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Analysis of TNF- α and interleukin-6 in seroma of patients undergoing mastectomy with or without flap fixation: is there a predictive value for seroma formation and its sequelae?

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ABSTRACT

Background: Seroma formation is a common complication after mastectomy. Flap fixation has the potential to prevent seroma formation, but identifying patients that are at risk of developing seroma, remains challenging. The aim of this study was to assess the association between pro-inflammatory cytokines in seroma fluid one day after surgery and seroma formation and its sequelae.

Methods: Patients undergoing mastectomy were randomized into one of three groups: no flap fixation, flap fixation using sutures or flap fixation using tissue glue. Seroma samples from 40 consecutive patients undergoing mastectomy were collected on the first postoperative day for analysis of interleukin-6 and tumor necrosis factor- α . Seroma formation and its sequelae were assessed in the outpatient clinic ten days, six weeks and three months after surgery.

Results: TNF- α concentrations were not detectable in the seroma samples of any of the 40 patients. BMI ($p = 0.001$) and weight of the resected surgical specimen ($p = 0.003$) were associated with higher IL-6 levels in seroma on the first postoperative day after mastectomy. A higher seroma concentration of IL-6 was associated with significantly fewer patients with clinical seroma formation three months after surgery ($p = 0.027$).

Conclusion: IL-6 is associated with clinical seroma formation three months after surgery. There is however no evident association between IL-6 and complications related to seroma formation. Higher IL-6 levels are predictive of less long-term seroma formation. Application of flap fixation does not seem to influence the level of IL-6.

1. Introduction

Seroma formation following mastectomy remains a frequent and persistent problem in breast cancer surgery. Many see it as a surgical nuisance rather than as being an avoidable surgical complication. Seroma formation can lead to (frequent) seroma aspirations, infection, skin flap necrosis, secondary surgical interventions, repeated outpatient visits and even delay in instituting adjuvant treatment [1–3]. It is

suspected that patients suffering from seroma and its sequelae have a lower quality of life and that patients and society are confronted with higher costs [4].

Many articles have been published on techniques for reducing seroma formation and its complications [4–12]. The key to achieving this goal lies in reduction of the dead space [13], but closure of the dead space unfortunately does not guarantee a seroma free recovery. Certain factors have been associated with seroma formation, such as extent of

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List of abbreviations;

BMI	body mass index
CON	conventional wound closure
DCIS	ductal carcinoma in situ
FF-S	Flap fixation using sutures

FF-G	Flap fixation using tissue glue
IL-6	Interleukin 6
MRM	modified radical mastectomy
SNLB	sentinel lymph node biopsy
SSI	surgical site infection
TNF- α	tumor necrosis factor alpha

surgical procedure, axillary clearance, use of electrocautery, smoking and BMI [1,6,14]. It is, however, not yet possible to accurately predict which patients are at risk for developing seroma and its sequelae.

Interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are released by leucocytes that extravasate out of blood vessels [15–17]. They are crucial in initiating, controlling and terminating the cellular events of wound healing. These two cytokines can induce the production of proteases, elastases and matrix metalloproteinases [18]. Levels of proinflammatory cytokines in wounds or wound fluid can potentially be used as a reliable marker of tissue damage [19,20]. IL-6 has previously been shown to be an early marker of the systemic inflammatory response and tissue damage in patients undergoing mastectomy [21–23]. Previous studies have shown a relationship between pro-inflammatory cytokines in seroma and seroma formation [24], but no studies thus far have shown an association between IL-6 and TNF- α levels and post-operative complications related to seroma formation after mastectomy. Seroma formation is associated with the extent of the surgical procedure and therefore seems to be related to tissue trauma. Pro-inflammatory cytokines such as IL-6 and TNF- α are related to tissue damage; one could expect that patients with higher levels of the early systemic inflammatory response markers in seroma would also suffer from increased seroma formation. The aim of this study was to assess if there was any association between IL-6 and TNF- α levels in seroma fluid measured on the first postoperative day and seroma and seroma related complications in patients undergoing mastectomy with or without flap fixation.

