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SHORT DURATION OF UPPER EXTREMITY LYMPHEDEMA CORRELATES WITH A FAVORABLE CYTOKINE RESPONSE AFTER LYMPH NODE TRANSFER SURGERY

E.H. Rannikko,* I.-M. Leppäpuska,* M. Laukka, A. Saarikko, P. Hartiala

Department of Plastic and General Surgery (EHR,I-ML,ML,PH) Turku University Hospital, Turku, Finland; Institute of Biomedicine (ML,PH), University of Turku, Turku, Finland; Department of Plastic Surgery (AS), Helsinki University Hospital, Helsinki, Finland

*contributed equally

ABSTRACT

Vascularized lymph node transfer surgery (VLNT) can provide benefit to lymphedema patients. Cytokines may play a role in the development of lymphedema and in the regeneration of lymphatic vessels after VLNT. Our primary aim was to investigate whether the VLNT patients have a specific cytokine profile. Our secondary aim was to see whether the preoperative lymphedema or severity affects the postoperative cytokine response. Wound exudate was gathered from 18 patients undergoing VLNT on the first and sixth postoperative day (POD). The concentrations of IL-10, TNF-α, TGF-β1 and VEGF-C were analyzed using enzyme-linked immune-sorbent assays. A general score was generated to assess the benefit of the surgery. The changes in cytokine concentrations (1st POD-6th POD) were correlated with the pre- and postoperative lymphedema related factors. A shorter duration of lymphedema preoperatively correlated with an increase in the concentration of IL-10 and TNF-β during the first six PODs (IL-10: r=0.495, p=0.051; TNF-α: r=0.737, p=0.006) and a decrease in the concentration of TGF-ß1 (r= -0.613, p=0.020). The increase of the concentration of TNF-α during the first six PODs also correlated with a greater total general score (r=0.775, p=0.005) and hence indicated a better response to the surgery. The patients with a shorter duration of lymphedema preoperatively had a more favorable cytokine response during the first six PODs after VLNT.

Keywords: lymphedema, inflammatory response, lymph node transfer, cytokine

Breast cancer is the most common cancer in women and the incidence of melanoma is rising (1,2). The treatment of these cancer types often includes sentinel lymph node biopsy or lymph node evacuation in the axillary or groin area (3-5). This iatrogenic damage to the lymph nodes and lymphatic pathways is a common cause for lymphedema (6). Other factors increasing the risk of postoperative lymphedema are radiotherapy, chemotherapy, obesity, and seroma fluid formation (7). According to a recent study, the cumulative incidence of clinically manifested lymphedema after breast cancer treatment is 41.1% (8). Chronic lymphedema is a progressive disease characterized by the accumulation of interstitial fluid leading to pitting edema of the affected limb, later accompanied by the proliferation of irreversible fibroadipose tissue and non-pitting edema (9). These changes cause pain and decrease the function of the affected arm (8).

The pathophysiology of lymphedema has

been under research for several years. Fibrosis and scarring are known to be key inhibitors of lymphatic regeneration (10-12). Therefore, the factors related to chronic inflammation and fibrosis, like transforming growth factor β1 (TGF-β1) and interleukin 10 (IL-10) are a major topic of interest (12). Some studies have implied that lymphedema could partly be caused by an immunological response towards a Th2 shift (10,13,14). Cytokines and growth factors, such as, tumor necrosis factor α (TNF-α) and vascular endothelial growth factor C (VEGF-C) have been shown to have pro-lymphangiogenic properties. TNF-α, a pro-inflammatory cytokine, has been shown to stimulate the production of VEGF-C by fibroblasts (14). On the contrary, lymphatic stasis is known to initiate chronic inflammation and tissue fibrosis resulting in worsening of the lymphatic function (15). Further, lymphedema has been associated with a similar gene expression profile as inflammation in a mouse model (16).

Vascularized lymph node transfer (VLNT) can improve lymphatic function and drainage in some patients (17). Our group has shown that it can easily be combined with routine microvascular breast reconstruction (BR) (18). In the VLNT technique, the lymphatic vessel anastomoses are expected to form spontaneously. Interestingly, breast reconstruction surgery without VLNT has also been reported to reduce lymphedema symptoms in some patients and reduce the arm volume (19). The hypothesis is that flap transfer alone modifies the local wound environment favorable to lymphangiogenesis (20,21). Our group has previously shown in a limited patient population that flap surgery hinders the immediate postoperative pro-inflammatory response and that VLNT surgery also seems to promote an anti-inflammatory and anti-fibrotic effect (22).

