforator (flap)
DLT	Decongestive lymphatic therapy
FOXC2	Forkhead box C2 gene
g	gram
ICG	Indocyanine green
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-13	Interleukin-13
LN	Lymph node transfer
LN-BR	Combined lymph node transfer and breast reconstruction
MBq	megabecquerel
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
msTRAM	Muscle-sparing transverse rectus abdominis musculocutaneous (flap)
POD	Postoperative day
SCIA	Superficial circumflex iliac artery
SIEA	Superficial inferior epigastric artery
TDC	Tissue dielectric constant
TGF-β	Transforming growth factor beta
Ti	Transport index
TNF-α	Tumor necrosis factor alpha
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
VEGFR-3	Vascular endothelial growth factor receptor 3
VEGFs	Vascular endothelial growth factors

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (**I - IV**).

I Saaristo AM, Niemi TS, **Viitanen TP**, Tervala TV, Hartiala P, Suominen EA (2012) Microvascular breast reconstruction and lymph node transfer for postmastectomy lymphedema patients. *Annals of Surgery 255(3):468-73.*

II **Viitanen TP**, Visuri MT, Hartiala P, Mäki MT, Seppänen MP, Suominen EA, Saaristo AM (2013) Lymphatic Vessel Function and Lymphatic Growth Factor Secretion after Microvascular Lymph Node Transfer in Lymphedema Patients *Plastic and Reconstructive Surgery - Global Open. 1(2):1-9.*

III **Viitanen TP**, Mäki MT, Seppänen MP, Suominen EA, Saaristo AM (2012) Donor-site lymphatic function after microvascular lymph node transfer. *Plastic and Reconstructive Surgery 130(6):1246-53*

Viitanen TP, Suominen EA, Saaristo AM (2013) Reply: Donor-site lymphatic function after microvascular lymph node transfer should be followed using indocyanine green lymphography. *Plastic and Reconstructive Surgery 131(3):444e*

IV **Viitanen TP***, Visuri MT*, Sulo EH, Saaristo AM, Hartiala P (2013) Microvascular lymph node transfer promotes an immunoregulatory and anti-fibrotic response in lymphedema patients *Plastic and Reconstructive Surgery, Submitted*
*Equal contribution

1 INTRODUCTION

Chronic lymphedema, commonly caused by surgery or radiation, offers a challenging clinical problem. The incidence of lymphedema varies from 9 to 41 % in patients that have undergone axillary lymph node dissection and from 4 to 10 % in patients with sentinel node biopsy (Suami, Chang 2010, McLaughlin et al. 2008, Clark, Sitzia & Harlow 2005). According to the American Cancer Society, there are currently 2.6 million breast cancer survivors in the USA alone (American Cancer Society) and 15 - 20% of them suffer from postmastectomy lymphedema (Suami, Chang 2010, Erickson et al. 2001). In Finland, there are approximately 58 000 breast cancer patients (Engholm et al. 2013), and it can be estimated that 8 700 - 11 600 of them have or will develop lymphedema.

Conventional treatment options for chronic lymphedema aim at alleviating symptoms and are mainly based on physiotherapy and compression therapy, whereas reconstructive surgical treatment options have been limited (Suami, Chang 2010). This is related to the fact that identifying and preserving lymphatic vessels is difficult even with modern microsurgical techniques. Late-stage lymphedema, which is accompanied by adipose tissue hypertrophy and fibrosis, can be managed with liposuction as a symptomatic treatment (Damstra et al. 2009a). However, a prerequisite to maintaining the effect of liposuction is the continuous use of compression garments (Brorson, Svensson 1998).

Recent studies have shown that autologous microvascular lymph node transfer from the groin area into the axillas or wrists of lymphedema patients may improve lymphatic drainage of the affected limb (Becker et al. 2006, Gharb et al. 2011, Lin et al. 2009). In the lymph node transfer technique the lymphatic vessel anastomoses are expected to form spontaneously, which has raised skepticism towards this technique. However, the biological mechanisms of lymphangiogenesis have been significantly elucidated in the recent years (Tammela, Alitalo 2010). The lymphatic vasculature is known to have a tremendous capacity to regenerate (Slavin et al. 1997, Tobbia et al. 2009, Shesol et al. 1979, Oden 1960, Paavonen et al. 2000, Slavin et al. 1999). Vascular endothelial growth factor C (VEGF-C) is known to be the key factor for inducing lymphangiogenesis (Tammela, Alitalo 2010). Results from the preclinical lymphedema models have demonstrated that VEGF-C or VEGF-D specifically induce the growth of new lymphatic vessels (Rissanen et al. 2003, Karkkainen et al. 2001, Saaristo et al. 2002, Baker et al. 2010, Tammela et al. 2007, Lahteenvuo et al. 2011).

Even though VEGF-C is necessary for lymphangiogenesis, in some circumstances lymphatic regeneration can be impaired despite a normal level of this growth factor (Rutkowski et al. 2006, Goldman et al. 2005). Immunological responses have

been found to play an important role in the chain of events, which results in either lymphatic regeneration or fibrosis and lymphedema. Recent studies have shown that lymphangiogenesis is regulated by a coordinated expression of pro-inflammatory and anti-inflammatory cytokines (Alitalo 2011, Ristimaki et al. 1998, Zampell et al. 2012). Further, fibrosis and scarring have been found to be key inhibitors of lymphatic regeneration (Ververs et al. 2001, Hinrichs et al. 2004). There is also evidence that CD4 T cell inflammation and namely a Th2-type inflammatory response is crucial for postsurgical lymphedema development (Avraham et al. 2009, Avraham et al. 2013).

Many lymphedema patients in western countries are postmastectomy patients who also require a breast reconstruction. The deep inferior epigastric perforator (DIEP) flap and muscle- sparing transverse rectus abdominis musculocutaneous (msTRAM) flap are the most popular free flaps for reconstructing the missing breast (Koshima, Soeda 1989, Allen, Treece 1994, Chevray 2004, Nahabedian et al. 2002). This study describes a modified lower abdominal wall flap containing lymph nodes from the groin area to reconstruct both the breast and the lymphatic anatomy of the operated axilla. The effect of this surgery on the lymphatic function of the recipient arm is analyzed, and also the influence of lymph node flap harvest on the donor area is evaluated.

To clarify the biological background of the lymph node transfer surgery, the production of VEGF-C and VEGF-D after lymph node surgery is studied. Further, the cytokines affecting chronic inflammation, T cell responses and fibrosis (IL-1α, IL-1β, TNF-α, TGF-β1, IL-4, IL-10, IL-13), are also major topics of this study.

2 REVIEW OF THE LITERATURE

2.1 Development and degeneration of the lymphatic system

The first lymph sacs in humans can be detected in 6 to 7 week old embryos (van der Putte 1975). These lymph sacs are suggested to generate from embryonic veins when a subpopulation of the endothelial cells in the embryonic veins differentiate into lymphatic endothelial cells (Sabin 1902, Dumont et al. 1994, Kaipainen et al. 1995, Wigle, Oliver 1999). Later during the development the lymph sacs form lymphatic vessels and primary lymph nodes (Clark 1912). Blood is removed from the lymphatic system and lymphatic vessels are further remodelled into the mature lymphatic capillaries and larger collecting lymphatic vessels.

Lymph nodes are fully developed in childhood, and after puberty their size starts to decrease (Denz 1947). As people age, the lymph nodes gradually degenerate. Senile involution affects all elements of the lymph node, including the cortex and medulla, and the amount of active lymphoid tissue decreases (Pan, Suami & Taylor 2008). In the elderly, the inactive lymph nodes contain only coils of lymphatics and fibrous connective tissue (Pan, Suami & Taylor 2008). Similarly, the function of the lymphatic vessels decreases during aging (Conway et al. 2009).

2.2 Anatomy and function of the lymphatic system

The lymphatic system regulates tissue fluid homeostasis, immune cell trafficking, and absorption of dietary fats (Olszewski 2003). Lymphatic tissue in lymph nodes, lymphatic vasculature, the spleen, the thymus, bone marrow and the digestive system forms the lymphatic system of the human body. Lymphatic vessels can be found in all vascularized tissues, with the exception of bone marrow and the central nervous system (Tammela, Alitalo 2010). The network of thin-walled lymphatic capillaries can be found in all vascularized tissues, with the exception of bone marrow and the central nervous system (Tammela, Alitalo 2010, Suami, Taylor & Pan 2005). These blind-ended lymphatic capillaries with a diameter of 30-80 μm collect tissue fluid, macromolecules, and immune cells (Casley-Smith 1980, Suami, Taylor & Pan 2005). Unlike blood capillaries, lymphatic capillaries lack pericytes and continuous basal lamina. They also have anchoring filaments, which connect the vessel walls to the surrounding extracellular matrix and regulate the permeability of capillary walls (Casley-Smith 1980). Lymphatic capillaries flow to larger collecting lymphatic vessels, which contain connective tissue and smooth muscle cells (Ross, Romrell &

Kaye 1995). There are also numerous valves inside the collecting lymphatic vessels to prevent backflow (Suami, Taylor & Pan 2005, Ross, Romrell & Kaye 1995). The lymphatic collectors drain lymph into the regional lymph nodes. The lymph nodes process foreign antigens, which are found soluble in the lymph, but they are also transported and presented to T cells in lymph nodes by antigen- presenting cells (Banchereau et al. 2000). The normally functioning lymphatic system transports approximately 1–2 liters of interstitial fluid with 20–30 g of protein per liter to the venous circulation every day (Tammela, Alitalo 2010).

The human lymphatic anatomy has been studied already in the eighteenth century (Mascagni 1787, Cruikshank 1786, Sappey 1874). Sappey published a detailed anatomy of the lymphatic system in 1874. He divided the human body into four territories by a sagittal midline and horizontal line at the L2 level. Lymph from these territories was found to drain into the ipsilateral axillar or inguinal lymph nodes. Lymph from the left side of the body, the abdomen, and both lower limbs ends up in the thoracic duct, the largest lymphatic vessel in the body. The thoracic duct connects with the left subclavian vein. Lymph from the right upper arm, the thorax, and the head flows to the right subclavian vein via the right lymphatic trunk (Jeltsch et al. 1997). In the era of sentinel node mapping and modern anatomical studies, the knowledge of lymphatic anatomy has been further defined (Uren, Thompson & Howman-Giles 1999, Suami, Taylor & Pan 2005, Suami et al. 2008a, Suami et al. 2008b). Lymphoscintigraphy has become a routine examination for identifying the sentinel node in cancer patients. This method has revealed unique patterns of lymphatic draining pathways that differ from Sappey´s anatomical drawings (Uren, Thompson & Howman-Giles 1999).

2.2.1 The lymphatic anatomy of the thoracic wall

Suami et al. have studied the lymphatic system of the upper thoracic wall and upper arm by radiography and dissection of cadavers. The lymph from the anterior side of upper torso is drained into the external mammary or axillary vein lymph nodes, and the posterior side flow is directed into the scapular nodes. A single sentinel node, which drains the majority of upper limb, also drains a large area of the anterior upper torso (Suami et al. 2008a).

2.2.2 The lymphatic anatomy of the upper arm

In a normal upper limb, superficial and deep lymphatic vessels can be identified. The superficial lymphatic system consists of a wavy network which parallels the cephalic and basilic veins. The most superficial lymph vessels flow into the regional lymph nodes of the axilla. In contrast, the deep lymphatic vessels flow to several interval lymph

nodes before reaching the axilla. In a normal situation, there are no connections between superficial and deep lymphatic vessels, except in the epitrochlear area. All the lymph vessels running the anterior surface of the arm are drained to one main sentinel node in the axilla. However, the lymphatics on the posterior surface of the arm may flow to other lymph nodes in the axilla or bypass all the regional axillary lymph nodes (Suami, Pan & Taylor 2007, Suami et al. 2008a).

2.2.3 The lymphatic anatomy of the lower abdominal wall and inguinal area

The superficial lymphatic collectors of the lower abdominal wall are located above the Scarpa´s fascia, below the subdermal venules. They run evenly across the abdominal wall in the direction of the large superficial veins and finally flow to the lymph nodes in the superolateral inguinal area (Tourani, Taylor & Ashton 2013). In contrast, the deep lymphatic system drains lymph from deep abdominal structures and flows to the iliac lymph nodes (Felmerer et al. 2002).

2.2.4 The lymphatic anatomy of the lower limb

The lymph from the lower limb is drained through deep and superficial lymphatic routes. The superficial lymphatic vessels follow the great saphenous vein and the small saphenous vein. The lymph from the calf is drained to the deep popliteal lymph nodes. In contrast, lymph from the anterior surface of the leg is drained to the inferior quadrant of the superficial inguinal nodes (Caplan 1978, Pan, le Roux & Levy 2011). In the thigh, there are three main lymphatic pathways: superficial lymphatic vessels on the medial side of the thigh, deep lymphatics accompanying the superficial femoral vessels, and deep lymphatics between the sciatic nerve and profunda femoris vessels (Pan, le Roux & Levy 2011). A recent study demonstrated lymphatic drainage patterns in the groin in 41 patients with lower limb melanoma (van der Ploeg et al. 2009). This study showed that 93% of the lower limb sentinel nodes were found from the superficial inguinal area, whereas the remaining 7% were found from deep zones of the groin. The majority of the superficial sentinel nodes were located in the superomedial, inferomedial or central inguinal area.

2.3 Regulation of lymphatic vessel growth

2.3.1 Vascular endothelial growth factors

The molecular mechanisms of lymphangiogenesis have been clarified in recent years (Tammela, Alitalo 2010). Vascular endothelial growth factors (VEGFs) are important regulators of angiogenesis and lymphangiogenesis. (Lohela et al. 2009, Oliver 2004). VEGF-C and VEGF-D mediate their lymphangiogenetic effects by binding to tyrosine

kinase receptor 3 (VEGFR-3), which is expressed in lymphatic endothelial cells. Even though VEGF-C and VEGF-D both bind VEGF-receptors-3 and -2, their *in vivo* effects in animal models differ from each other (Tammela, Alitalo 2010, Olsson et al. 2006). VEGF-C is critical for the development of lymphatic vasculature, whereas VEGF-D has also more pronounced angiogenic effects (Karkkainen et al. 2001, Baldwin et al. 2005). VEGF-C is produced by macrophages and granulocytes during normal wound healing and inflammation (Alitalo, Tammela & Petrova 2005, Baluk et al. 2005). In adults, new lymphatic vessels are formed by sprouting from pre-existing vessels (Alitalo, Tammela & Petrova 2005). However, recent studies have also shown that macrophages may transdifferentiate into lymphatic endothelium, adapt the lymphatic endothelial cell phenotype and form new lymphatic vessels (Maruyama et al. 2007, Kerjaschki et al. 2006).