2. Methods

The Seroma reduction After Mastectomy (SAM) Trial is a double blind randomized controlled trial evaluating seroma formation and its sequelae in patients undergoing mastectomy with or without flap fixation. (<https://clinicaltrials.gov/ct2/show/NCT03305757>). A subgroup analysis was performed within this trial in 40 consecutive patients in order to perform IL-6 and TNF- α analyses. Patients were allocated to one of three groups, (A) no flap fixation and placement of a low suction drain (CON), (B) flap fixation using sutures and placement of a low suction drain (FF-S), (C) flap fixation using ARTISS tissue glue and placement of a low suction drain (FF-G).

All participants were recruited from a large breast cancer clinic (Zuyderland Medical Center, Sittard-Geleen, the Netherlands) treating 500 newly diagnosed breast cancer patients annually. Female patients older than 18 years of age suffering from invasive breast cancer or ductal carcinoma in situ (DCIS) with an indication for mastectomy with or without sentinel lymph node biopsy (SNLB) or modified radical mastectomy (MRM), were eligible for inclusion. Patients undergoing breast conserving therapy or direct breast reconstruction or patients unable to sign for informed consent were not eligible for inclusion.

Randomization was achieved during surgery using a web based randomization programme 30 min before wound closure. Patients were blinded throughout the trial and the surgeon performing the follow-up assessments was also blinded.

Five experienced breast surgeons performed all procedures. All patients received 2 g of cefazolin prior to surgery. The nipple-areola complex was removed and dissection of the skin flaps was performed using electrocautery. Removal of breast tissue from the pectoral muscle included removal of the pre-pectoral fascia. All patients received a low

suction drain before closure of the skin.

2.1. Conventional wound closure

After mastectomy and placement of a low suction drain, the extent of the skin flaps was measured (in cm's) from medial to lateral and from cranial to caudal. The skin edges were sutured using absorbable monofilament sutures (Monocryl 3.0 or V-loc 30 cm), depending on the surgeon's preference.

2.2. Flap fixation using sutures

After mastectomy and placement of a low suction drain, the extent of the skin flaps was measured (in cm's) from medial to lateral and from cranial to caudal. The skin flaps were sutured on to the pectoral muscle using interrupted polyfilament absorbable sutures (Vicryl 3.0), placed at 4–5 cm intervals in two or three rows, depending on the extent of the skin flaps. The distance between all sutures was 4–5 cm. Care was taken to prevent dimpling of the skin. The number of rows and total number of Vicryl sutures were noted. The axillary area was not approximated using sutures. The skin edges were sutured using absorbable monofilament sutures (Monocryl 3.0 or V-loc 30 cm), depending on the surgeon's preference.

2.3. Flap fixation using ARTISS tissue glue

After mastectomy and placement of a low suction drain, the extent of the skin flaps was measured (in cm's) from medial to lateral and from cranial to caudal. ARTISS tissue glue (Baxter Healthcare, Australia) [25] was applied as a 2 mL spray and used on both skin flaps. Care was taken to make sure that the skin flap and pectoral muscle surfaces were dry before application of the glue. After the spray had been applied, compression on both skin flaps to the underlying muscle was applied for 3 min. The skin edges were sutured using absorbable monofilament sutures (Monocryl 3.0 or V-loc 30 cm), depending on the surgeon's preference.

2.4. Drains

The drain was connected to a low suction drain bottle and drain output was noted daily. In patients undergoing mastectomy without axillary clearance, the drain was removed when drain output was less than 50 mL or after a maximum of 48 h, irrespective of drain output. In patients undergoing modified radical mastectomy (including axillary clearance) the drain was removed if daily production was less than 50 mL or after a maximum of 5 days, irrespective of drain output.