In this article, we studied the postoperative inflammatory status of patients undergoing VLNT surgery by evaluating the postoperative production of cytokines and growth factors (IL-10, TNF-α, TGF-β1 and VEGF-C) in 18 patients. Our aim was to analyze whether a certain cytokine profile could be observed in patients benefitting from the surgery. We also aimed to analyze whether the duration or severity of lymphedema would affect the inflammatory response after VLNT surgery.

MATERIALS AND METHODS

Patients and Collection of Samples

This study and the collection of patient samples were approved by the Ethical Committee of the Turku University Hospital (ETMK:11/1801/2017 and ETMK:53/180/2011). All patients signed an approval for the sample collection and approved the use of their patient information in the study. The study was conducted according to STROBE guidelines. Postoperative wound exudate samples were collected on the first and sixth postoperative day (POD) from 18 postmastectomy patients. The patients had a history of lymphedema of 35±21months (range 6-84 months) previous to the VLNT. Eighteen of the patients received the VLNT-flap to the axilla. All patients were operated on between May 2011 - February 2013. Four patients had simultaneous liposuction (LIPO). The VLNT with or without BR from groin to axilla was performed as previously described (9,18).

Lymphoscintigraphy

Pre- and postoperative lymphatic function of the affected arm was evaluated using lymphoscintigraphy. For semiquantitative evaluation of lymphatic drainage, a numerical transport index (TI) was used as described previously (23). Arm lymphoscintigraphy was performed preoperatively and at 6, 12, and 24 months postoperatively as previously described (13). The TI-value of over 10 is thought to be pathological (24).

Circumference and Volume Measurements

The circumference measurements of the arms were taken preoperatively and at 6, 12, 24, and 36 months postoperatively. Arm circumference was measured at every 4 cm between the wrist and the axilla. The edema volume compared to the contralateral arm was derived from the patients with circumference

measurements from all the measurement points using the truncated cone model as described by Håkan Brorson (25).

The General Score

We generated a general score to assess the benefit of the surgery. The general score was the total of the change of the TI-value, the change of the mean circumference difference, and the change in use of compression garments during the follow-up period (*Tables 1,2*).

TABLE 1
The Component Values Used to Generate the General Score of Change for the Subjects.

Points	Transport Index change	Circumference diff. change (cm)
0	<0.0	<0.0
1	0.1-10.0	0.1-2.0
2	>10.0	>2.0

The patients with a poorer TI-value after the follow-up than preoperatively, were given 0 points. The patients that had slight improvement of the TI-value during the follow-up (between 0-10) were given 1 point. The patients with a significant improvement of the TI-value during the follow-up (over 10), were given 2 points.

The patients with no improvement of the mean arm circumference difference after the follow-up, were given 0 points. The patients with a slight improvement in the mean arm circumference difference (0-2cm) during the follow-up, were given 1 point. The patients with a significant improvement of the mean arm circumference difference (over 2cm) after the follow-up, were given 2 points. Volumetry results were recorded, when available (*Table 2*). If the volume of the operated arm increased during the follow-up, it confirmed the score of 0 points. If there was no change of volume or only slight improvement, it confirmed the score of 1 point. If the reduction of volume after the follow-up was over 200ml, it confirmed the score of 2 points.

All of the patients used compression garments regularly 23h/d before the operation as it was a prerequisite for the surgery. The patients that needed to continue the regular use of compression garments, were given 0 points. If the patient was able to reduce the time of compression garment use compared to the preoperative usage, 1 point was given. The patients able to discontinue (permission given by their treating physician) the use of compression garments, were given 2 points.

These points from the three categories were summarized and a general score was calculated. For the analyses, the following groups were formed: Group 1 (0-2 points) did not benefit from the operation, Group 2 (3-4 points) had some benefit and group 3 (5-6 points) benefitted most of the operation. Sufficient results from all three categories were available for 15/18 patients.