2.3.2 Pro-inflammatory and anti-inflammatory cytokines

In addition to VEGFs, also pro-inflammatory and anti-inflammatory cytokines have an important role in the regulation of lymphangiogenesis (Alitalo 2011, Ristimaki et al. 1998, Zampell et al. 2012). Inflammation has a diversified and not completely understood role in lymphangiogenesis. Inflammation seems to be closely related to lymphangiogenesis, and pro-inflammatory cytokines (IL-1α, IL-1β and TNF-α) have been shown to induce VEGF-C expression in experimental settings (Alitalo 2011, Ristimaki et al. 1998). On the contrary, lymphatic stasis is known to induce chronic inflammation and tissue fibrosis, which results in decreased lymphatic function and worsening of lymphedema symptoms (Avraham et al. 2010). Interestingly, anti-inflammatory pharmacotherapy with ketoprofen has been shown to reduce post-surgical lymphedema in experimental models (Nakamura et al. 2009).

2.3.3 Transforming growth factor-β

Fibrosis and scarring are known to inhibit lymphatic regeneration (Avraham et al. 2009). The factors that promote scarring and fibrosis, such as radiation therapy, infections or extensive surgical procedures, are also associated with increased incidence of lymphedema (Ververs et al. 2001, Hinrichs et al. 2004). Transforming growth factor beta (TGF-β**)** is an anti-inflammatory cytokine which inhibits the initiation of inflammation (Li et al. 2012), but it also has a major role in regulation of tissue fibrosis and scarring (Clavin et al. 2008, Penn, Grobbelaar & Rolfe 2012). TGF-β can prevent lymphatic endothelial cell proliferation, tubule formation and migration and result in markedly decreased lymphatic function despite high levels of VEGF-C (Avraham et al. 2009, Clavin et al. 2008, Yan et al. 2011b).

In light of these recent studies, it is clear that lymphatic vessel regeneration is regulated not only by VEGFs but also by several cytokines and immunological responses.

2.4 Lymphedema

Lymphedema is a pathological condition which is a result of impaired lymphatic fluid transport. Reduced or absent lymphatic flow leads to accumulation of protein-rich interstitial fluid to subcutaneous tissue and progressive swelling of the extremity. This leads to tissue fibrosis, impaired immune functions and subcutaneous fat hypertrophy (Witte, Witte 1987, Brorson et al. 2006, Brorson 2004, Rockson 2001). Lymphedema patients are prone to develop soft tissue infections as extravasated fluid and proteins and an impaired local immune response offer an opportunity for microbial growth (Rockson 2001). Lymphedema is classified into primary (congenital) and secondary (acquired) lymphedema based on the pathogenesis.

2.4.1 Primary lymphedema

Primary lymphedemas are rare developmental disorders. It has been estimated that 1:6000 newborns develop primary lymphedema (Dale 1985). Symptoms in primary lymphedema may be present in early childhood, but sometimes the symptoms develop in puberty or in early adulthood. Defects of lymphatic vasculature in primary lymphedema are typically systemic, whereas in secondary lymphedema the impairment occurs locally. Depending on the gene mutation, primary lymphedema can be caused by lymphatic vessel hypolasia, hyperlasia or abnormally structured lymphatic vessel walls or valves (Tammela, Alitalo 2010, Schulte-Merker, Sabine & Petrova 2011, Ferrell, Finegold 2008) (See **Figure 1** on page 18). There are several known gene mutations which result in primary lymphedema. For example, congenital hereditary lymphedema (Milroy´s disease) has been linked to mutations in VEGFR-3 (Ferrell et al. 1998). Mutations in the FOXC2 gene result in the rare lymphedema-distichiasis syndrome, which usually occurs at puberty (Finegold et al. 2001, Fang et al. 2000, Bell et al. 2001).

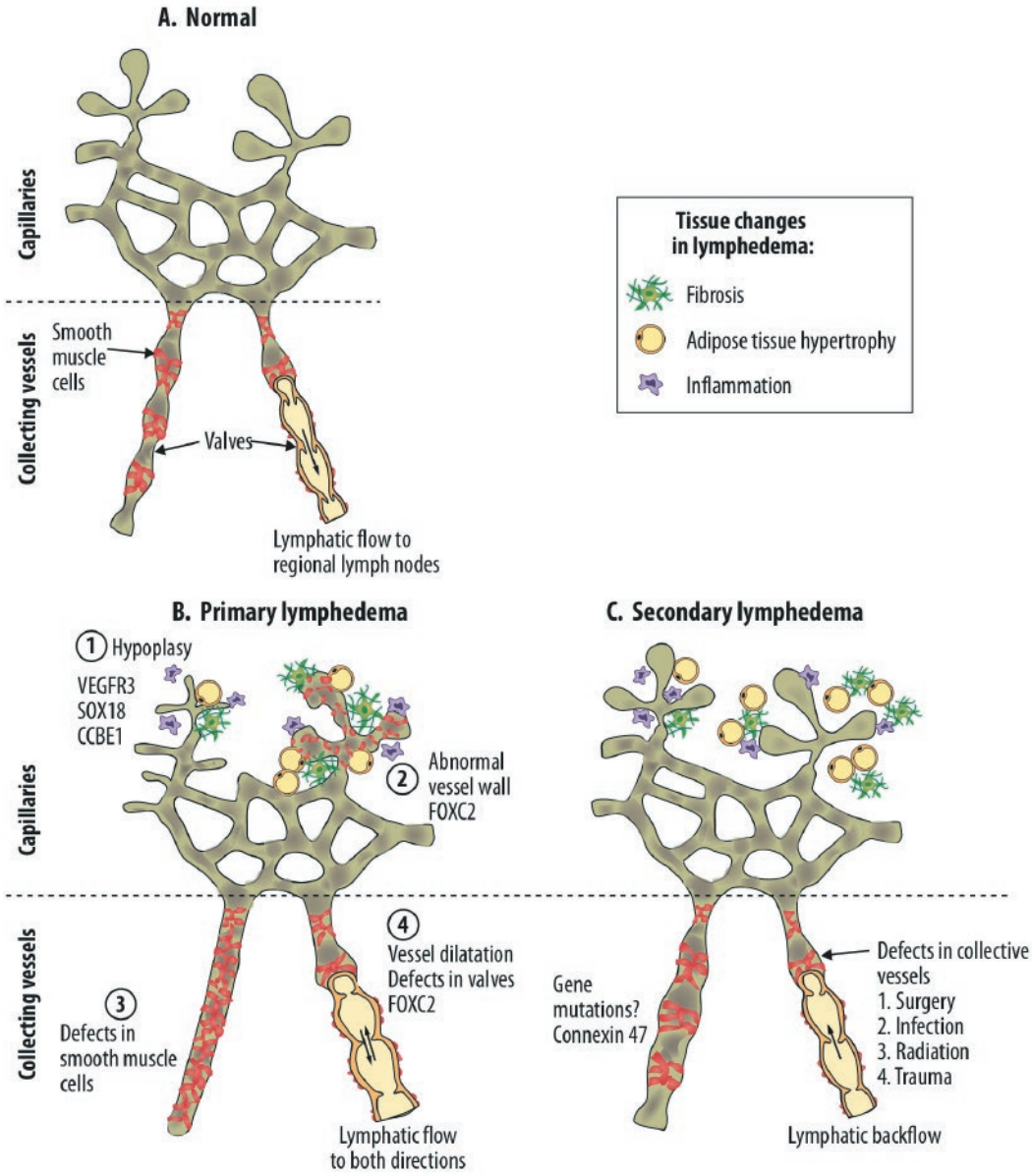


Figure 1. The background of primary and secondary lymphedema. A. Normal lymphatic capillaries, collective lymphatic vessels and lymphatic flow. B. Typical defects in primary lymphedema and related gene mutations. C. Secondary lymphedema is caused by damage in collective lymphatic vessels. Impaired lymphatic flow leads to fibrosis, adipose tissue hypertrophy and inflammation. Gene mutations that may predispose to secondary lymphedema have been studied recently. (Figure modified from Saaristo et al., Duodecim, in press)

2.4.2 Secondary lymphedema

2.4.2.1 Filariasis

The most common cause for secondary lymphedema is filariasis and other infectious diseases. Approximately 120 million people suffer from lymphedema caused by filariasis (Michael, Bundy & Grenfell 1996). This infection of the lymphatic system leads to obstruction and scarring of lymphatic vessels, and finally to an extremely severe form of lymphedema (elephantiasis).

2.4.2.2 Iatrogenic lymphedema

In industrialized countries, the most common cause of lymphedema is treatment of breast cancer (Radhakrishnan, Rockson 2008). Surgical management of breast cancer involves an operation of axillary lymph nodes; either removal of sentinel nodes or, in the case of lymph node metastasis, radical excision of all axillary lymph nodes and adipose tissue. Breast cancer patients with axillary metastasis also receive axillary radiotherapy. The incidence of lymphedema varies from 9 to 41 % in patients that have undergone radical axillary lymph node dissection. In patients with sentinel node biopsy the incidence is smaller, from 4 to 10 % (Suami, Chang 2010, McLaughlin et al. 2008, Clark, Sitzia & Harlow 2005). In addition to the extent of the axillary surgery, another treatment-related risk factor for lymphedema is radiation therapy (Meek 1998, Aitken et al. 1989). It is not fully understood why some patients develop lymphedema after axillary clearance while in other patients the lymphatic vasculature is able to regenerate and maintain the lymphatic transport from the upper limb. According to recent studies, some patients may have a genetic predisposition to develop lymphedema after breast cancer surgery (Finegold et al. 2012, Newman et al. 2012). For example, connexin 47 gene mutations increase the risk of postmastectomy lymphedema (Finegold et al. 2012). Immunological functions also seem to play a major role in the development of postoperative lymphedema (Ji 2012, Maruyama et al. 2007). In the literature, there are controversial results about the possible risk factors (age, coexisting medical disease, dominant hand involvement, obesity) for lymphedema. However, several studies have found that the risk and severity of lymphedema is statistically related to a high BMI (Segerstrom et al. 1992, Johansson et al. 2002, Goffman et al. 2004, Soran et al. 2006).

Lymphedema may occur from within days up to 30 years after the initiating factor (typically surgery, trauma or radiation) (Petrek et al. 2001). However, 80% of patients develop lymphedema symptoms within 3 years of surgery and the rest of the patients are affected at a rate of 1% per year. Patients usually complain about swelling, heaviness, tension, weakness and pain in the affected limb (Brorson et al. 2008). Further, erysipelas and other soft-tissue infections are typical due to impaired immune reactions of the limb (Rockson 2001). In rare cases, chronic lymphedema may lead to malignant diseases,

such as lymphangiosarcoma, Kaposi sarcoma and Stewart-Treves syndrome (Ruocco, Schwartz & Ruocco 2002).

2.4.2.2.1 Changes in lymphatic vasculature after axillary lymphadenectomy

A recent study by Mihara et al. presents pathological steps of cancer-related lymphedema. In early stages of lymphedema, ectasis of the collecting vessels can be detected. Gradually the increased endolymphatic pressure promotes transformation of smooth muscle cells and proliferation of collagen fibers in vessel walls. This results in contraction and finally to lymphosclerosis, which can be seen in late stage lymphedemas. Further, changes in intercellular attachments between endothelial cells result in leakage of the lymphatic fluid out of the vessel. In the sclerosis type, the vessel lumen is markedly narrowed or occluded and the lymphatic vessels have lost their ability to contract and transport lymphatic fluid. (Mihara et al. 2012a)

In addition to histological changes in lymphatic vessels, also structural changes in lymphatic vasculature can be seen after axillary lymphadenectomy. Recently, Suami et al. have studied postmastectomy changes of lymphatic vasculature of both upper limbs of a cadaver who did not develop clinical lymphedema (Suami, Pan & Taylor 2007). They found several changes in the lymphatic pathways of the operated arm; atrophy of superficial lymphatic vessels, incompetent valves between precollectors and collecting lymphatic vessels enabling dermal backflow. Interestingly, they also found unusual communications between superficial and deep lymphatic vessels, and spontaneous formation of a lymphovenous shunt. These latter changes may explain why the patient in question did not develop lymphedema.

2.4.3 Clinical staging of lymphedema

The International Society of Lymphology has published a classification of lymphedema (International Society of Lymphology 2003):

- Stage 0. Latent or subclinical condition where swelling is not evident despite impaired lymph transport.
- Stage I. Early accumulation of fluid relatively high in protein content (e.g. in comparison with “venous” edema) that subsides with limb elevation. Pitting may occur (**Figure 2A**, page 21).
- Stage II. Pitting may or may not occur as tissue fibrosis develops. Limb elevation alone rarely reduces tissue swelling (**Figure 2B**, page 21).

- Stage III. Lymphostatic elephantiasis where pitting is absent. Hypertrophic skin changes, such as acanthosis, fat deposits, and warty overgrowths, often develop (**Figure 2C**).

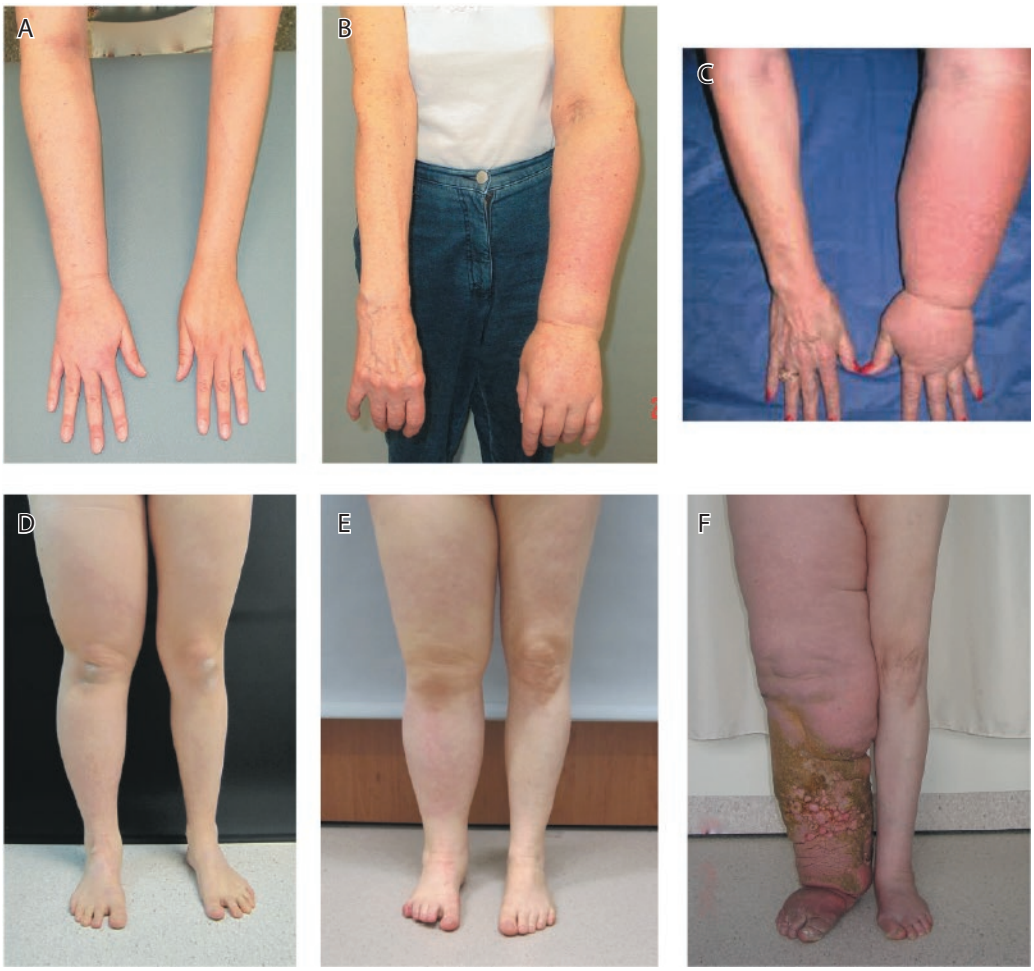


Figure 2. A. Stage I lymphedema. B. Stage II lymphedema. C. Stage III lymphedema (Photos from the Turku University Hospital, Department of Plastic and General Surgery, except the photo of stage 3 arm lymphedema which is from www.pmhtherapy.com)

2.4.4 Quality of life among breast cancer patients with lymphedema

It has been shown that without treatment, lymphedema progresses and leads to physical and functional deficits, chronic infections, reduced quality of life and high health care costs (Piller, Thelander 1998, Ridner 2005, Cormier et al. 2012). Recently Pusic et al. (Pusic et al. 2013) have reviewed 39 studies comparing quality of life in breast cancer patients with or without lymphedema. The majority of these reviewed studies reported

significantly decreased physiological functioning and diminished psychosocial and social well-being. However, recent studies have shown that some patients suffer from subjective symptoms of lymphedema without objective changes in arm volume (Hormes et al. 2010). Bulley et al. used both objective and subjective criteria in diagnosis of lymphedema and studied the quality of life among breast cancer related lymphedema patients (Bulley et al. 2013). They found that quality of life did not differ between patients with or without lymphedema when using objective classification, but when using the subjective tools, the quality of life was significantly decreased in lymphedema patients. These findings suggest that lymphedema clearly affects the physical, psychosocial and social health of the lymphedema patients, but the objective clinical measurements may underestimate the impact of lymphedema (Hormes et al. 2010).