2.5. Interleukin-6 and TNF- α sampling

Seroma samples were collected on the first postoperative day for analysis of IL-6 and TNF- α . Drain fluid (10 ml) was collected between 8 and 10 a.m. and the samples were consequently centrifuged at 1300 revolutions per minute for 10 min and stored at -80°C . IL-6 and TNF- α concentrations were determined using in-house developed standard enzyme linked immunosorbent assays (ELISA). The sampling was performed in one batch to ensure reliability of the sampling process.

2.5.1. IL-6 ELISA

A 96-well microplate (Greiner, 655061) was coated overnight at 4 °C with mouse anti-human IL6 (5E1). Hu IL-6 (R&D Systems) was used for standard titration curve. Standard and samples were incubated for 2 h at room temperature. Biotinylated polyclonal rabbit anti-human IL-6 was bound to captured human IL-6. Streptavidin-peroxidase conjugate was bound to the biotinylated antibody and reacted with the substrate Tetramethylbenzidine (TMB). The enzyme reaction was stopped by the addition of 1M H₂SO₄. Spectrophotometry was performed at 450 nm.

2.5.2. TNFα ELISA

A 96-well microplate (Greiner, 655061) was coated overnight at 4 °C with mouse anti-human TNFα (61E71; Celltech). rHu TNF-α was used for standard titration curve. Standard and samples were incubated for 1 h at room temperature. Polyclonal rabbit anti-human TNF-α was bound to captured human TNF-α. Goat anti rabbit peroxidase (IgG HRP Jackson Immuno Research) was bound to the secondary antibody and reacted with TMB. The enzyme reaction was stopped by the addition of 1M H₂SO₄. Spectrophotometry was performed at 450 nm.

2.6. Follow-up

Patients were evaluated in the outpatient clinic ten days, six weeks and three months postoperatively. Follow up in this subgroup was ended three months after surgery. Assessment of seroma and its sequelae in the outpatient clinic was always performed by a breast surgeon that did not perform the surgery. Special attention was given to the presence of clinical seroma, (surgical skin) infection or the need for seroma aspiration. This information was duly recorded in patients' medical charts.

2.7. Outcome measures

- Seroma:** a breast surgeon clinically assessed the presence of seroma. A patient was considered to have seroma if there was a swollen lump or fluid collection under the skin flaps.
- Seroma aspiration:** there were strict criteria for seroma aspiration. The need for seroma aspiration was always assessed by a breast surgeon in the breast clinic. The mere presence of seroma did not warrant seroma aspiration. Seroma aspirations were performed if A) wound healing was at risk due to seroma (wound break down, seroma leakage, necrosis), B) if there was discomfort or pain caused by large amounts of seroma, characterised by tenseness of the skin or C) if there was contaminated/infected seroma and aspiration was necessary to treat infection. All patients undergoing seroma aspiration due to infection were also treated with a 1-week course of amoxicillin/clavulanate 500/125 mg, three times daily.
- Infection:** surgical site infection (SSI) was defined as redness, pain, heat or swelling at the site of the incision or by the drainage of pus. Infection rate was measured by A) the need for antibiotics, B) seroma aspiration due to infection or C) surgical drainage during the first three postoperative months.

2.8. Statistics

Statistical analyses were performed using SPSS, version 21 (IBM Corporation, Armonk, NY, USA). Patient characteristics that are continuous variables are presented as means with standard deviations; categorical characteristics are presented as percentages. Continuous variables were compared between study groups with one-way analysis of variance (ANOVA). Categorical variables were compared between study groups with Pearson's Chi-squared test. Linear regression analysis was performed to assess the association between baseline demographics and IL-6 levels. To analyze the association between IL-6 and the presence of seroma and seroma related complications, binary logistic

Table 1

Linear regression IL-6 and baseline demographics. * = p < 0.05.