Evaluation of Wound Exudate IL-10, TNF-α, TGF-ß1, and VEGF-C Concentrations

To determine the immunological profile of wound healing after VLNT, 10 ml of wound exudate was taken from the suction drainage system on the 1st and 6th POD from the recipient site. A protease inhibitor (cOmplete EDTA-free Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Mannheim, Germany) was added to the wound exudate samples, followed by centrifugation to separate the supernatant and cell pellet (13). Enzyme-linked immunosorbent assay (ELISA) for the quantitative detection of the respective cytokine in the supernatant (IL-10, TNF-α, TGF-β1, VEGF-C) was performed using commercial kits according to the manufacturer's instructions (Human IL-10, TNF-α, TGF-ß, VEGF-C ELISA KIT, Thermo Fisher Scientific, Bender MedSystems GmbH, Austria). The absorbance was measured at 450nm and 540nm with a microplate reader (Victor multilabel counter, Wallac, Finland) and the result from 540nm was subtracted from the result from 450nm. The final result (pg/mL) was read from a standard curve using the subtracted value. The concentration of the cytokine at 6th POD was subtracted from the concentration of the 1st POD to derive a temporal trend of cytokine expression (concentration change).

TABLE 2
Summary of Patients and Study Data. Details for Generation of General Store Are in the Text

ID	Age at operation	Type of operation	Follow-up (months)	Duration of lymphedema preoperatively (months)	General Score				Change of volume difference (ml)	Cellulitis (n)		Function of the limb	Pain symptoms
					TI-value	Circumference difference	Compression garment use	Total	6 - 24 months	preop	postop	improved (n=17/18)	improved (n=16/18)
1	38	VLNT	90	6	1	1	0	2		1	0	X	X
2	74	VLNT + LIPO	90	31	2		2	4		3	1	X	X
3	61	VLNT-BR + LIPO	86	48	1	2	0	3		0	0	X	X
4	53	VLNT + LIPO	85	16		1	2	3		2	0		
5	49	VLNT-BR	85	18	0	0	2	2		0	0	X	X
6	50	VLNT-BR	84	72	1	0	2	3		0	0	X	X
7	56	VLNT	82	84	1	0	1	2		0	0	X	X
8	60	VLNT-BR	81	36	0		2	2		0	0	X	X
9	55	VLNT + LIPO	80	60	2	1	1	4		0	0	X	X
10	47	VLNT-BR	80	69	2	1	2	5	159	0	0	X	X
11	68	VLNT-BR	72	51	0	2	0	2	520	0	0	X	X
12	50	VLNT-BR	71	27	0	1	2	3	81	0	0	X	X
13	49	VLNT-BR	73	16	0	1	0	1		0	0	X	
14	31	VLNT-BR	73	20	1	1	2	4		0	0	X	X
15	47	VLNT-BR	73	27	0	2	0	2	394	0	0	X	X
16	52	VLNT-BR	73	35	2	0	0	2		0	0	X	X
17	44	VLNT	69	30	2	2	1	5	65	0	0	X	X
18	52	VLNT-BR	69	24	2	1	2	5		0	0	X	X
MEAN ± SD	52±10		79±7	37±21	1.0±0.9	1.0±0.7	1.2±0.9	3.0±1.2	244±181	0.4±0.9	0.1±0.2		

Statistical Analysis

The statistical analysis was performed with GraphPad Prism 8 software. The data normality was determined by Kolmogorov-Smirnov test. Operation and lymphedema related parameters (Preoperative TI-value, change of TI-value during follow-up, preoperative circumference difference, change of circumference difference during follow-up, duration of preoperative lymphedema, age at operation and cellulitis incidence) were correlated with the concentration change of IL-10, TNF-α, TGF-ß1 and VEGF-C during the first six PODs (1st POD-6th POD) using Pearson's correlation analysis as the data was normally distributed. The p-value of ≤0.05 was considered to be statistically significant. To see whether the cytokine response differed between general score groups (categorical data), Kruskall-Wallis variance analysis followed by Dunn's multiple comparison test was performed.