2.4.5 Diagnostics of lymphedema

2.4.5.1 Clinical diagnosis

Diagnosis of lymphedema is mainly clinical. The most commonly used diagnostic tool is limb volumetry. The limb volume can be measured with the water displacement method or with circumferential measurements and geometric formulae to calculate the estimated volume. The limb volume difference indicating lymphedema varies, but commonly it is stated to be equal to or greater than 10% or a 200 mL or 2 cm - 5 cm difference in limb circumference (Armer, Stewart 2005, Hayes, Cornish & Newman 2005). In the early stage of lymphedema, the accumulation of tissue fluid can be easily detected by a positive pitting test (pitting edema). As the disease progresses, the amount of subcutaneous fat and fibrotic tissue increases (Brorson 2012, Brorson et al. 2006, Brorson 2004). Therefore, in the later stages of the disease, as the swelling is dominated by hypertrophic adipose and fibrotic tissue, the limb shows only little or no pitting (non-pitting edema).

2.4.5.2 Lymphangiography

Earlier, the commonly used imaging technique was lymphangiography. In this technique, a positive x-ray contrast agent (Lipiodol) is injected directly into the lymphatic vessels, which enables the visualization of the lymphatic vessels and the nodes. Nowadays the use of lymphangiography has been abandoned due to several complications and lymphatic vessel damage induced by Lipiodol (Sharma et al. 2008).

2.4.5.3 Lymphoscintigraphy

Nowadays lymphoscintigraphy has replaced the use of lymphangiography. Lymphoscintigraphy is a radionuclide-based imaging technique that enables two-dimensional visualization of the lymphatic network. Evaluation of lymph flow with this technique remains qualitative or semiquantitative on the basis of clearance rates and

dermal backflow parameters (Sharma et al. 2008). Transport index is a semiquantitative method of analyzing lymphoscintigraphy images. This index is based on five criteria: lymphatic transport kinetics, the distribution pattern of the radiopharmaceutical, time to appearance of lymph nodes and visualization of lymph nodes and lymph vessels (Kleinhans et al. 1985, Weiss, Baumeister & Hahn 2003).

2.4.5.4 Other imaging techniques

There are also other imaging techniques available, but they have not gained popularity in clinical use in Finland thus far. MR lymphangiography offers high-resolution imaging of lymph vessels with detailed anatomical and morphological information (Notohamiprodjo et al. 2012, Sharma et al. 2008), but high costs and reduced availability limits the use of MRI. A fairly new non-invasive method is the measurement of local tissue water from the tissue dielectric constant (TDC). It can be used as a diagnostic tool as well as a method for following the effect of the treatment (Birkballe et al. 2013). Optical imaging techniques (patent blue or indocyanine green (ICG) lymphography) offer possibilities to assess the peripheral anatomy and function of lymphatic vessels preoperatively (Sharma et al. 2008, Yamamoto et al. 2011c).

2.5 Conservative treatment of lymphedema

2.5.1 Compression therapy

The standard treatment for lymphedema is compression therapy (Warren et al. 2007, Cormier et al. 2012). Complete decongestive therapy (CDT) or decongestive lymphatic therapy (DLT) includes compression with garments or bandaging, manual lymph drainage, exercise and skin care. CDT can be divided into two phases: the reductive phase and the maintenance phase. The reductive phase aims at removal of tissue fluid and reduction of limb volume. The compression garments' size is reduced regularly as the limb volume decreases (controlled compression therapy) (Warren et al. 2007). The maintenance phase of CDT means a life-long treatment of lymphedema with compression garments, skin care and exercise. With these methods, limb volume can be reduced 31 - 46% (Warren et al. 2007). It has been clearly shown that optimally executed compression therapy can prevent the progression of lymphedema (Brorson 2003, Brorson et al. 2012, Tenenbaum et al. 2005), but, the conservative treatment does not improve the lymphatic function permanently.

2.5.2 Manual lymphatic drainage

There is no evidence of the effectiveness of manual lymphatic drainage in the treatment of lymphedema (Preston et al, Huang et al). In the recent Cochrane review, Preston et al. assessed the effect of physical treatment programs on the long-term control of lymphedema.

No difference in reduction of excess arm volume or symptoms was found between groups with compression sleeves alone or compression sleeves and manual lymphatic drainage received eight times over two weeks. Despite these facts, in practice manual lymphatic drainage or self-administered massage is commonly recommended to lymphedema patients and it is often combined with compression therapy, as in CDT introduced above.

2.6 Surgical treatment options

2.6.1 Reductive surgery and liposuction

The first reported surgical technique in the treatment of lymphedema was Charles´ procedure in 1912. In this technique, all lymphedematous skin and subcutaneous tissue is removed above the deep fascia and the limb is covered with skin grafts taken from the excised specimen (Cormier et al. 2012). In 1927, Sistrunk published a surgical procedure specifically for upper extremity lymphedema (Sistrunk 1927). In the Sistrunk technique, the excess skin, soft tissue and deep fascia are removed from the medial side of the arm and the wound is closed primarily. Thompson modified this technique by raising a de-epithelialized skin flap from the lateral aspect of the arm (Thompson 1967). The edge of the flap is embedded beside the neurovascular bundle in order to create a lymphatic bridge between deep and superficial lymphatic systems. Later, several modifications of Charles´ procedure and other resective techniques have been introduced (Suami, Chang 2010). Charles` procedure and its variations can be used in reduction of extremely bulky limbs in severe lymphedema. However, the cosmetic result is poor, and the patients often have delayed wound healing, remarkable scaring, sensory nerve loss and lymph fistulas (Gloviczki 1999).

The modern reductive technique in the treatment of lymphedema is liposuction (Brorson 2010). Even when lymphedema is optimally treated with compression therapy, there can be several liters of excess volume in the lymphedema limb. In these patients the excess volume consists of hypertrophied adipose tissue and fibrosis, and clinically non-pitting edema can be detected (Brorson et al. 2009). Importantly, liposuction does not decrease the already impaired lymphatic flow (Brorson et al. 1998). Typically there is no need for skin excisions even after large volume liposuction (Brorson 2012, Brorson 2000). It has been shown to be an effective reductive treatment option when performed on a selected patient group. The result of liposuction has been shown to be permanent in long-term follow-up. However, life-long meticulous usage of compression is a prerequisite to prevent recurrences (Brorson 2003).

2.6.2 Lymphovenous anastomoses (LVA)

The idea of redirecting lymph from impaired lymphatic vessels to the venous system by lymphovenous anastomoses was introduced already in 1963 (Laine, Howard 1963). In

the LVA technique, the superficial lymphatic vessels in the lymphedema limb are located with ICG lymphography or with Patent Blue, and the lymphatic vessels and adjacent venous vessels are dissected through small incisions. Usually one to three lymphovenous anastomoses are performed (**Figure 3**). The use of LVA on lymphedema patients started in the 1970s, and since then, several groups have published their patient series (O´Brien, Shafiroff 1979, Damstra et al. 2009b, Koshima et al. 2004, Suami, Chang 2010, Campisi et al. 1995). Damstra et al. reviewed 9 studies on the effect of LVA. The majority of these studies have reported reduction in limb volumes after the LVA procedure. However, there are only a few prospective studies, and the patency of the performed LVA is often not shown. In addition, the majority of the studies have not reported whether the compression garment is needed after surgery (Damstra et al. 2009b). According to a review article by Lee et al., the compression therapy is needed to maintain the effect of reconstructive surgery (Lee, Laredo & Neville 2011). In practice, this technique can be used only in the early stage lymphedema, as there have to be functioning lymphatic vessels available. Another challenge in the LVI technique is backflow from the venous system. Lymphatic endothelial cells are known to express podoplanin, a protein, which interacts with platelets and results in platelet aggregation (Uhrin et al. 2010). As a result, blood backflow into the lymphatic vessel leads to a thrombosis of the anastomosis. Therefore, the recipient venous vessels should be very small with low pressure and with functioning venous flaps.

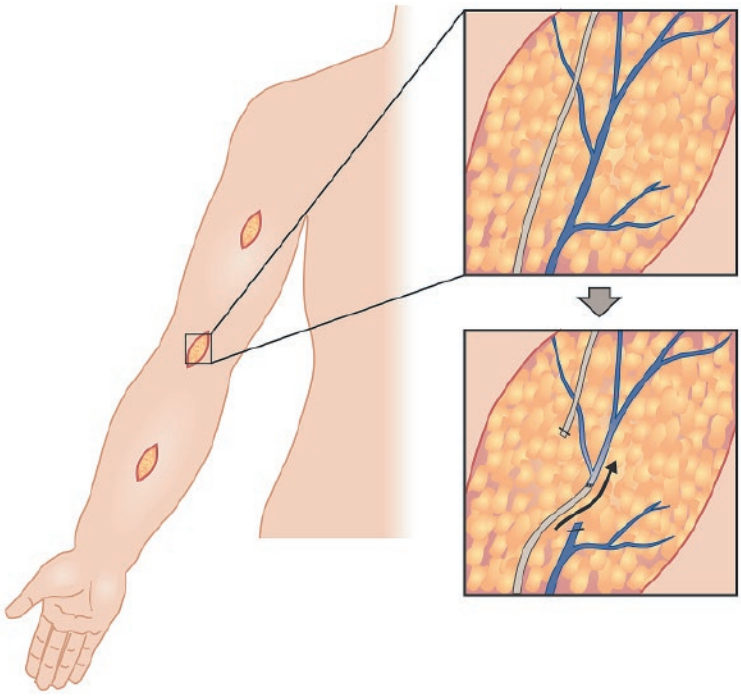


Figure 3. Lymphovenous anastomosis to redirect lymphatic flow to venous circulation.

2.6.3 Lymphatico-lymphatic bypass

Lymphatic grafting, published by Baumeister, has been used in the treatment of lymphedema patients since 1980 (Baumeister, Seifert & Hahn 1981). In this technique, the healthy lymphatic vessels from the medial thigh are used as grafts to bypass the lymphatic flow from the upper extremity to the supraclavicular region (**Figure 4**) or from the lower extremity to the lower abdominal area. The patency of the lymphatic graft and improved lymphatic flow function have been demonstrated with postoperative lymphoscintigraphy (Baumeister, Seifert & Hahn 1981). Postoperative volume reduction of the affected arm has been shown to persist in long-term follow-up (Kleinhans et al. 1985). Felmerer et al. published a small series of stage 2 lymphedema patients with lymphatic vessel transfer (Felmerer et al. 2012). They reported a full recovery of 3/14 patients and reasonable reduction in swelling in 9/14 of the operated patients. Four patients in this study were able to discontinue compression therapy. In practice, the use of this technique requires functioning lymphatic vessels at the brachial level of the recipient arm to enable the surgical anastomosis of the graft. In the lower extremity, the lymphatic graft can reach the upper proximal part of the affected thigh. There have been no reports of postoperative donor limb lymphedema related to this technique. According to Baumeister's reports, the lymphatic function of the donor limb has to be proven normal before collection of the graft in order to avoid postoperative lymphatic dysfunction (Baumeister, Siuda 1990).

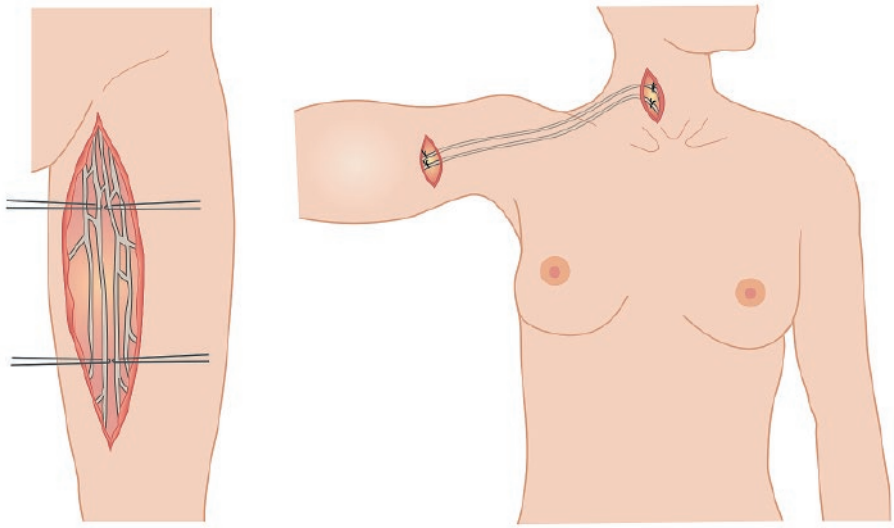


Figure 4. Lymphatic vessel graft to bridge the impaired lymphatic flow over the axilla

2.6.4 Lymph node transfer

Lymph node transfer aims to reconstruct the lymphatic anatomy and lymphatic function of the swollen extremity. In the lymph node transfer technique, the lymphatic anastomoses are not performed surgically since the lymphatic vasculature is expected to form spontaneously from the transferred lymph nodes and residual lymphatics. The therapeutic effect of the lymph node transfer method has been evaluated in different experimental lymphedema models (Chen et al. 1990, Blum et al. 2010, Tobbia et al. 2009, Hadamitzky, Blum & Pabst 2009). Chen et al. demonstrated that a normal architecture and size of the lymph node is preserved six months after vascularized lymph node transplantation in a canine model (Chen et al. 1990). In their study, the circumference of the limb was reduced after transfer, and postoperative lymphangiography demonstrated regeneration of the lymphatic system six months after surgery. Blum et al. found that transplantation of lymph node fragments in minipigs increased the lymphatic flow and that larger node fragments were more often integrated into the lymphatic system than small lymph node slices (Blum et al. 2010). Tobbia et al. compared the effect of vascularized and non-vascularized lymph node transplants in the treatment of post-surgical lymphedema in a sheep model and found that the vascularized transplants were associated with a better clinical result (Tobbia et al. 2009).