Parameter	Regression coefficient	CI (95%)	Significance
Age	-0.086	-0.02 - 0.20	0.196
Comorbidity (CI)	0.016	-0.00 - 0.04	0.112
Neoadjuvant chemotherapy	-0.003	-0.01 - 0.00	0.097
Anticoagulants	-0.001	-0.01 - 0.00	0.566
Smoking	0.000	-0.00 - 0.00	0.922
BMI	0.084	0.04-0.13	0.001*
Type of operation	0.000	-0.01 - 0.01	0.966
OR time	0.088	-0.11 - 0.28	0.361
Bloodloss	0.050	-0.51 - 0.61	0.856
Wound surface	0.084	-0.15 - 0.32	0.464
Randomization group	-0.003	-0.01 - 0.01	0.492
Drain time	-0.014	-0.03 - 0.00	0.075
Drain output	-2.706	-6.29 - 0.88	0.134
Axillary lymph node clearance	-0.001	-0.01 - 0.00	0.617
Specimen weight	5.014	1.81- 8.22	0.003*

regression analysis was performed. A p-value ≤0.05 was considered to indicate statistical significance. The association between IL-6 and the presence of seroma was subsequently adjusted for BMI and weight of the resected surgical specimen.

3. Results

Forty consecutive patients undergoing mastectomy and spending the first postoperative night in hospital in a short time frame within the study, were included. Characteristics of these patients at baseline are shown in Table 1.

Baseline demographics associated with IL-6 were identified by linear regression analysis. BMI (p = 0.001) and weight of the resected surgical specimen (p = 0.003) were associated with higher IL-6 levels in seroma on the first postoperative day after mastectomy (Table 1). There was a high correlation between BMI and weight of the resected specimen (Pearson correlation coefficient 0.805, 2-tailed significance, p < 0.001). The wound surface was considered to be shaped like a horizontal diamond and was calculated from the measurements of the skin flaps (from medial to lateral and from cranial to caudal). IL-6 levels were not significantly associated with the calculated wound surface (p = 0.464). Binary logistic regression was used to assess the association between IL-6 and postoperative complications. Remarkably, higher IL-6 levels were associated with significantly less clinical seroma three months after surgery (OR 0.952 [0.90–0.99], (p = 0.027)) Table 2. Fig. 1 depicts an error bar for seroma formation at three months in relation to the level of IL-6 in seroma. The association between IL-6 and the presence of seroma at three months was subsequently adjusted for BMI and weight of the resected surgical specimen and revealed similar

Table 2

Binary logistic regression IL-6 and outcome. NA = not applicable. OR = odds ratio. * = p < 0.05.

Outcome	OR	CI (95%)	Significance
Clinical seroma 10 days	0.999	0.98–1.02	0.947
Seroma aspiration 10 days	NA	NA	NA
Infection 10 days	NA	NA	NA
Earlier unplanned visit 6 weeks	0.971	0.91–1.04	0.410
Clinical seroma 6 weeks	0.984	0.96–1.01	0.141
Seroma aspiration 6 weeks	0.978	0.90–1.07	0.627
Infection 6 weeks	0.972	0.92–1.02	0.268
Earlier unplanned visit 6 weeks	1.00	0.98–1.02	0.888
Clinical seroma 3 months	0.952	0.90–0.99	0.027 *
Seroma aspiration 3 months	NA	NA	NA
Infection 3 months	0.981	0.90–1.07	0.657
Earlier unplanned visit 3 months	1.00	0.97–1.04	0.791