RESULTS

Patient Characteristics

The clinical results of some of the patients have been published previously (13,26). The mean±sd age of the patients was 52±10 years and the mean±sd follow-up time 79±7 months. The mean±sd body mass index (BMI) was 28.2±3.2. Preoperative TI values (mean±sd) were 21.3±14.5 and preoperative duration of lymphedema was 37±21 (mean±sd) (*Supplementary Table*). The mean±sd incidence of cellulitis was preoperatively 0.06±0.15/year and postoperatively 0.01±0.03/year. Pain due to lymphedema was preoperatively reported by 61% (11/18) of the patients and the pain was reported to be relieved in 89% (16/18) of the patients. As a subjective report by the patients, 94% (17/18) of the patients reported the function of the affected arm to be improved. Patient characteristics and results are summarized in *Table 2.*

Lymphoscintigraphy, Circumference Difference, and Compression Therapy

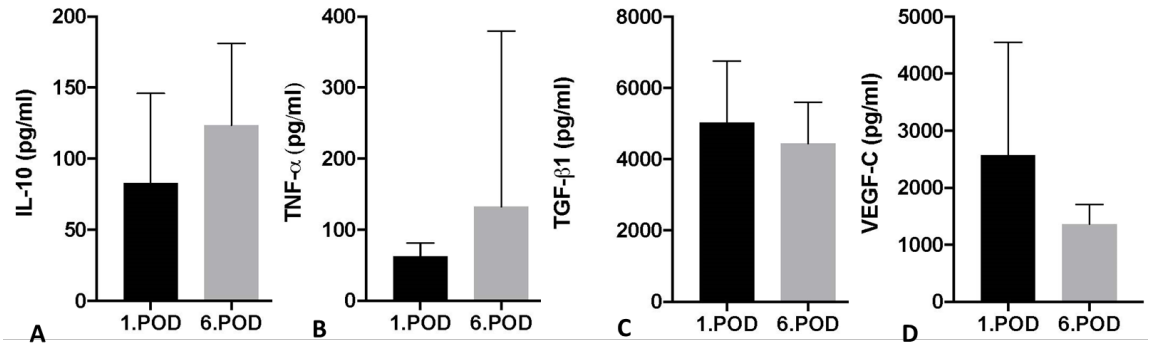

Fig. 1. *Concentrations measured in wound exudate mean±sd (pg/mL) of IL-10 (A), TNF-α (B), TGF-ß1 (C), and VEGF-C (D) at 1st POD and 6th POD.*

Lymphoscintigraphy was performed in 94% (17/18) of the patients both pre- and postoperatively. The TI-value was improved in 65% (11/17) of the patients. The mean±sd general score regarding the lymphoscintigraphy change was 1.0±0.9 *(Table 2)*.

The mean±sd circumference difference preoperatively was 2.5±2.1cm. It was reduced to 1.1±1.3cm after the follow-up time of 25.1±1.4 months. The mean±sd general score regarding the circumference difference change was 1.0±0.7. The change of circumference difference was statistically significant in the patients who continued the use of compression garments (p=0.026). The mean±sd volume difference was preoperatively 522.1±449.6 ml. It reduced to 233.0±388.2 ml after the follow-up time of 25.1±1.4 months.

67% of patients (12/18) were able to reduce the use of compression therapy after the operation. 58% (7/12) of them discontinued the compression therapy on average 15±8 months after the surgery and the rest were able to reduce the use of compression garment to occasional use (25%, 3/12) or under 12 hours a day (17%, 2/12). The mean±sd general score regarding the use of compression garments was 1.2±0.9. The mean±sd general score (total) of the patients was 3.0±1.2.

The Wound Exudate IL-10, TNF-α, TGF-ß1, and VEGF-C Concentrations and Concentration Change

The concentrations of IL-10, TNF-α, TGF-ß1, and VEGF-C at 1st and 6th POD in the wound exudate are seen in *Figs. 1A-D*. The concentration of IL-10 and TNF-α increased during the first six PODs and that of TGF-ß1 and VEGF-C decreased during the first six PODs. However, these results were not statistically significant. The mean±sd concentration change of IL-10 (1st POD - 6th POD) was -53.9±68.7pg/mL and of TNF-α -0.45±16.2 pg/mL. The mean±sd concentration change of TGF-ß1 (1st POD - 6th POD) was 782.1±2081.6 pg/mL. The mean±sd concentration change of VEGF-C (1st POD - 6th POD) was 1335.9±2215.6, but it did not correlate with the outcome of the surgery. The patients with the shorter duration of lymphedema preoperatively had a correlation with the increase of the concentration of IL-10 and TNF-α (IL-10: r=0.495, 95% CI -0.001-0.795, p=0.051; TNF-α: r=0.737, 95% CI 0.282-0.921, p=0.006) and the decrease of TGF-ß1 (r= -0.613, 95% CI -0.863- -0.123, p=0.020) during the first six PODs (*Figs 2A-C*). There was also a correlation of greater age at operation with increase of TGF-ß1 during the first six PODs (r=-0.584, 95% CI -0.851-0.077, p=0.029). Other statistically significant correlations were not found between the preoperative measurements (TI-value, circumference difference, preoperative duration of lymphedema) and the change of cytokine concentrations during the first six PODs (*Table 3*).