Autologous microsurgical lymph node transfer in human patients was first introduced by Dr. Corinne Becker on 2006 (Becker et al. 2006). In this technique, a free flap from the inguinal area was transferred to the axilla of a lymphedema patient (**Figure 5**, page 28). According to Becker´s long-term follow-up study, this form of lymph node transfer improves the lymphatic function of the affected arm in 31% of patients. The majority of the patients were reported to clinically benefit from this surgery; 62.5% of patients were able to discontinue physiotherapy and the lymph node transfer also seemed to prevent infectious episodes of the lymphedema arm (Becker et al. 2006). Further, the lymph node transfer was found to have a favorable effect on postmastectomy related neuropathic pain (Becker et al. 2008). However, the lack of surgically performed lymphatic anastomosis in this lymph node transfer technique raised skepticism and the procedure did not gain popularity during the first few years. Recently, knowledge of the biological mechanisms of lymphatic regeneration has increased markedly (Tammela, Alitalo 2010, Alitalo, Tammela & Petrova 2005), and importantly the spontaneous lymphatic vessel regeneration after lymph node transfer has been demonstrated in large animal models (Honkonen et al. 2012, Lahteenvuo et al. 2011).

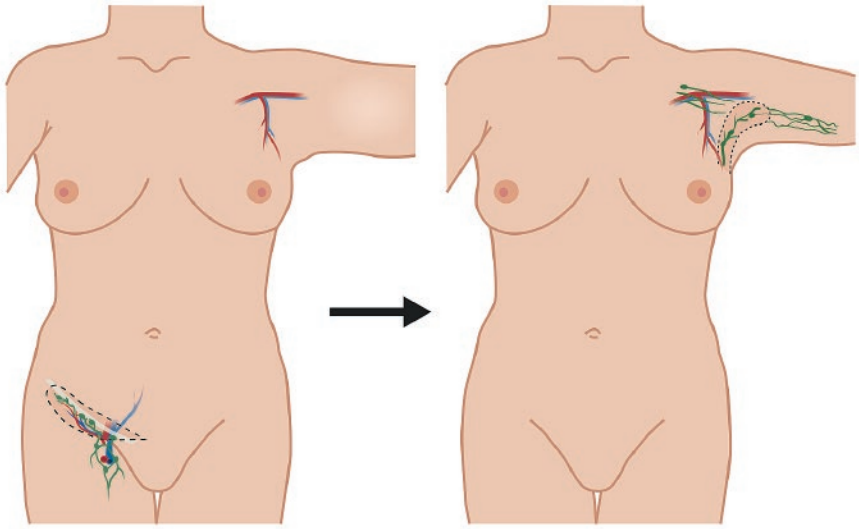


Figure 5. Microvascular lymph node transfer from the inguinal area to the axilla. Lymphatic flap pedicle vessels (SCIA) are anastomosed with thoracodorsal vessels. Surgical lymphatic anastomosis is not performed. Lymphatic vessels regenerate spontaneously after lymph node transfer.

3 AIMS OF THE STUDY

1 To evaluate whether lymph node transfer operation can be performed simultaneously with standard microvascular breast reconstruction (**I**).

2 To evaluate the clinical effects of microvascular lymph node transfer on the affected arm in postmastectomy lymphedema patients (**I, II**).

3 To evaluate the effect of microvascular lymph node transfer on the lymphatic function of the flap donor site (**III**).

4 To analyze whether human lymph nodes produce VEGF-C (**I**) and to measure the seroma fluid concentrations of the lymphangiogenic growth factors after the lymph node transfer surgery in order to clarify the biological background of the lymph node transfer operation (**II, IV**).

5 To measure the seroma fluid concentrations of anti- and pro-inflammatory and Th2 cytokines after the lymph node transfer surgery in order to clarify the biological background of the lymph node transfer operation (**IV**).

4 PATIENTS AND METHODS

All the patients in Studies **I-IV** were operated in Turku University Hospital during 5/2007 - 3/2012 and followed in the plastic surgery outpatient clinic. Postmastectomy patients in the plastic surgery outpatient clinic were asked about upper extremity lymphedema symptoms. Lymphedema patients who were suitable for microvascular breast reconstruction were offered simultaneous lymph node transfer (combined breast reconstruction and lymph node transfer (LN-BR-group)). For postmastectomy patients without lymphedema symptoms, a traditional breast reconstruction with a lower abdominal wall flap was recommended (BR-group). For lymphedema patients who did not need breast reconstruction, a sole lymph node transfer (LN) was offered if the patient complained about neuropathic pain in the affected arm and/or recurrent erysipelas infections. The ALND group consisted of patients who underwent a routine breast cancer operation with removal of axillary lymph nodes. All patients in this study were females, and the majority of them had secondary iatrogenic lymphedema due to a treatment of breast cancer; axillar lymph node removal and postoperative radiation. Only one patient in the LN group had developed lymphedema after diagnostic lymph node removal and oncological treatment of Hodgkin´s disease. All patients in the BR, LN and LN-BR groups were previously examined by an oncologist and were considered cancer free. Patients with a long history (>10 years) of severe lymphedema were excluded. Lymph node transfer patients with severe non-pitting edema were offered a simultaneous liposuction to reduce the arm (**Figure 6**). All patients signed an approval for sample collection and approved the use of their patient information in the study. (See summary of the patients in **Table 1**, page 36)

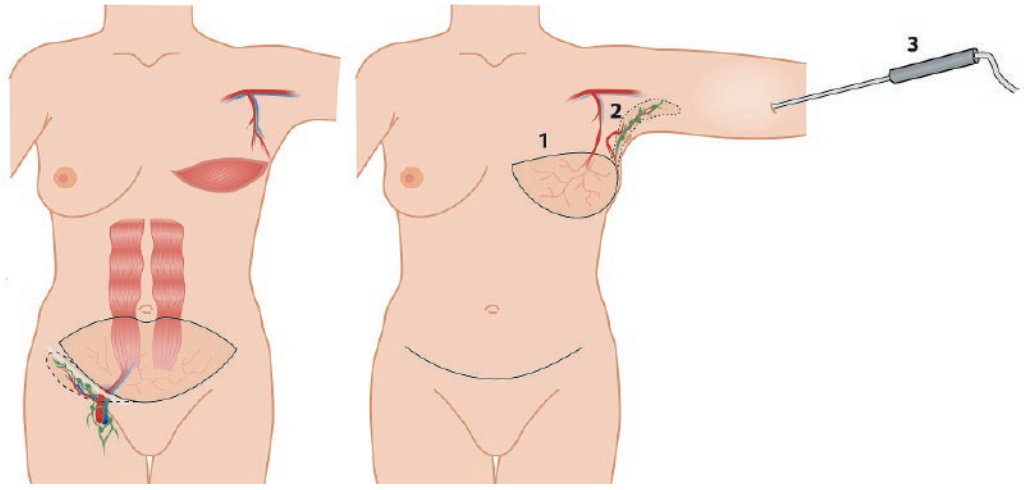


Figure 6. The combined breast reconstruction and lymph node transfer. 1. Microvascular breast reconstruction with a lower abdominal flap, 2. Lymph node transfer to target pitting edema, 3. Liposuction to target non-pitting edema.

4.1 Surgical technique and postoperative care

Lower abdominal wall perforator vessels were preoperatively searched and marked with a Doppler ultrasound device. 0.5 – 1 ml of Patent Blue was injected above the iliac crest to visualize the lymphatic tissue in the groin area. The dissection was started with preparation of the lymphatic flap. Preparation was performed by starting laterally and then proceeding medially in order to identify the superficial circumflex iliac artery (SCIA). In the first patients, also the superficial inferior epigastric vessels (SIEA) were included in the lymphatic flap. The adipose tissue and Patent Blue stained lymphatic tissue around the SCIA vessels were included in the flap. The flap pedicle vessels were ligated at the level of their origin from the femoral vessels (see **Figure 7**). In the LN-BR patients, the lymphatic flap was left connected with the msTRAM/DIEP flap at the level of SIEA vessels, and then the normal msTRAM/DIEP flap was dissected as previously described (Allen, Treece 1994, Chevray 2004, Nahabedian et al. 2002).

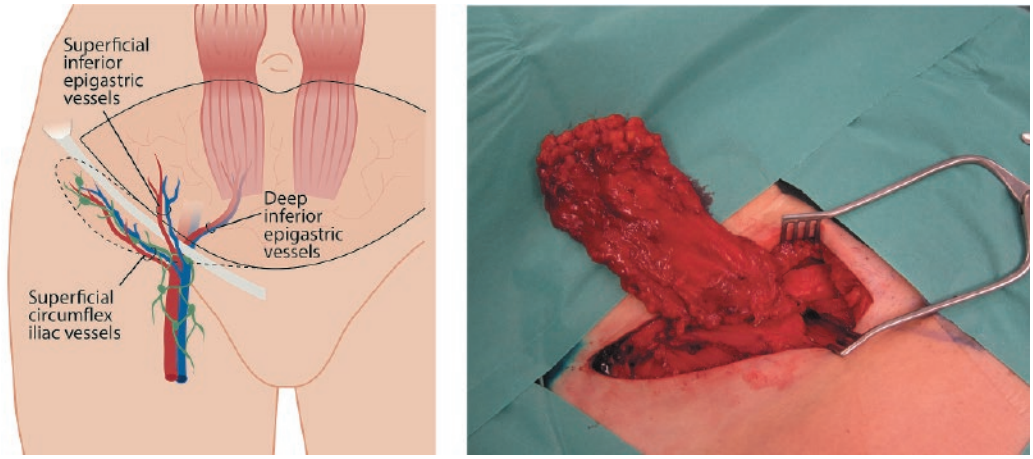


Figure 7. The anatomy of the lymphatic flap. The flap is based on a superficial circumflex iliac artery (SCIA).

In the LN-BR patients, the abdominal wall flap was used to reconstruct the breast. The thoracodorsal vessels were used as recipient vessels in all patients. All scar tissue was removed from the recipient axillary area. In the LN patients, the SCIA pedicle of the lymphatic flap was anastomosed to the thoracodorsal artery and vein. In the LN-BR patients, the blood vascular anastomosis of the msTRAM/DIEP flap was first performed and then the vitality of the lymph node flap was evaluated. If there was concern about sufficient perfusion in the lymphatic flap, the SCIA pedicle was also anastomosed with the retrograde thoracodorsal artery and vein. The lymphatic tissue was placed to cover the axillary plexus in the lymphedema arm.

The blood perfusion of the flap was monitored clinically and with a Licox (Integra, New Jersey) oxygen measurement device for 5 days. Compression was used in the lower abdominal and inguinal area for 4 weeks to prevent seroma formation in the donor area. In most lymphedema patients, the manual lymphatic drainage of the lymphedema arm was started on the second postoperative day and was continued 2 times a week, 3 months postoperatively. Lymph node transfer patients with simultaneously performed liposuction started manual lymphatic drainage 14 - 21 days postoperatively. All lymphedema patients were recommended to use compression garments at least 6 months postoperatively.

4.2 Evaluation of lymphatic vessel function

4.2.1 Lymphoscintigraphy

For lymphoscintigraphy, 40 MBq of technetium-labeled sulfur nanocolloid (99mTc-Nanocoll, GE Healthcare) in a volume of 0.1 - 0.2 ml was injected intradermally in the first interdigital space of both feet or hands. Anterior and posterior planar images of lower extremities were scanned in a supine position at 5, 15, 30, 45, 60 and 120 minutes with the Infinia Hawkeye SPECT/CT (General Electric Medical Systems, Milwaukee, WI, USA). For semiquantitative evaluation of lymphatic drainage, a numerical transport index (Ti) was used. This index is based on five criteria: lymphatic transport kinetics, distribution pattern of the radiopharmaceutical, time to appearance of lymph nodes and visualization of lymph nodes and lymph vessels. A borderline Ti of 10 is used to differentiate between normal and pathological lymph drainage (Kleinhans et al. 1985).

4.2.2 Circumference measurements

The amount of edema was assessed by limb circumference measurements. Pre- and postoperative arm circumferences were measured at 10cm below the elbow and 10 cm above the elbow. The results were compared with diameter measurements of the contralateral limb. At the lower extremity, the sites measured were at the ankle, calf, knee and thigh. The average circumference difference between the donor lower limb and the contralateral lower limb was calculated.

4.3 Study I

In Study **I** the combined microvascular breast reconstruction and lymph node transfer (LN-BR) was performed on 9 postmastectomy lymphedema patients on 2008-2010. To compare this new combined technique to standard breast reconstruction, we collected

data from all patients who underwent traditional unilateral microvascular breast reconstruction (BR) with a lower abdominal wall flap (msTRAM or DIEP, n=78) in 2008-2010 (Table 1, page 44). We compared the operation time, the need for donor site drainage, and the donor site healing process. The effect of lymph node transfer was analyzed with lymphoscintigraphy and arm circumference measurements. The long-term results of these patients were analyzed in Study **II**.

To clarify the biological background of the lymph node transfer technique, the VEGF-C expression of human lymph nodes and other tissues was compared. The multiple human tissue Northern blot (Clontech, Saint-Germain-en-Laye, France) containing RNA from fetal liver, bone marrow, peripheral blood leukocytes, thymus, and spleen was hybridized with the VEGF-C probe. The Northern blot prehybridization, hybridizations and the filter detection were performed as previously described (Saaristo et al. 2000).

4.4 Study II

In Study **II** the clinical efficacy of lymph node transfer on the lymphedema recipient site was evaluated in 19 patients (13 LN-BR patients and 6 LN patients) operated in 5/2007 - 3/2012 (including the long-term results of patients presented in Study **I**) (Table 1, page 44). Seven patients also had at least one episode of erysipelas infection preoperatively. Five patients had neuropathic pain in the lymphedema arm. Combined liposuction was performed on 5 patients with deposition of fat and fibrotic tissue in the affected arm (non-pitting edema). The clinical effect of surgery was analyzed with lymphoscintigraphy and upper limb circumference measurements, which were executed postoperatively at 3 months, 6 months, 1 year, 1,5 years and 2 years, with some exceptions. To assess the necessity for postoperative compression therapy, the patients were encouraged to reduce or discontinue using the compression garment after 6 months of surgery.