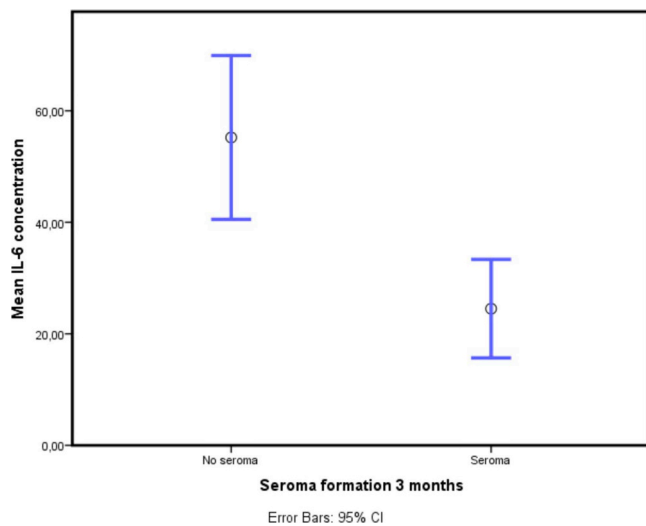


Fig. 1. Plot with error bars expressing mean IL-6 concentration and seroma formation at 3 months.

results, OR 0.94 [0.90–0.99], $p = 0.024$. No seroma related complications were associated with IL-6 levels in seroma fluid.

Thirteen patients were treated in the conventional closure group (CON), thirteen in the flap fixation group using sutures (FF-S) and fourteen patients were treated in the flap fixation group using ARTISS tissue glue (FF-G). Baseline characteristics of these patients are represented in Table 3. There were no significant differences in patient demographics between the three groups. Study results, specified per method of closure, are listed in Table 4. There was a significant difference in mean closure time between the three groups (CON = 14.9 min (range 5–25), FF-S = 24.8 min (range 13–37), FF-G = 15.8 min (range 12–32), $p < 0.001$). The mean total drain production was significantly higher in the conventional closure group (CON = 522 ml (range 50–1575), FF-S = 174 ml (range 25–505), FF-G = 178 ml (range 5–1080), $p = 0.037$). The presence of seroma six weeks after surgery was significantly reduced in the flap fixation groups (CON = 69.2%, FF-S = 7.7% and FF-G 35.7%, $p < 0.001$). There was a strong tendency towards fewer seroma aspirations in the flap fixation groups (CON = 15.4%, FF-S and FF-G 0%, $p = 0,064$). Patients undergoing conventional wound closure had significantly more seroma formation three months after surgery compared to the other groups (CON = 46.2%, FF-G = 0%, FF-S = 28.6%, $p = 0.008$).

Seroma IL-6 concentrations on the first post-operative day were not significantly different between the three groups (CON = 54.21 ng/ml, FF-S = 47.71 ng/ml, FF-G = 44.58 ng/ml, $p = 0.785$). Fig. 2 depicts an error bar per group (CON, FF-S, FF-G) in relation to the level of IL-6 in seroma. TNF-alpha concentrations were not detectable in the seroma samples; the concentration of all samples was below the minimally detectable value of 39 ng/ml. The occurrence of surgical site infections (SSI) showed no significant differences at any given time during follow up.

4. Discussion

Preventing seroma formation and its complications after mastectomy remains challenging. Flap fixation has been shown to reduce seroma formation and its sequelae in patients undergoing mastectomy [13]. In the literature, several descriptive studies can be found describing the levels of pro-inflammatory cytokines and matrix metalloproteinases in seroma of patients after mastectomy [21–23,26]. In a study by Szecsi et al. a broad spectrum of cytokines were measured in the seroma fluid of 42 patients after mastectomy. Very high levels of IL-6 and IL-8 were detected in seroma, however, these levels did not

predict the cause of seroma formation [27]. No studies, however, have been performed on the association between pro-inflammatory cytokine levels and seroma related complications after mastectomy.