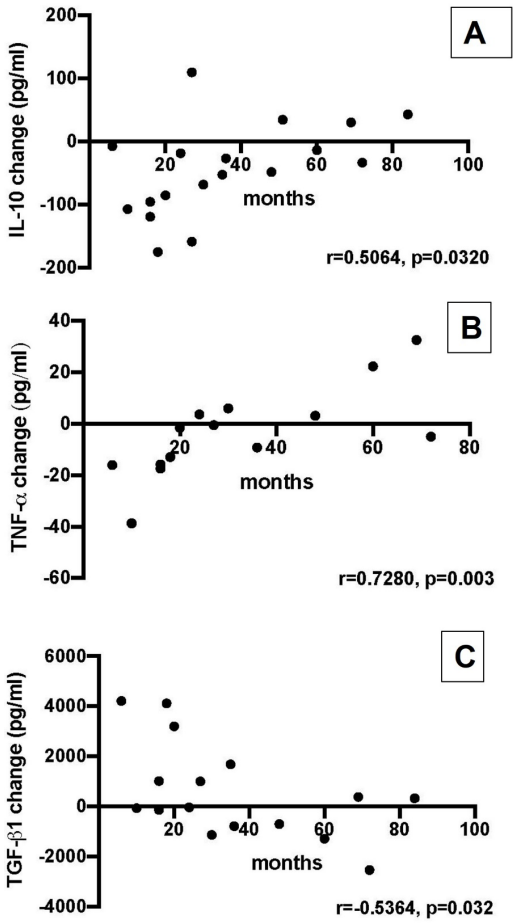

Fig. 2. *Correlation between change (1st POD – 6th POD) and duration of preoperative lymphedema (months) for IL-10 (r=0.495, p=0.051) (A), TNF-α (r=0.737, p=0.006) (B), and TGF-ß1 (r=-0.6133, p=0.020) (C).*

A greater total of the general score, indicating possibly a better response to the surgery, correlated with an increased concentration of TNF-α during the first six PODs (r=0.775, 95% CI 0.327 - 0.939, p=0.005) (*Fig. 3*). No significant correlation was found between the general score groups and the change of cytokine concentration (IL-10: p=0.935; TNF-α p=0.037 (Dunn's test no significant results); TGF-ß1 p=0.143; VEGF-C p=0.246). Statistically significant correlations were not found between the operation outcome (change of compression garment use, change of TI-value, change of circumference difference, change of cellulitis incidence) and the change of cytokine concentrations during the first six PODs.

DISCUSSION

In this study, we analyzed the inflammatory profile from wound exudate samples after VLNT. We found that a shorter duration of lymphedema preoperatively correlated with a more beneficial inflammatory response during the first six PODs. Among these patients, the concentration of the anti-inflammatory and anti-fibrotic (27-29) cytokine IL-10 and the pro-inflammatory cytokine TNF-α increased within six PODs and the concentration of pro-fibrotic TGF-β1 decreased within six PODs, indicating a favorable response. TNF-α increased during the first six PODs in the patients with a greater total general score, also

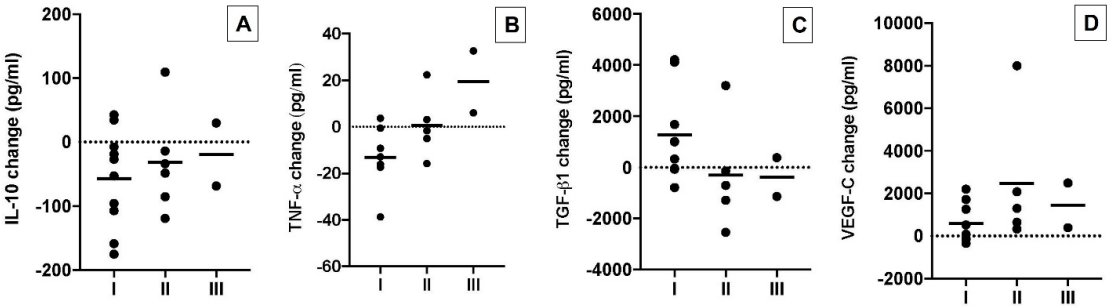

Fig. 3. *Correlation between TNF-α change (1st POD – 6th POD) and the total general score (r=0.7747, p=0.0051).*