4.4.1 Analysis of the seroma samples

Seroma samples from the lymph node flap recipient and donor sites were collected to analyze the postoperative production of VEGF-C. Permission for using patient seroma samples was approved by the Ethical Committee of the Turku University Hospital. The 15 ml samples were collected from axillary drains of all voluntary LN-BR (n=9) and LN (n=5) patients. For controls we used samples collected from axillary lymph node dissection (ALND) patients and (n=10) BR patients (n=10). The BR group included patients without lymphedema symptoms who underwent normal microvascular lower abdominal breast reconstruction. The ALND group consisted of

patients who underwent a routine breast cancer operation with removal of axillary lymph nodes (Table 1, page 44). VEGF-C protein concentrations were measured using enzyme-linked immunosorbent assays (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Optical densities were read at 450 nm (using wavelength correction set to 540 nm) with a microplate reader (Infinite 200; Tecan Group Ltd., Männedorf, Switzerland) and converted to pg/ml using a standard curve.

4.5 Study III

The effect of lymph node transfer on the flap donor area was evaluated in the first 13 patients who had undergone lymph node transfer in 5/2007 - 5/2011 (10 LN-BR patients and 3 LN patients) (Table 1, page 44). The donor site lymphatic function was evaluated 3 - 52 months after surgery with lower extremity lymphoscintigraphy and circumference measurements. Permission to examine the lower limb lymphatic function by lymphoscintigraphy was approved by the Ethical Committee of the Turku University Hospital. The lymphoscintigraphy was performed on 10 patients, as 3 patients did not give permission for this imaging. The scintigraphies were analyzed with the semiquantitative transport index method (Kleinhans et al. 1985). None of the patients had previous edema symptoms in their lower limbs or previous operations on inguinal areas. The results were compared to the contralateral lower extremity. The donor site healing process was followed and all patients were asked about the symptoms in their donor site inguinal area and lower limb.

4.6 Study IV

In Study **IV** we analyzed the effect of lymph node transfer on postoperative production of growth factors and pro- and anti-inflammatory and Th2 cytokines (VEGF-D, IL-1α, IL-1β, TNF-α, TGF-β, IL-4, IL-10, IL-13), which are involved in lymphangiogenesis and wound healing. The growth factor and cytokine concentrations were measured from axillary seroma fluid collected from LN-BR (n=8), LN (n=8), BR (n=8) and ALND (n=8) patients 1 and 6 days postoperatively (Table 1, page 44). Growth factor and cytokine protein concentrations were measured using enzyme-linked immunosorbent assays (R&D systems, Minneapolis, MN, USA) as described above (Chapter 4.4.1, page 33).

4.7 Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics 21.0.0.0 (IBM Corporation, Armonk, NC, USA), JMP Pro 10.0.0 (SAS Institute, NC, USA) or SAS 9.1.3 (SAS Institute, NC, USA). In the case of non-normality or heterogeneity of variances, the data were logarithmic transformed before analysis and again back-transformed to the original scale for presentation. For data not following normal distribution even after transformation, non-parametric tests were used. Differences between groups were tested separately on the first and sixth postoperative days using either one-way analysis of variance (ANOVA) (VEGF-c, VEGF-D, IL-1α, IL-1β, TNF-α, TGF-β1 and IL-10) or Kruskal-Wallis one-way analysis of variance (IL-4 and IL-13)). In the case of statistically significant group-wise differences, this was followed by Dunn's, Gabriel's, Games-Howell's or Tukey's HSD pairwise comparisons test. P-values < 0.05 were considered statistically significant. For normally distributed data, effect sizes for pairwise comparisons are reported as Hedges' G statistic with a 95% confidence interval (CI), whereas Cliff's delta statistic with a 95 % CI is used for non-normally distributed data. The results are interpreted as a combination of both the p-value and the effect size CI: With a significant p-value and an effect size CI excluding the zero, the result is interpreted as significant and substantial. In the case of a non-significant p-value and an effect size CI excluding the zero, the result is interpreted as statistically non-significant, but still substantial. The results are expressed as arithmetic means (± SD) for normally distributed data and geometric means (95 % CIs) for log-transformed data, with error bars representing 95% CIs in the respective graphs. For the non-normally distributed and non-transformed data, the results are reported as medians (interquartile ranges).

Table 1. Summary of the patients in Studies I - IV

Patients in StudyI	
BR group (n)	78
ms-TRAM (n)	52
DIEP (n)	26
Age (mean, range)	49, 25-64
BMI (mean, range)	26, 21-32
Smokers (n)	7
Hypertension (n)	8
Diabetes (n)	1
Previous axillary lymphadenectomy and radiation (n)	48
LN-BR group (n)	9
LN-msTRAM (n)	5
LN-DIEP (n)	4
Age (mean, range)	49, 25-64
BMI (mean, range)	28, 21-33
Smokers (n)	0
Hypertension (n)	2
Diabetes (n)	0
Duration of symptoms, months (mean, range)	43, 6-120
Previous axillary lymphadenectomy and radiation (n)	9

Patients in Study II	
Lymph node transfer patients (n)	19
LN (n)	6
LN-BR (n)	13
Combined liposuction (n)	5
Duration of symptoms, months (mean, range)	48, 6-120
Previous axillary lymphadenectomy and radiation (n)	18
Previous erysipelas infections (n)	7
Neuropathic pain of the arm (n)	5
Age (mean, range)	54, 38-74
BMI (mean, range)	29, 21-37
Patients for the seroma sample analysis (n)	34
ALND (n)	10
BR (n)	10
Previous axillary lymphadenectomy and radiation (n)	6
LN (n)	5
Previous axillary lymphadenectomy and radiation (n)	5
LN-BR (n)	9
Previous axillary lymphadenectomy and radiation (n)	9

Patients in Study III	
Lymph node transfer patients (n)	13
LN (n)	3
LN-BR (n)	10
Age (mean, range)	54, 38-74
BMI (mean, range)	28, 21-37
Smokers (n)	0
Hypertension (n)	2
Diabetes (n)	0

Patients in Study IV	
Patients for the seroma sample analysis (n)	32
ALND (n)	8
BR (n)	8
Previous axillary lymphadenectomy and radiation (n)	5
LN (n)	8
Previous axillary lymphadenectomy and radiation (n)	8
LN-BR (n)	8
Previous axillary lymphadenectomy and radiation (n)	8

5 RESULTS

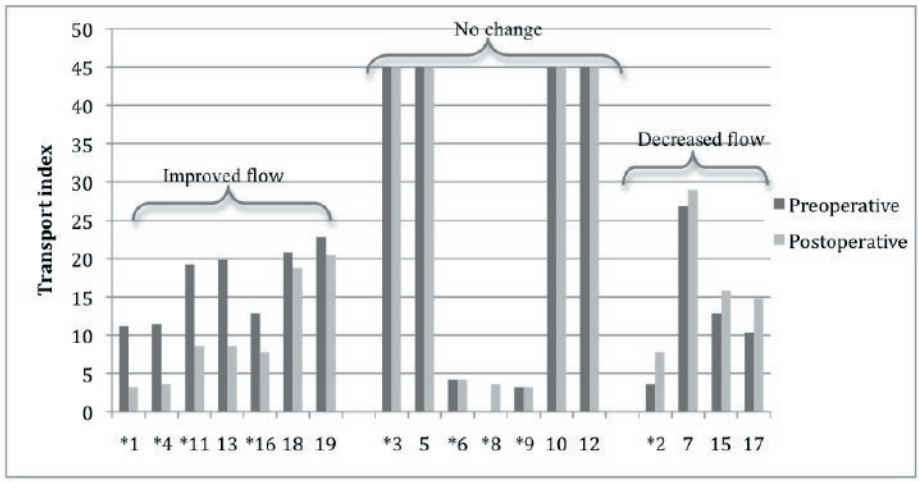
5.1 Comparison of BR and LN-BR

The average operation time was 426 minutes (range, 385–495) in the LN-BR group (n=9) and 391 minutes (range, 220–675) in the BR group (n=78). Drainage in the flap donor site was needed on average for 4.6 days (range 3–6 days) in the LN-BR group and on average for 3.6 days (range 2–7 days) in the BR group. Two LN-BR patients and six BR patients had a local complication in the abdominal wound leading to delayed wound healing. (Study **I**)

5.2 Effect of lymph node transfer on lymphatic vessel function and lymphedema symptoms

The clinical efficacy of lymph node transfer on the lymphedema recipient site was evaluated in 19 patients. The postoperative lymphatic transport index was improved in 7 patients. A minor reduction in arm circumference was detected in 12 patients at 6 months to 4.5 years during follow-up (patients with liposuction were excluded). Five patients were able to reduce compression garment usage and 5 were able to discontinue compression treatment at 6 - 24 months postoperatively. Two lymph node transfer patients with simultaneous liposuction were also able to reduce compression treatment. Six erysipelas patients did not have upper limb infections postoperatively during the 8 – 65-month follow-up. Neuropathic pain was relieved in all 5 patients. There was no correlation between postoperative lymphatic function and patient age or BMI. (**Figure 8**, page 38 and **Table 2**, page 39) (Study **II**)

A



B

Patient	Preoper	3months	6months	1year	1,5years	2years
1	11.2	11.8		3.2		3.2
4	11.4		6.6		3.6	
11	19.2	45	24.8		8.6	
13	19.8			8.6		
16	12.8		7.8			
18	20.8	15.8	18.8			
19	22.8	20.4				
3	45		15.4	45		
5	45	45	41	45		
6	4.2	4.2		4.2		
8		3.6			3.6	
9	3.2	3.2				
10	45		45			
12	45		45			
2	3.6		3.6	3.2		7.8
7	26.8	45		29		
15	12.8	11.4		15.8		
17	10.4		14.8			

Figure 8. A. Pre- and postoperative transport index. *=Patient was able to reduce or discontinue using compression garment. B. Transport index at 3 months to 2 years postoperatively. The result is often seen with delay. Patients with improved lymphatic function (marked with blue) tend to have a fairly low preoperative transport index. Patients with reduced lymphatic function are marked with red.

Table 2. Summary of the clinical results of lymph node transfer patients. P=Neuropathic pain relieved, E=No erysipelas infections postoperatively. nd= no data.

Patient	Duration of symptoms (months)	Age at operation	Operation type	Effect on lymphatic flow	Average circumference difference		Liposuction (ml)	Use of compression	Additional effect of treatment	Follow-up (months)
					Preop	Postop				
1	84	56	LN	improved	2	1.9		Reduced	P, E	67
2	80	40	LN-BR	decreased	0.3	0.8		Reduced	P	60
3	49	50	LN-BR	no change	2	1.3		Discontinued		59
4	45	62	LN-BR	improved	2.7	1.3		Discontinued	E	41
5	30	55	LN-BR	no change	4.2	7.7		No change		39
6	26	51	LN-BR	no change	1.7	0.3		Discontinued	P	33
7	120	65	LN-BR	decreased	6	5.3		No change	E	29
8	6	43	LN-BR	no change	1.3	1		Discontinued		27
9	62	60	LN-BR	no change	0.8	0.5		Discontinued		24
10	120	38	LN	no change	2.9	0.5	900	No change	E	19
11	17	74	LN	improved	4.8	-1.4	1500	Reduced	E	19
12	19	56	LN-BR	no change	2.7	0		No change		20
13	45	61	LN-BR	improved	3.6	2.5	700	No change		16
14	28	53	LN	nd	4.3	1.4	550	Reduced	P,E	15
15	70	50	LN-BR	decreased	2.5	2.3		No change	P	12
16	73	56	LN	improved	0.2	-0.9		Reduced		11
17	26	59	LN-BR	decreased	0	-0.6		No change		11
18	51	55	LN	improved	3.9	1	750	No change		10
19	69	47	LN-BR	improved	2.9	2		No change		10

5.3 Lymphatic flap donor site

The lymphatic function of the donor site lower limb was evaluated in 13 patients. Four patients developed a postoperative seroma that was treated with aspiration. A donor site wound infection developed in 2 patients and 3 patients had delayed wound healing. Four patients complained about numbness or pain in the superficial femoral nerve skin area related to dissection or preparation of the cutaneous femoral nerve during flap harvest. In 2 patients the symptoms were relieved after 9 weeks, whereas in 2 patients the sensitivity of the femoral nerve area remained slightly decreased. Three patients did not give permission for lower limb lymphoscintigraphy. Lymphatic flow was normal in both lower extremities in 4 patients. In 6 patients the lymphatic flow was slightly decreased in the donor site limb compared to the non-operated limb. Two patients showed a slightly abnormal lymphatic function (Ti >10) of the donor site limb (**Table 3** and **Figure 9**, page 40). None of the patients developed any symptoms in their lower extremities during the 8 – 56-month follow-up. Further, there were no differences detected in the circumferences of the donor site lower limbs compared to the non-operated sides (Table 3). (Study **III**)

Table 3. Summary of the donor site results. Both lower limb circumferences were measured from three different points, and the average was compared to the non-operated lower limb. The lower limb lymphoscintigraphy was performed 3 - 52 months postoperatively. dwh= delayed wound healing

Patient	Average circumference difference (cm)	Donor limb transport index	Contralateral transport index	Donor site wound
1	0.5	8.4	4.6	infection, seroma
2	-0.38	12.8	0.2	dwh
3	0.5	nd	nd	
4	-1	10.8	0.2	seroma
5	-0.38	5.4	5.8	
6	-0.38	nd	nd	
7	-0.38	0.2	0.2	infection, dwh
8	0	0.2	0.2	dwh
9	0.13	nd	nd	
10	-1.13	4.2	0.2	seroma
11	0	0.2	0.2	seroma
12	0.13	9.2	0.2	
13	0.38	4.2	0.2	

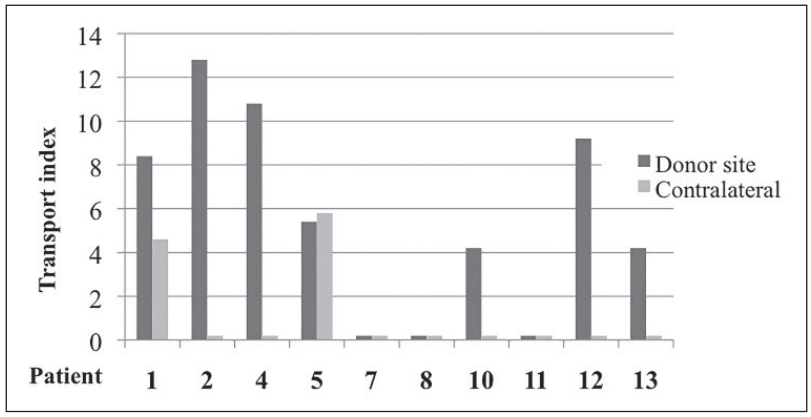


Figure 9. The postoperative transport index of the donor lower limb and the contralateral limb. Values over ten are considered abnormal. None of these patients developed lymphedema symptoms in the 8 – 56-month follow-up.