BMI and weight of the resected surgical specimen were all significantly associated with the level of IL-6 on day 1. A higher BMI and larger breast size (larger bust line and larger total breast area) have been shown to be correlated [28]. If the extent of tissue damage is more extensive in women with larger breasts, higher levels of IL-6 could be accounted for in these patients. It remains surprising that the calculated wound surface was not related to the IL-6 levels. The wound surface corresponds with the surface of the skin flaps and it would be reasonable to assume that larger skin flaps are directly related to extent of surgical dissection. A possible explanation could be that in patients with a larger bust size, more breast tissue is removed but with a much smaller effect on the extent of the skin flaps. In a study published on patients receiving neoadjuvant chemotherapy for locally advanced breast cancer, serum IL-6 levels were significantly higher before, during and after termination of chemotherapy. This demonstrates a higher pro-inflammatory cytokine release in these patients [29]. In our group of patients there was however no clear association between neoadjuvant chemotherapy and IL-6 ($p = 0.097$). Surprisingly, higher IL-6 levels were associated with significantly less clinical seroma three months after surgery. This seems to be the most important finding in this study. A higher level of IL-6 one day post-operatively is directly related to less long-term seroma formation. In this respect one could conclude that higher IL-6 levels are predictive of less long-term seroma in mastectomy patients. This is in contrast to all reports currently available in the scientific literature. All studies to date have demonstrated that higher IL-6 levels in seroma are related to more wound seroma. The induced inflammatory response after mastectomy releases wound repair cytokines such as IL-6, in turn promoting seroma formation [27]. No plausible explanation could be found for this surprising finding in this study. In this relatively small group of patients there was no association between IL-6 and seroma related complications.

TNF- α was not detectable in any of the seroma samples as concentrations were too low to be detected by the analytic method used (< 39 pg/ml) on the first postoperative day after mastectomy. A study published by Chow et al. evaluated 29 patients undergoing mastectomy with IL-6 and TNF- α sampling from drained seroma on day 1, 2 and 5. TNF- α levels were low on day 1, showing a progressive rise on day 5. These results indicate that IL-6 may be a main cytokine in the early stage, whereas TNF- α may be mainly involved in the later stage of wound healing [21]. As all samples were collected from drained fluid on day 1, TNF- α was not detectable. Only nine out of forty patients had a drain in situ on day 5, making measurement of TNF- α impossible with our current drain policy.

IL- 6 levels in seroma were not significantly different between the three groups (flap fixation versus conventional wound closure). This could be explained by the fact that the extent of tissue damage was

Table 3

Patient demographics. Continuous variables are presented as means \pm SD. Categorical variables in percentages.

Demographics	Conventional	FF– sutures	FF-glue
Number of patients	13	13	14
Age (years)	67.2 (\pm 12.2)	70.2 (\pm 11.5)	68.9 (\pm 13.7)
Comorbidity (CI)	4.5 (\pm 2.0)	5.0 (\pm 2.2)	5.2 (\pm 2.7)
Anticoagulant drugs (yes)	3 (23.1%)	2 (15.4%)	4 (28.6%)
Smoking (yes)	0%	3 (23.1%)	1 (7.2%)
BMI (kg/m ²)	29.0 (\pm 7.2)	27.0 (\pm 4.6)	28.0 (\pm 5.6)
Bloodloss (ml)	78 (\pm 48)	68 (\pm 49)	91 (\pm 79)
Neoadjuvant chemotherapy (yes)	4 (30.8%)	2 (15.4%)	3 (21.4%)
Axillary clearance (yes)	7 (53.8%)	3 (23.1%)	6 (42.9%)
Radiotherapy (yes)	6 (46.2%)	4 (30.8%)	3 (21.4%)

Table 4

Outcome per group. Continuous variables are presented as means ± SD, one-way ANOVA. Categorical variables in percentages. * = p < 0.05.