TABLE 3
A Summary of the Correlation Analysis Results of the Preoperative Measurements and the Cytokine Concentration Changes During the First Six PODs

Correlation (p- value)	IL-10	TNF-α	TGF-β1	VEGF-C	Preop Ti index	Preop circ. diff.	Preop Lymph-edema	Age at operation
IL-10		0.075	0.263	0.892	0.964	0.964	0.051	0.308
TNF-α	0.075		0.240	0.227	0.433	0.830	**0.006**	0.819
TGF-β1	0.263	0.240		0.227	0.653	0.771	**0.020**	**0.028**
VEGF-C	0.892	0.227	0.227		0.284	0.638	0.635	0.451
Preop Ti-index	0.964	0.433	0.653	0.284				
Preop circ. diff.	0.964	0.830	0.771	0.638				
Preop lymphedema	0.051	**0.006**	**0.020**	0.635				
Age at operation	0.308	0.819	**0.028**	0.451				
General score total	0.482	**0.005**	0.190	0.174				

Statistically significant results are bolded.
(Abbreviations: *IL-10, TNF-α, TGF-ß1 and VEGF-C* = the concentration change of the cytokine during the first six PODs; Preop *TI-index* = the preoperative TI-index; Preop *circ. diff.* = the preoperative circumference difference; *Preop lymphedema* = the duration of lymphedema preoperatively). P values for the correlation analyses are reported and statistically significant results are bolded.

indicating a more favorable surgery outcome.

A shorter duration of lymphedema leads to more favorable results

Lymphedema has been associated with a fibrotic Th2 type inflammatory response orchestrated by Th2 type helper T cells and macrophages. It has been suggested that macrophages have an anti-fibrotic role in lymphedema, and either directly or indirectly macrophages regulate Th2 differentiation (30). The study by Ghanta et al provided evidence that lymphedema-associated macrophages are a major source of VEGF-C and that impaired macrophage responses after lymphatic injury result in decreased lymphatic function (30). IL-10 is an anti-inflammatory cytokine produced primarily by regulatory macrophages (31). The major physiological importance of IL-10 is the prevention of uncontrolled harmful immunologic reactions (31). IL-10 is thought to be responsible for an anti-inflammatory- and anti-fibrotic effect (27,28). TGF-β1 is a regulator of tissue fibrosis and scarring in the later stages of wound healing (32). TGF-β1 expression has been shown to decrease with age (33) and our results were in line with this as a correlation was found bet-ween age and cytokine change. It has been demonstrated that inhibition of TGF-β1 leads to increased lymphatic repair during wound healing (32). IL-10 is also known to protect from TGF-β1-induced fibrosis (27). It has also been shown that IL-10 down-regulates fibrosis, promoting pro-inflammatory cytokines (28). In both preclinical and clinical studies IL-10 has been found to be efficient in scar reduction and scar-improving therapies (27,29,34). It is known, that one of possible causes for lym-

phedema is scar constriction of the blood and lymphatic vessels (35,36). Previously we have shown that VLNT surgery induces higher IL-10 production compared to regular breast reconstruction surgery (22). In light of the previous evidence, it is possible that some effects of VLNT are mediated by IL-10 and its anti-inflammatory and anti-fibrotic properties. In the present study, the favorable IL-10 and TGF-β1 response correlated with the shorter duration of lymphedema. It can be deduced that the timing of the surgery might be better in patients with a shorter duration of lymphedema even though direct correlation was not found between duration and operation outcome.