5.4 Lymphatic growth factors and cytokines in lymph node transfer patients

5.4.1 VEGF-C and VEGF-D

The VEGF-C expression of human lymph nodes and other tissues were studied with Northern blot analysis. Lymph nodes were found to express the highest amounts of VEGF-C mRNA. (Study **I**)

The seroma VEGF-C concentration was found to be higher in the BR, LN and LN-BR groups compared to the ALND group on the first postoperative day. Compared to ALND, the level of VEGF-C was significantly higher in the BR ($p < 0.001$) and LN-BR ($p < 0.001$) groups, and non-significantly but still substantially higher in the LN group ($p = 0.099$, $g = 1.51$, 95% CI [0.42, 2.61]). On the sixth POD, there were no significant differences in the VEGF-C concentrations between the ALND and flap transfer groups. (Studies **II** and **IV**) Further, the level of the VEGF-C protein was significantly higher in seroma fluid samples obtained from axillary recipient wounds compared to abdominal donor (abdominal) wounds ($p = 0.003$). (Study **II**)

The VEGF-D production differed markedly from the VEGF-C production; the VEGF-D concentration in the ALND group was significantly higher compared to the lymph node transfer groups on the first postoperative day (ALND vs. BR p=ns, ALND vs. LN p=0.017, ALND vs. LN-BR p=0.047). On the sixth POD, the VEGF-D concentration in the ALND group was clearly higher compared to all flap transfer groups ALND vs. BR, $p = 0.004$; ALND vs. LN, $p = 0.001$; ALND vs. LN-BR, $p < 0.001$. **(Figure 10)** (Study **IV**)

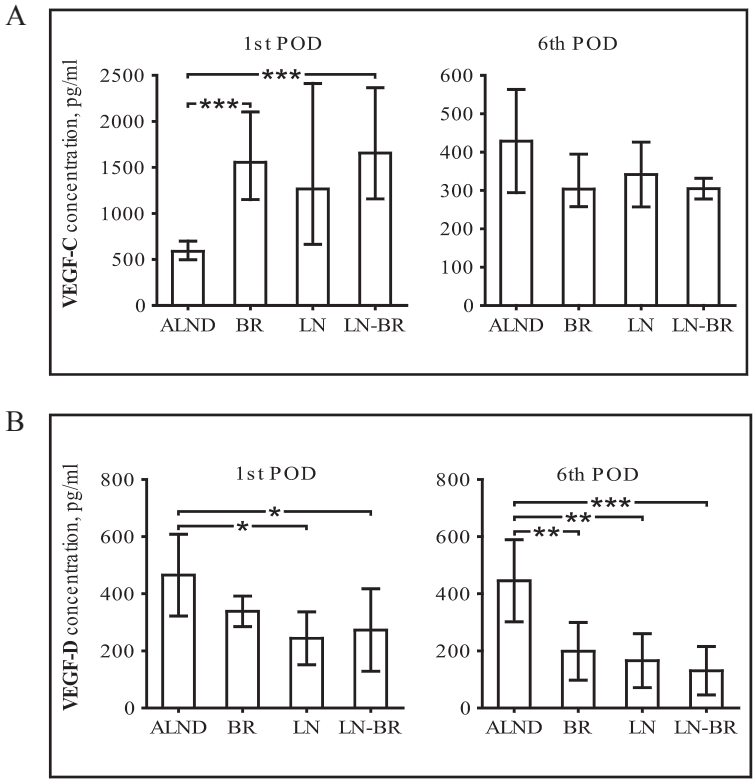


Figure 10. Lymphatic growth factor concentrations in axillary seroma fluid on the first and sixth POD. A. VEGF-C. B. VEGF-D. *: p < 0.05; **: p < 0.01; ***: p < 0.001.

5.4.2 Pro-inflammatory cytokines Interleukin 1α (IL-1α), Interleukin 1β (IL-1β) and Tumor necrosis factor (TNF-α)

There was no difference in the IL-1α concentration between the groups on the first or sixth POD (data not shown). The IL-1β concentration was found to be higher in the ALND group compared to the BR group on the first POD (p= 0.001). On the sixth POD, no significant differences in the IL-1β concentration were found between the groups. The TNF-α concentration was substantially higher in the ALND group compared to the LN-BR group on the sixth POD (p=0.052, g= -1.44, 95% Cl [-2.54, -0.34], whereas on the first POD the concentrations were low in all groups. A common finding for all pro-inflammatory cytokines was large variation inside the ALND group on the sixth POD. **(Figure 11)** (Study **IV**)

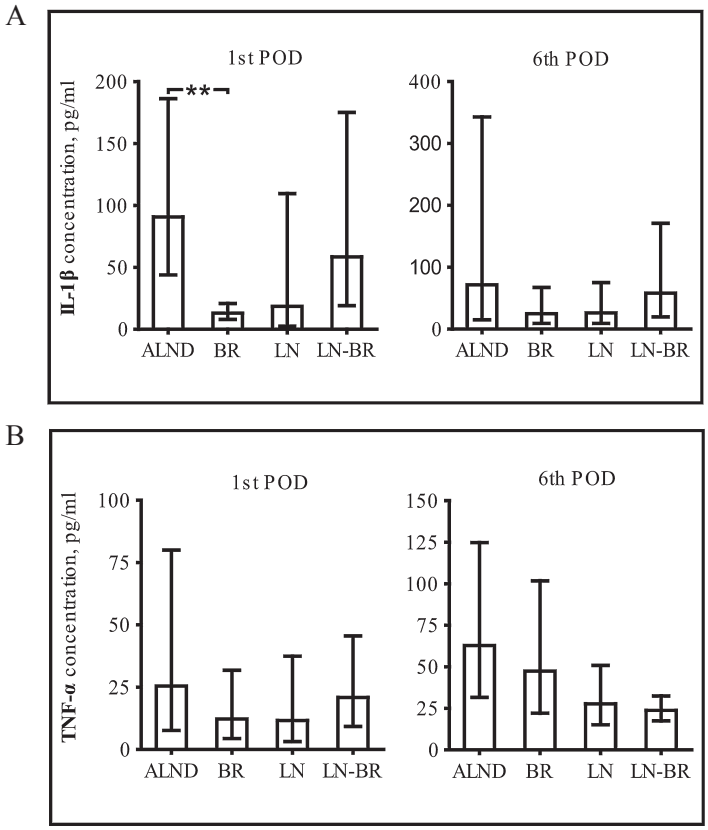


Figure 11. Pro-inflammatory cytokine concentrations in axillary seroma fluid on the first and sixth POD. A. IL-1β. B. TNF-. *: p < 0.05

5.4.3 Immunoregulatory cytokine Interleukin 10 (IL-10)

There were no differences in the IL-10 concentrations on the first POD, but interestingly on the sixth POD, the IL-10 concentration was the highest in the LN and LN-BR groups. The IL-10 concentration was significantly higher in the LN-BR group compared to the ALND group (p=0.004). The difference was statistically substantial between the BR and LN-BR groups (p= 0.165, g=1.36, 95% Cl [0.28, 2.45]. **(Figure 12)** To define whether this difference between the lymph node transfer groups and the other groups was related to the transferred lymph nodes or to the characteristics of a patient group (lymphedema vs. non-lymphedema patient), also the IL-10 concentration from the donor site seroma fluid was measured from all flap groups. We found no differences in donor site IL-10 production between the groups on the first POD (p = 0.318) or the sixth POD (p = 0.346). (Study **IV**)

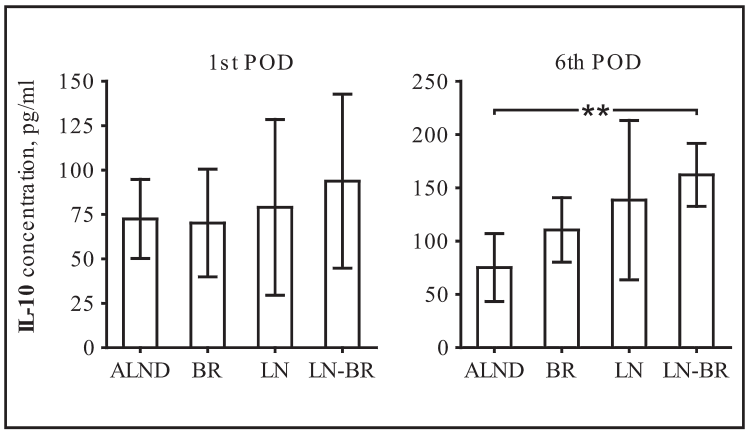


Figure 12. IL-10 concentrations in axillary seroma fluid on the first and sixth POD. **: p < 0.01

5.4.4 Transforming growth factor β1 (TGF-β1)

The concentration of TGF-β1 was significantly higher on the first POD in all flap transfer groups compared to the ALND group (ALND vs. BR p=0.003, ALND vs. LN p=0.01 ALND vs. LN-BR p=0.001). On the sixth POD, there were no differences between the groups as the level of TGF-β1 was low also in flap transfer groups. **(Figure 13**, page 44**)** (Study **IV**)

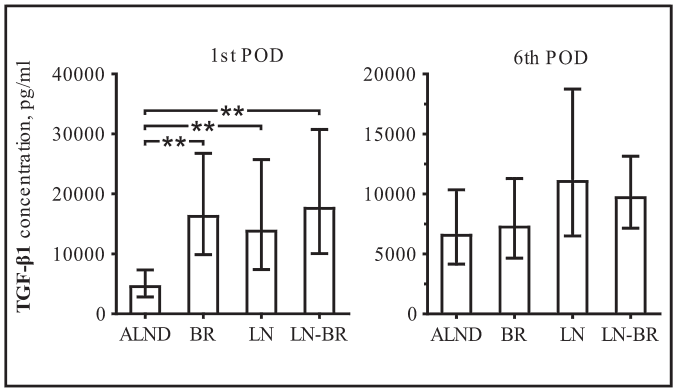


Figure 13. TGF-β1 concentrations in axillary seroma fluid on the first and sixth POD. **: p < 0.01. Note the different scale between the figures.

5.4.5 Th2-related cytokines Interleukin 4 (IL-4) and Interleukin 13 (IL-13)

The concentrations of both IL-4 and IL-13 were very low on the first POD in the LN and ALND groups, whereas in the BR group the level of IL-13 was found to be higher compared to the other groups (BR vs. ALND p=0.006, BR vs. LN p=0.006, BR vs. LN-BR p=0.04). On the sixth POD, the IL-4 level seemed to be higher in all flap transfer groups compared to the ALND group, although the concentrations were quite low in all groups and the results were not statistically significant. The IL-13 concentrations were very low on the sixth POD and no differences between the groups were therefore noticed. **(Figure 14)** (Study **IV**)

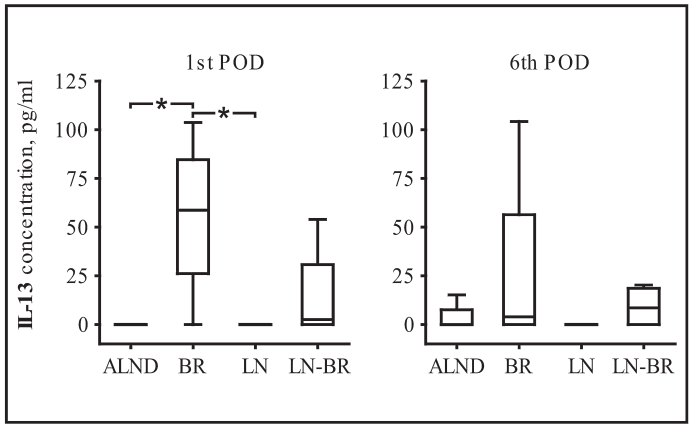


Figure 14. IL-13 concentrations in axillary seroma fluid on the first and sixth POD. *: p < 0.05

To summarize the results of separate pro-inflammatory, Th2-related and anti-inflammatory cytokine concentrations, group-wise cytokine profiles were generated from the relative

concentrations (group-wise mean divided by the grand mean). On the first POD, the pro-inflammatory cytokines dominated the profile of the ALND group. The level of Th2-related cytokines was, in contrast, low for the ALND group and high for the BR group, particularly with IL-13. Interestingly, the TNF-α and IL-13 levels were low in the LN group on both the first and sixth POD, and the IL-10 levels were slightly elevated on the sixth POD in the LN-BR group and to a smaller degree in the LN group. The results on the sixth POD were similar to those of the first POD, with the exception of TNF-α, which was elevated also in the BR group. (**Figure 15**) (Study **IV**)

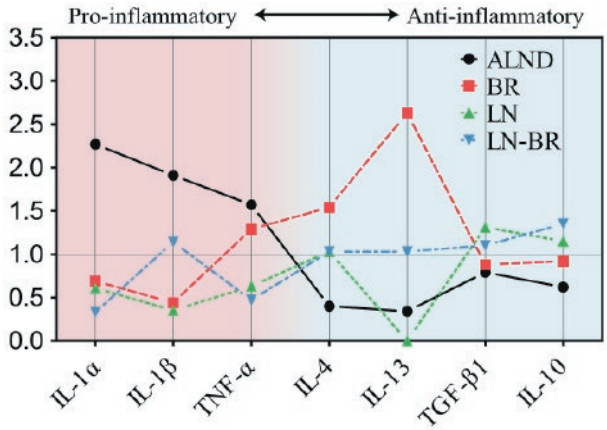


Figure 15. Summary of the relative cytokine concentrations in different patient groups on the sixth POD. Cytokines are arranged in the figure according to their type: pro-inflammatory cytokines are located on the left, Th2-cytokines are in the middle, and anti-inflammatory cytokines on the right side of the figure.

6 DISCUSSION

The current treatment of lymphedema aims at alleviating symptoms and preventing the progression of swelling with life-long compression treatment and physiotherapy (Suami, Chang 2010). Reductive surgical techniques can be used to remove the excess volume of the lymphedema limb, but the result can be maintained only with continuous compression therapy (Brorson, Svensson 1998, Warren et al. 2007). Animal model studies and recent results in lymphedema patients have shown that microvascular lymph node transfer may improve the compromised lymphatic function of the affected extremity (Chen et al. 1990, Tobbia et al. 2009, Lin et al. 2009, Becker et al. 2006). Other options for reconstructing lymphatic function are lymphovenous anastomoses and lymphatic vessel transfer (Suami, Chang 2010, Warren et al. 2007). In both of these techniques, it is essential to find functioning lymphatic vessels to perform surgical anastomosis. This is often difficult or impossible in late stage lymphedema patients. In the lymph node transfer technique, the lymphatic anastomoses are assumed to form spontaneously. This has raised questions and scepticism towards this fairly new technique. However, the lymphatic vasculature is known to have an exceptional capacity to regenerate (Slavin et al. 1997, Tobbia et al. 2009, Slavin et al. 1999, Oden 1960, Paavonen et al. 2000). The lymphatic growth factor VEGF-C is the most important regulator of lymphangiogenesis (Alitalo 2011). In this study we have found that human lymph nodes express high levels of VEGF-C. This might be the biological explanation for the recovery of the lymphatic function of the affected arm after lymph node transfer. However, VEGF-C was also found in the axillary seroma fluid of the breast reconstruction patients without lymph node transfer. Residual axillary lymph nodes (Szuba et al. 2007) and the revision of the scar might explain the high production of VEGF-C in these patients. The anti-inflammatory and anti-fibrotic response was the most prominent in lymph node transfer patients. This might also have a significant effect on the recovery of the patients.