Item	Conventional	FF– sutures	FF-glue	P-value
Operative time (min)	94.2 (± 20.5)	88.3 (± 14.9)	96.3 (± 27.0)	0.615
Closure time (min)	14.9 (± 5.6)	24.8 (± 6.0)	15.8 (± 5.5)	< 0.001*
Specimen weight (grams)	991 (477)	796 (304)	825 (357)	0.422
Total drain production (ml)	522 (510)	174 (186)	178 (286)	0.037*
Seroma 10 days (yes)	76.9%	53.8%	78.6%	0.065
Seroma aspiration 10 days (yes)	0	0	0	–
SSI 10 days (yes)	0	0	0	–
Seroma 6 weeks (yes)	9 (69.2%)	1 (7.7%)	5 (35.7%)	< 0.001*
Seroma aspiration 6 weeks (yes)	2 (15.4%)	0	0	0.064
SSI 6 weeks (yes)	2 (15.4%)	0	2 (14.3%)	0.264
Seroma 3 months (yes)	6 (46.2%)	0	4 (28.6%)	0.008*
Seroma aspiration 3 months (yes)	0	0	0	–
SSI 3 months (yes)	0	0	1 (7.1%)	0.430
IL-6 (ng/ml)	54.21 (± 48.59)	47.71 (± 29.59)	44.58 (± 27.83)	0.785

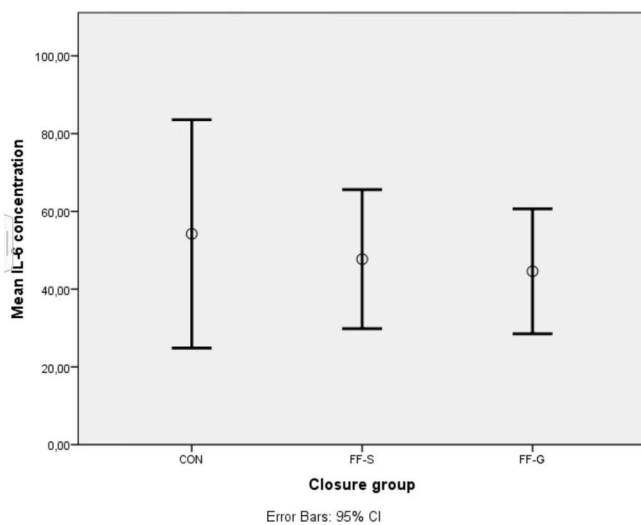


Fig. 2. Error bar depicting the relation of IL-6 levels in seroma per method of wound closure group (CON, FF-S, FF-G).

similar in all groups. Wound surface, percentage of patients undergoing axillary clearance or neoadjuvant chemotherapy were not significantly different between the groups. The final results of the SAM-trial are being awaited. This is a well-powered study in which 336 patients will be included. This will provide us with further information on the true value of flap fixation in mastectomy patients with regard to seroma and seroma related complications as well as cost-effectiveness and quality of life.

5. Conclusion

Higher interleukin-6 levels are associated with less clinical seroma formation three months after surgery but there is no apparent association between IL-6 and seroma related complications up to three months after mastectomy. Higher IL-6 levels in this group of patients seem indicative of less long-term seroma. Flap fixation does not seem to influence postoperative levels of IL-6 in seroma fluid on the first day after mastectomy.

Ethics approval and consent to participate

Written informed consent was obtained from all trial participants. This trial was approved by the hospitals’ joint medical ethical committee (METC-Zuyd, 14-T-21, 2 June 2014).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors declare that they have no competing interests.

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Trial registration

Trial registration: [NCT03305757](https://clinicaltrials.gov/ct2/show/NCT03305757). Registered 10 October 2017 - Retrospectively registered, <https://clinicaltrials.gov/ct2/show/NCT03305757>.

CRediT authorship contribution statement

J. van Bastelaar: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **R. Granzier:** Data curation, Formal analysis, Project administration. **L.M. van Roozendaal:** Data curation, Formal analysis, Project administration. **S.M.J. van Kuijk:** Investigation, Methodology, Validation. **A.V. Lerut:** Investigation, Project administration. **G. Beets:** Supervision. **M. Hadfoune:** Validation, Visualization. **S. Olde Damink:** Supervision. **Y.L.J. Vissers:** Conceptualization, Formal analysis, Supervision.

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Appendix A. Supplementary data

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