Inflammation and Duration of Lymphedema

Several studies have demonstrated that inflammation is closely related to lymphangiogenesis, and pro-inflammatory cytokines like TNF-α induce VEGF-Cs expression in experimental settings (14,37). On the contrary, lymphatic stasis is known to initiate chronic inflammation and tissue fibrosis resulting in worsening of the lymphatic function (12). VEGF-C is produced by macrophages and lymph nodes (30). We have previously shown increased VEGF-C secretion in wound exudate samples of both BR and VLNT patients suggesting a role in tissue remodeling after flap surgery (13). However, in this study we did not detect a correlation with the operation outcome and VEGF-C expression during the first six PODs suggesting that postoperative lymphangiogenesis after VLNT is coordinated by many factors. TNF-α is a pro-inflammatory cytokine needed in initiation of wound healing. In our results, patients with a better response to the surgery according to the total general score, had a greater increase in the concentration of the TNF-α during the first six PODs. According to our results, the postoperative Th1 type immune response is hampered as lymphedema duration increases.

Clinical Relevance of the Study

Understanding the pathophysiology behind lymphedema is fundamental when determining a curative method for lymphedema. Also, the prevention of lymphedema after cancer-related surgery would be more achievable and the incidence of lymphedema could be reduced. It has been thought that VLNT surgery would be more beneficial in early-stage lymphedema patients but to our knowledge, there is no direct evidence supporting it (38). Overall, we did not find a correlation between lymphedema duration and benefit of the surgery. However, we found evidence that with time the immunological response to surgery shifts to an unfavorable pro-fibrotic Th2 type in lymphedema patients. This will help to determine the right patient material for VLNT surgery. It seems that the pathophysiology of lymphedema is multifactorial, but part of it is immunological (39). Although this study is based on a small number of patients, the findings suggest a beneficial anti-inflammatory IL-10 and TNF-α profile related to shorter lymphedema duration. A more comprehensive study regarding inflammatory responses after axillary lymph node removal surgery is in progress and we hope that it will light the pathophysiology of lymphedema.

Limitations

Limitations of this study were the smaller number of patients enrolled in the study, and also that the patient cohort included small subgroups of patients with varying surgery types (VLNT and VLNT-BR; with or without LIPO). The data analyses were also performed based on the subgroups, but due to the small group size, no statistically significant or meaningful results were found. However, based on our previous data, the cytokine profiles are very similar after these operations (22). Another limitation is that we did not collect data of the wound exudate volume of these patients and thus could not normalize the cytokine concentration based on the produced volume. How-ever, in our clinical experience the amounts of the wound exudate are in the same range.

CONCLUSIONS

A favorable inflammatory response during the first six PODs after VLNT is seen in patients with a shorter preoperative duration of lymphedema. This result should be taken into account in the patient selection protocol of VLNT. The results are not conclusive and hence further research is needed.

CONFLICT OF INTEREST AND DISCLOSURE

PH and AS are research scientists involved in a clinical trial with Herantis Pharma and have received Honoria for participating in advisory boards of Herantis Pharma. Other authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Author contributions:

Eeva H Rannikko: writing of the article, data and sample collection, data analysis; Ida-Maria Leppäpuska: writing of the article, data and sample collection, ELISA-analyses, data analysis; Mervi Laukka: writing of the article, sample collection, ELISA-analyses; Anne Saarikko: design of the study, writing of the article; Pauliina Hartiala: design of the study, sample collection, data analysis, writing of the article

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Eeva H. Rannikko, MD
Department of Plastic and General Surgery,
Turku University Hospital
P.O. Box 52
Kiinamyllynkatu 4-8
FI 20251 TURKU, FINLAND
Tel: +358-44 3302044
Fax: 358-2-3132215
E-mail: ehsulo@utu.fi

SUPPLEMENTARY TABLE
Summary of the Preoperative Results for the Study Patients

ID	Age at operation	Preoperative TI-value	Preoperative circumference difference (cm)	Preoperative duration of lymphedema (months)
1	38	41.0	2.7	6
2	74	19.2		31
3	61	22.8	3.8	48
4	53	6.6	4.3	16
5	49	0.2	1.3	18
6	50	8.6	2.2	72
7	56	12.8	-0.8	84
8	60	8.4		36
9	55	20.8	3.8	60
10	47	45.0	2.5	69
11	68	20.4	7.2	51
12	50	11.4	1.2	27
13	49	45.0	2.0	16
14	31	5.6	0.8	20
15	47	45.0	2.8	27
16	52	37.0	5.7	35
17	44	19.8	0.2	30
18	52	14.2	0.8	24
MEAN ± SD	52±10	21.3±14.5	2.5±2.1	37±21