6.1 The clinical effect of lymph node transfer surgery

For the majority of the patients in this study, the lymph node transfer was performed in combination with routine breast reconstruction. The timing of breast reconstruction in our clinic is typically one to two years after finalization of the oncological treatments of breast cancer. Lymphedema usually develops relatively slowly after surgery or radiation (Petrek et al. 2001). Therefore, some of our patients had early stage lymphedema at the time of surgery. According to the results of this study, it seems that a fairly low preoperative transport index may predict a better outcome of the operation. It can be

proposed that the optimal timing for the lymph node transfer is during the early stage of the disease, before the secondary changes of lymphedema occur. However, we do not know what the prognosis of these patients would have been without the lymph node transfer.

Results from experimental animal models have demonstrated that lymphatic vessel maturation after surgical operation is a rather slow process. It has been shown that lymphangiogenesis occurs in two weeks, but the newly formed lymphatic vessels stabilize and maturate into true collecting lymphatic vessels spontaneously over the course of six months (Tammela et al. 2007). This may be the reason for the finding that the clinical benefits of the surgery are often seen with a delay. Patients may need to wait for 1 to 2 years before they can reduce the use of compression garments. Similarly, the improvement in lymphoscintigraphy may be detected even after a year postoperatively.

The lymphoscintigraphies were analyzed with the semiquantitative transport index method. This method takes into account not only the isotopic marker flow velocity but also the visualization of vessels, nodes and the distribution pattern of the marker. The sensitivity of this technique is 97.4% and the specificity is 90.3% in 122 investigations (Kleinhans et al. 1985). However, the result in scintigraphy does not always seem to correlate with the clinical benefit. Some patients were able to reduce or stop using compression therapy even though the postoperative lymphatic function in lymphoscintigraphy was decreased or showed no change. To provide more information about the lymphatic function after lymph node transfer, it would be beneficial to analyze the lymphatic function also with additional imaging methods, such as MRI lymphography.

Minor reduction in arm circumference was measured in over half of our patients. However, the amount of edema varies during the day and it is dependent on temperature, amount of physical work, the quality of the compression garment used on the day of the examination, and several other factors. For more accurate information, the arm volumetry should be performed first with proper preceding compression treatment. After that, the patient removes the compression garment for a 1 – 2-week time period and the increase in arm volume is measured at several time-points. The change in the arm volume can be compared to the postoperative situation.

For lymphedema patients without the need for breast reconstruction, the indications for lymph node transfer were recurrent erysipelas infections and/or neuropathic pain of the arm. Lymphedema patients are prone to soft tissue infections as accumulation of protein-rich fluid in subcutaneous tissue favors the growth of microbes (Rockson 2001). In addition, antigen-presenting cells have no direct route to local lymph nodes to induce immune responses needed for effective clearance of pathogens. In our data, six out of seven patients with erysipelas infections have not had infectious episodes after

the lymph node transfer. Similarly, Becker et al. have reported decreased episodes of erysipelas and lymphangitis after lymph node transfer (Becker et al. 2006). This suggests that transferred lymph nodes may have resumed the immunological defense barrier of the arm. However, liposuction also seems to decrease the incidence of soft tissue infections (Brorson, Svensson 1997). This may be related to increased local skin blood flow after liposuction (Brorson, Svensson 1997). Three of our seven erysipelas patients had combined liposuction and lymph node transfer.

All patients with neuropathic pain benefited from the operation. This finding is compatible with the results of Becker et al. (Becker et al. 2008). However, it is possible that removal of the axillary scar and transfer of any tissue flap to cover the axillary plexus relieves the pain symptoms.

In addition to improvement in the lymphatic flow and immunological functions of the affected arm, lymph node transfer offers a possibility that other reconstructive options are lacking. Previous experimental studies have shown that the sentinel node function of transferred lymph nodes are regained and intracutaneously injected lung carcinoma cells are trapped in the transferred lymph node (Tammela et al. 2007). Transplanted lymph nodes also retain the ability to mount a cytotoxic immune response against tumor cells (Fu et al. 1998, Rabson et al. 1982). Clinically this means that transferred lymph nodes may work as new sentinel nodes if the patient develops a recidive or new cancer.

After the publication of Study **I**, the interest towards lymph node transfer has increased rapidly since it is conveniently performed simultaneously with breast reconstruction. Recently, a few groups have also published their patient series with positive results (Cheng et al. 2013, Dancey et al. 2013). Criticism has also been presented due to the fact that the outcomes of lymph node transfer have not been compared to flaps without lymph nodes (Schaverien, Munnoch 2013). To date, including our studies, there have been 4 published studies with a total of 61 patients reporting the outcomes of lymph node transfer to the axilla (Becker et al. 2006, Saaristo et al. 2012, Viitanen et al. 2013, Dancey et al. 2013). Three published studies with a total of 44 patients have reported the effect of lymph node transfer using the wrist or forearm as a recipient site (Lin et al. 2009, Gharb et al. 2011, Cheng et al. 2013). These preliminary results suggest that lymph node transfer may be beneficial for lymphedema patients. However, larger, randomized, prospective studies are needed to evaluate the clinical efficacy of the lymph node transfer surgery in the treatment of lymphedema.

6.2 The donor site morbidity

There are currently 2.6 million breast cancer survivors in the USA alone (American Cancer Society) and 15 - 20% of them suffer from postmastectomy lymphedema

(Suami, Chang 2010, Erickson et al. 2001). After 2012, the popularity of microvascular lymph node transfer has increased rapidly, since it is easily combined with routine breast reconstruction operation. However, the effect of this surgery on the flap donor site has not been studied before. Lymphedema patients that we operate on have already developed lymphatic dysfunction after the axillary clearance and radiation. Therefore, they might be more prone to develop donor site lower limb lymphedema as well. To evaluate the postoperative lymphatic function of the donor site lower limbs, we performed a lymphoscintigraphy to all voluntary lymph node transfer patients. Surprisingly, the transport index was considered abnormal in two patients. Despite the lymphoscintigraphy findings, we found no differences in the circumferences of the lower limbs. Further, none of the patients have complained about swelling or heaviness of the donor site limb during the 8 – 56-month follow-up.

The scintigraphy results were unexpected since it was assumed that the lymph nodes in the groin flap area would not be critical for the lymphatic drainage of the lower limb (Clodius et al. 1982, Becker, Hidden 1988, Assouad et al. 2002). After the results of this study, we have modified the design of the lymphatic flap (**Figure 16**, page 50). The modification was based on the knowledge that the majority of the lymph nodes draining the lower limb are located in the central or inguinal area, and that the major collecting lymphatic vessels from the lower limb are found medial to the femoral vessels (van der Ploeg et al. 2009, Caplan 1978). It is possible that any dissection in this area may damage the lower limb lymphatic flow (Hannequin et al. 1988, Haaverstad et al. 1995).

In the first patients, we also harvested the SIEA vessels as a second pedicle for the lymphatic flap. Interestingly, SIEA flaps are associated with increased seroma formation in comparison to other lower abdominal breast reconstruction techniques (Moradi et al. 2011). This might be related to the fact that the origin of the SIEA vessel pedicle is located close to the central and inferomedial inguinal area containing the main lymphatic vessels and sentinel nodes of the lower limb (van der Ploeg et al. 2009, Caplan 1978). To prevent any damage to this critical area, it might be reasonable to avoid the harvest of the SIEA as a second vascular pedicle in the lymphatic groin flap (Figure 16).

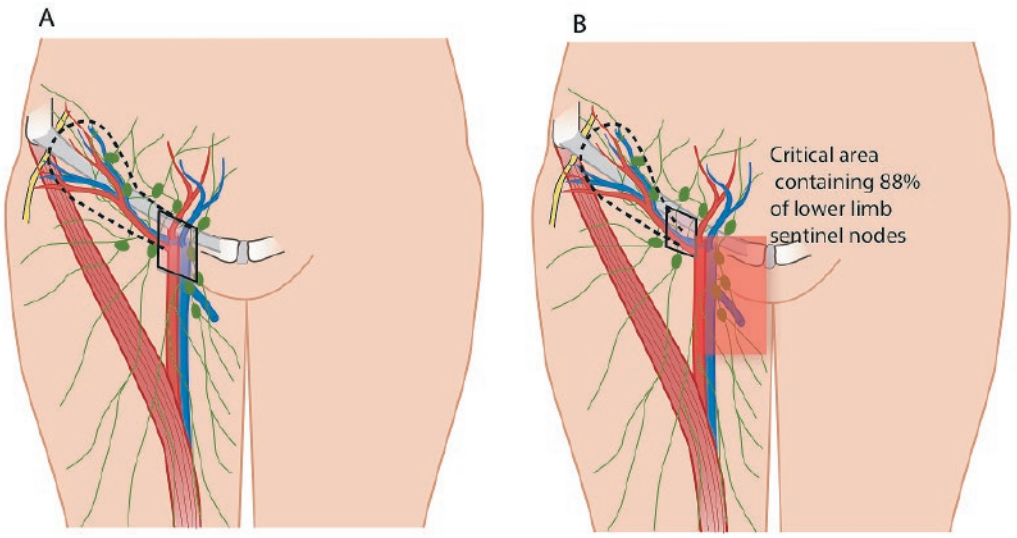


Figure 16. A. The lymphatic flap used for the first patients. The area of dissection is marked with a square. B. The defined flap design. The dissection is limited to the lateral border of the major inguinal vessels.

The clinical significance of these scintigraphy findings remains unclear, since none of the patients in this study have developed lymphedema symptoms so far. On the other hand, lymphedema is known to progress with delay. In addition, according to recent studies, some breast cancer related lymphedema patients might have a genetic predisposition to develop lymphedema (Newman et al. 2012, Finegold et al. 2012). Importantly, in 2013, the first case reports of donor site lymphedema were published (Vignes et al. 2013, Pons et al. 2013). Vignes et al. reported donor site complications of 26 lymph node transfer patients treated and followed in the center, which manages conservative treatment of lymphedema. They found six donor site lymphedemas (2 lower limbs and 4 upper limbs). The diagnosis was based on circumference measurements and the Kaposi-Stemmer sign (Stemmer 1976). However, these 26 patients represent only a small percentage of the estimated 1500 patients who have undergone lymph node transfer (Becker et al. 2012, Vignes et al. 2013). In addition, Pons et al. have performed 42 lymph node transfers and found one postoperative lower limb edema (Pons et al. 2013).

In addition, a recent anatomical study has revealed new information about the lymphatic drainage of the thigh. Tourani et al. found that the superficial lymphatic collectors of the thigh can be divided into two groups: the ventromedial bundle and the local collectors. The ventromedial bundle drains the majority of the anterior thigh and flows to the inferolateral inguinal lymph nodes. In contrast, the local collectors are widespread across the upper thigh immediately deep to the subdermal venules, and they are drained to the

superolateral group of the superficial inguinal lymph nodes – the area of the lymphatic flap. (Tourani, Taylor & Ashton 2013)

It would be interesting to examine our patients also with IGC lymphography, as Azuma et al. commented in their letter (Azuma, Yamamoto & Koshima 2013). ICG lymphography is suggested to reveal stage 0 lymphedema based on dermal backflow patterns (Yamamoto et al. 2011a, Yamamoto et al. 2011b). Baumeister and Siuda recommend that normal preoperative function of the donor thigh needs to be ensured with lymphoscintigraphy before harvesting lymph vessels from the thigh (Baumeister, Siuda 1990). This approach might be used in lymph node transfer patients as well. In addition, ICG lymphography could be used to identify the lymph nodes draining the lower limb which should not be harvested for the flap (Mihara et al. 2012b).

In conclusion, in the future it is important to also evaluate patients operated with our new, more conservative technique. In addition, all lymph node transfer patients need to be followed in order to detect any clinical symptoms in the donor lower limb, and it is of utmost importance to emphasize to the patients that there might be a risk of donor area swelling.

6.3 Lymphatic growth factors and cytokines

6.3.1 VEGF-C and VEGF-D

VEGFs are important regulators of angiogenesis and lymphangiogenesis (reviewed in Oliver 2004, Lohela et al. 2009). VEGF-C and VEGF-D both induce mainly lymphangiogenesis by enhancing proliferation, migration, and survival of endothelial cells (Tammela et al. 2005). In this study, we have found that human lymph nodes express endogenous VEGF-C mRNA, which might partly explain the beneficial effects of the microsurgical lymph node transfer method. Transfer of the lymph nodes and the resulting endogenous VEGF-C expression may enhance the regeneration of the lymphatic network in the axilla.

However, the increased concentration of the VEGF-C protein was found from the axillary seroma fluid of both LN and LN-BR patients as well as from BR patients on the first postoperative day. One reason for the high VEGF-C concentration in the BR patients might be the fact that patients who have not developed lymphedema after axillary clearance have actually functioning residual axillary lymph nodes (Szuba et al. 2007) which are producing VEGF-C (**Figure 17,** page 55). In addition, recruited macrophages have been shown to be the source of VEGF-C after flap transfer in a mouse model, suggesting another potential mechanism for the elevated VEGF-C level in the LN, LN-BR and BR groups (Yan et al. 2011a). VEGF-C is also known to play a role in normal

wound healing (Alitalo 2011, Paavonen et al. 2000), and for that reason it might be expected that the large wound surface area in the BR and LN-BR patients may produce higher amounts of growth factors. Therefore, we compared the VEGF-C production also on donor site wounds and the lymphatic flap recipient site. Importantly, the VEGF-C concentrations in the large abdominal donor wounds were lower than in the recipient site wounds.

Interestingly, we found that the VEGF-D production after lymph node transfer differs markedly from VEGF-C production (See Figure 17, page 55). In our material, the samples collected from the ALND patients had a significantly higher concentration of VEGF-D compared to the samples of the flap groups. VEGF-C and VEGF-D both bind VEGFR-3 and -2, but their *in vivo* effects in animal models differ from each other (Tammela, Alitalo 2010, Olsson et al. 2006, Karkkainen et al. 2001). VEGF-C is critical for the embryonic development of lymphatic vasculature, whereas VEGF-D has more pronounced angiogenic effects in addition to the lymphangiogenic effects (Rissanen et al. 2003, Lahteenvuo et al. 2011). The ALND patients in this study have undergone axillary clearance due to a breast cancer lymph node metastasis, while the patients in the other groups did not have active cancer at the time of surgery. Several studies have shown a direct correlation between VEGF-C and VEGF-D expression and lymphatic invasion and lymph node and distant organ metastasis (Achen et al. 1998, Alitalo, Tammela & Petrova 2005, Tobler, Detmar 2006). It is not known to what extent the recently operated carcinoma can alter the VEGF-D concentration in the axillary seroma.

6.3.2 The role of inflammation and fibrosis

The role of inflammation is not completely understood in the development of lymphedema. Several studies have shown that inflammation is closely related to lymphangiogenesis, and that pro-inflammatory cytokines (IL-1α, IL-1β and TNF-α) can induce VEGF-C expression in experimental settings (Alitalo 2011, Ristimaki et al. 1998). On the other hand, lymphatic stasis is known to initiate chronic inflammation and progressive tissue fibrosis, resulting in worsening of the lymphatic function and clinical lymphedema (Avraham et al. 2010). To clarify the biological effects of lymph node transfer surgery and the role of inflammation, the postoperative production of pro- and anti-inflammatory- and Th2-cytokines were studied.

According to our results, it seems that removal of axillary lymphatic tissue promotes a pro-inflammatory response, whereas transfer of healthy tissue to the axilla may reduce this effect. On the sixth postoperative day, the concentrations of pro-inflammatory cytokines (IL-1α, IL-1β and TNF-α) were the highest in the ALND group. The variation inside the ALND group was large. This variation might be related to minor disturbances

in the wound healing process (infection, delayed wound healing), which are known to promote a pro-inflammatory response. Another explanation for the large variation inside the ALND group could be the fact that some of these patients are going to develop lymphedema in the future, while the others are able to regenerate axillary lymphatic vasculature.

There were no significant differences in the production of pro-inflammatory cytokines IL-1α, IL-1β and TNF-α between the different flap transfer groups, with the only exception being the substantial difference in the IL-1β concentration between the LN-BR and BR groups on the first POD. It is possible that transfer of a healthy tissue flap, rather than transferred lymph nodes, are responsible for this lower production of pro-inflammatory cytokines. This assumption is compatible with the finding that a muscle flap decreases the TNF-α level in early wound healing in an experimental model (Brown et al. 2000). In practice, the transfer of healthy tissue is commonly used in the treatment of infectious wound complications (Cabbabe, Cabbabe 2009, Corten et al. 2013). In addition, according to recent reports, the transfer of a flap without lymphatic tissue (normal breast reconstruction) has improved the lymphatic function of the affected arm in some patients (Abbas Khan et al. 2011, Blanchard, Arrault & Vignes 2012). Similarly, immediate breast reconstruction seems to reduce the risk of developing lymphedema after breast cancer treatments (Card et al. 2012). However, the increased production of VEGF-C after flap transfer (also without lymph nodes) probably plays a major role in inducing these beneficial effects in relation to lymphedema.

The most interesting finding in our study was the fact that the concentration of IL-10 on the sixth POD was higher in the lymph node transfer groups compared to the BR and ALND groups. IL-10 is an immunoregulatory cytokine which is primarily produced by regulatory macrophages. The main function of IL-10 is the prevention of uncontrolled nonadequate immunologic reactions (Asadullah, Sterry & Volk 2003). IL-10 limits and terminates inflammatory responses and regulates the differentiation and proliferation of several immune cells. The therapeutic effects of IL-10 have been investigated in several autoimmune diseases (Asadullah, Sterry & Volk 2003). The effect of IL-10 on lymphangiogenesis has not been studied before. However, it has been shown that IL-10 can reduce scar formation and fibrosis (Shi et al. 2013) and down-regulate fibrosis-promoting pro-inflammatory cytokines IL-6 and IL-8 (Singer, Clark 1999). In pre-clinical studies, IL-10 has been found to be beneficial for scar-improving therapies (Shi et al. 2013, Peranteau et al. 2008). It would thus be possible that the clinical effects of lymph node transfer are partly mediated by the increased production of IL-10, which has anti-inflammatory and also anti-fibrotic properties.

TGF-β is an anti-inflammatory cytokine which inhibits the initiation of inflammation (Li et al. 2012). In our material, the TGF-β1 concentration in the axillary seroma fluid on the first POD was higher in all flap transfer groups compared to the ALND group. This finding is compatible with our conclusion above that a flap transfer may induce an anti-inflammatory response. In addition to its anti-inflammatory activities, TGF-β is an important regulator of tissue fibrosis and scarring (Clavin et al. 2008, Penn, Grobbelaar & Rolfe 2012). Clinical studies have demonstrated that fibrosis is a key player in the development of lymphedema (Goffman et al. 2004). In clinics, it is well-known that the factors causing scarring and fibrosis, such as radiation therapy, infections or extensive surgical procedures, are often related to development of lymphedema (Ververs et al. 2001, Hinrichs et al. 2004). In contrast to the first POD, there were no differences in the TGF-β levels between the groups on the sixth POD. It can be speculated that this decrease in the TGF-β concentration on the sixth POD may be related to the production of IL-10, as it is known that IL-10 down-regulates TGF-β expression in experimental settings (Nakagome et al. 2006). Importantly, it has been shown that IL-10 induces a protective effect against TGF-β-induced fibrosis (Shi et al. 2013) and that inhibition of TGF-β leads to acceleration of lymphatic repair (Clavin et al. 2008).

Inflammatory responses of T-cells are known to play an active role in the pathogenesis of many autoimmune and fibrotic diseases. Avraham et al. showed that Th2 differentiation is necessary for soft tissue fibrosis and development of lymphedema. Further, they showed that the lymphedema-initiating effect of the Th2 response is mediated by IL-4 and IL-13 (Avraham et al. 2013). Therefore, we were interested in studying the postoperative concentrations of the Th2 cytokines IL-13 and IL-4. Interestingly, in our study the BR group had the highest concentration of Th2-related cytokines, although the concentrations were quite low in all groups. It can be speculated that this finding may be related to the different biological nature of the surgery in all the flap groups, while in the lymph node transfer groups the increased production of IL-10 may prevent T cell inflammation and secretion of Th2 cytokines. Importantly, IL-10 is known to suppress the Th2 T cell polarization (Avraham et al. 2010, Mosser, Edwards 2008). (See summary in Figure 17, page 55)

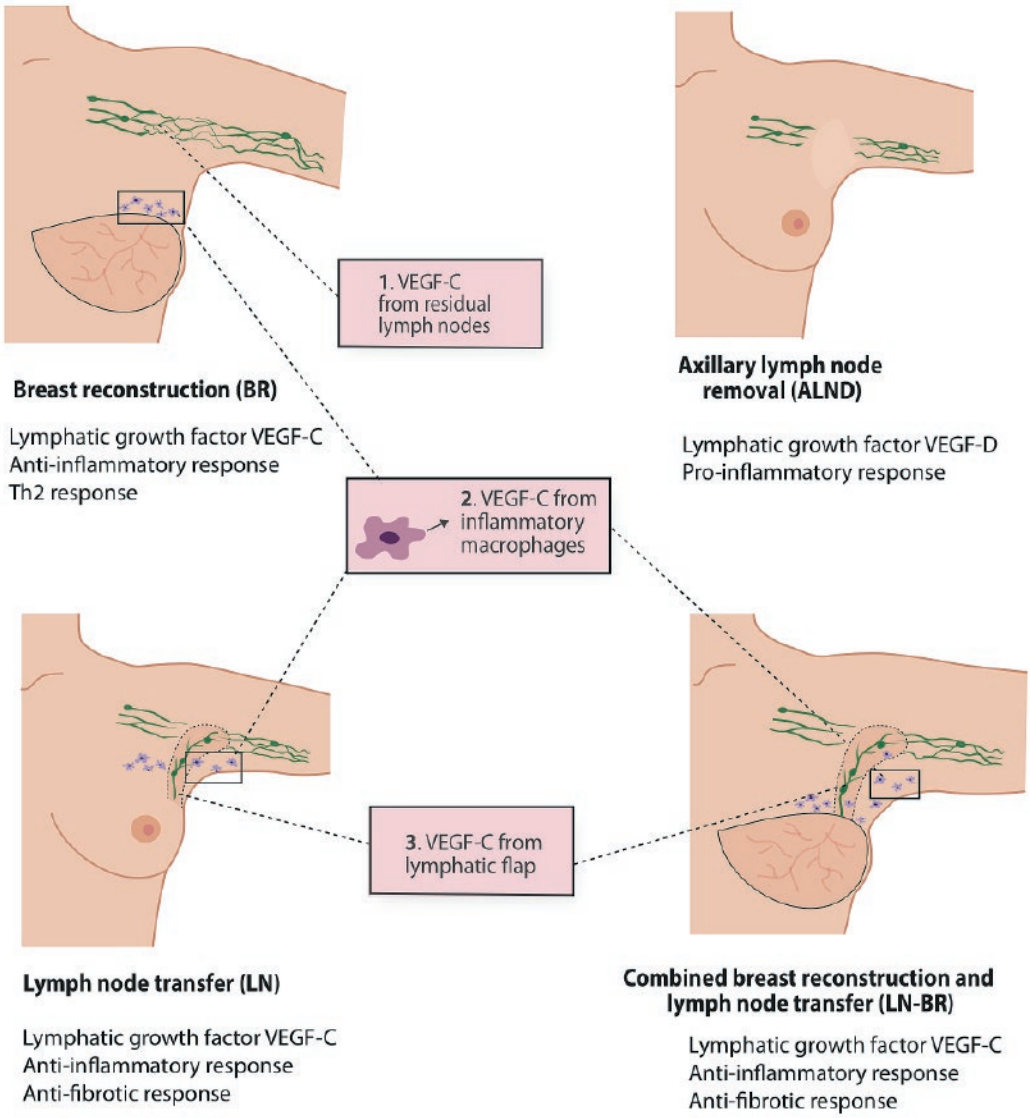


Figure 17. Summary of growth factors and immunological responses in different patient groups. The concentration of pro-inflammatory cytokines was low in all flap groups (anti-inflammatory response). In addition, in lymph node transfer groups IL-10 induces anti-inflammatory and anti-fibrotic response. 1. – 3.: Possible sources of VEGF-C production in the axilla.

6.4 Selection of the surgical treatment for a lymphedema patient

6.4.1 Liposuction for non-pitting edema

In late stage lymphedema patients, the edema is mainly dominated by adipose and fibrous tissue (Brorson et al. 2006). In these patients with non-pitting edema, liposuction is still a treatment of choice. It is clear that excess adipose tissue cannot be spontaneously

dissolved even if the lymphatic function could be normalized with reconstructive surgery. However, to maintain the results of the liposuction, the patients need to continue the use of the compression garment (Brorson 2003). The combination of liposuction and reconstructive surgery aims at a situation where the arm has been reduced with liposuction and lymphatic function has been improved with reconstructive methods in order to prevent further swelling even without compression treatment.

6.4.2 Reconstructive surgery to target pitting edema

The reconstructive surgery is targeted to treat pitting edema by improving the lymphatic flow from the extremity. In contrast to the lymph node transfer surgery, the use of a LVA technique or lymphatic vessel transfer requires functioning lymphatic vessels, which are visible with available imaging techniques. Therefore, in practice these latter techniques can only be used in the early stage lymphedema. However, lymph node transfer also seems to be most efficient in patients with a fairly low preoperative transport index.

Unlike both lymph node transfer and the lymphatic vessel transfer technique, the LVA surgery does not include the possibility of donor site lymphedema. On the other hand, there is lack of evidence of the long-term patency of the performed anastomosis. The main challenge is backflow from the venous system, which leads to a thrombosis of the anastomosis (Uhrin et al. 2010). In addition, LVA surgery cannot be recommended to patients with a previous malignancy of the limb. LVA may offer a direct pathway for cancer cells from the lymphatic vessels to the blood circulation.

According to the present study, lymph node transfer could be most suitable for lymphedema patients with previous axillary lymphadenectomy and mastectomy, with recurrent soft tissue infections and neuropathic pain of the arm. A minority of lymphedema patients may have developed lymphedema after trauma or surgery which have not involved the axillar or inguinal lymph nodes. Perhaps for these patients, who still have functioning lymph nodes in their axillas, the LVA or lymphatic vessel transfer would be the treatment of choice.

At present time, there is no consensus about the right reconstructive treatment option for lymphedema patients. Likely there is no a single treatment option available which would be suitable for all lymphedema patients. Further studies of all reconstructive techniques are needed to clarify the patient selection for each method.

6.5 Future directions

Half of our patients did not benefit from the lymph node transfer. This finding is comparable with the results of experimental large animal studies, which have shown

that even though spontaneous lymphangiogenesis after lymph node transfer does occur, the incorporation of the transferred lymph nodes into the existing lymphatic network may fail (Honkonen et al. 2012, Lahteenvuo et al. 2011). This might be partly explained by individual differences in the VEGF-C expression in lymph nodes and differences in axillary lymphatic vessel anatomy. Recent findings from the experimental animal models have demonstrated that the incorporation of the transferred lymph nodes into the resident lymphatic vascular tree can be enhanced with the use of growth factors (Lahteenvuo et al. 2011). Postoperative lymphatic drainage can be significantly improved in the VEGF-C/D-treated pigs compared with controls. Importantly, the structure of the transferred lymph nodes was best preserved in the VEGF-C-treated pigs. Therefore, in the future, one option for enhancing the therapeutic effect of lymph node transfer on lymphedema patients might be the induction of short-term overexpression of the patients' own endogenous lymphatic growth factors (Honkonen et al. 2012).

On the other hand, flap transfer without lymph nodes also seems to increase the production of VEGF-C and reduce the local pro-inflammatory response after surgery. These findings raise a question whether lymphedema could be surgically treated with traditional breast reconstruction without the transfer of the lymph nodes, avoiding the possible risk of donor site swelling. In fact, there are previous studies which suggest that immediate breast reconstruction reduces the risk of postmastectomy lymphedema (Card et al. 2012) and that delayed breast reconstruction may reduce lymphedema symptoms of the affected arm (Blanchard, Arrault & Vignes 2012, Abbas Khan et al. 2011, Chang, Kim 2010). It would thus be important to compare the effects of BR and LN-BR in a large prospective randomized study. However, lymph node transfer offers possibilities that traditional breast reconstruction and other reconstructive options are lacking. In an ideal situation, the lymphatic, immunological and sentinel functions may all be restored.

7 CONCLUSIONS

On the basis of the present study, the following conclusions can be made:

1 Microvascular lymph node transfer can be performed simultaneously with traditional msTRAM or DIEP flap breast reconstruction.

2 The lymphatic flow of the affected arm might be improved with microvascular lymph node transfer. Half of the patients benefit from lymph node transfer surgery as they have been able to reduce or discontinue the use of a compression garment postoperatively. Further, the majority of the patients with recurrent erysipelas infections or neuropathic pain benefit from lymph node transfer. However, larger randomized studies comparing the effects of BR and LN-BR on recipient site lymphatic function are needed.

3 The harvesting of the lymph node flap may affect the lymphatic function of the donor lower limb. Future studies and follow-up is needed to clarify this donor site issue.

4 Human lymph nodes produce high concentrations of VEGF-C mRNA. An increased concentration of VEGF-C protein can be found from the axillary seroma fluid after lymph node transfer and traditional breast reconstruction.

5 Lymph node transfer induces a local anti-inflammatory and anti-fibrotic response in the flap recipient site.

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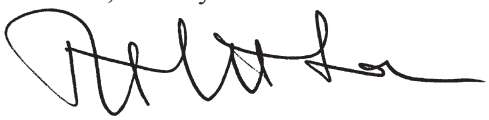
Turku, January 2014



Tiina Viitanen

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