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Serum NETosis expression and recurrence risk after regional or volatile anaesthesia during breast cancer surgery:
A pilot, prospective, randomised single-blind clinical trial

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All of the above authors contributed to this piece of work.

Running Head: Does the anaesthetic technique used effect NETosis expression in women undergoing breast cancer surgery?

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Abstract

Background

Some experimental and retrospective clinical studies signal an association between certain anaesthetic techniques and tumour metastasis following breast cancer surgery. Neutrophil Extracellular Trapping (NETosis) is an immunological process whereby neutrophils engulf tumour antigen then degranulate, leaving a serologic marker. NETosis expression among breast cancer patients is associated with an increased risk of metastasis. We investigated the effect of two distinct anaesthetic techniques on the expression of NETosis in women who underwent potentially curative breast cancer surgery.

Methods

In a parallel-group, randomised controlled trial, a subset of women (n=40), undergoing breast cancer resection surgery, who were partaking in a larger trial (NCT00418457), were randomly assigned to receive volatile general anaesthesia (GA) or propofol GA combined with paravertebral regional anaesthesia (PPA) for their surgery. Serum was taken and stored before and 24 hr post operatively. NETosis was measured by ELISA using Neutrophil Myeloperoxidase (MPO) and citrullinated histone H3 (H3Cit) biomarkers, which were the co-primary end-points.

Results

Patient and breast cancer characteristics did not differ significantly between groups. Recurrence occurred in 7.5% patients. GA patients received more opioids and reported higher post-operative pain than PPA. There was no difference in postoperative MPO in GA vs PPA (10.5+6.6 vs 11.5+4.7 ng ml^{-1}, p=0.60). Regarding CitH3, there was no difference postoperatively in GA vs

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PPA (3.6+2.3 vs 4.0+5.9, p=0.80). NET expression did not differ before or after anaesthesia and surgery in either group, for either biomarker.

Conclusion

Anaesthetic technique did not affect NETosis expression in breast cancer patients, indicating that it is not a viable marker of the effect of anaesthetic technique on breast cancer recurrence.

Key Words

Anaesthesiology, general; Anaesthesiology, regional; Breast cancer;
Neutrophil extracellular trapping; Cancer, metastasis.

Editorial Comment

Neutrophil extracellular trapping (NETosis) is associated with increased risk of metastasis. This perioperative study in a small breast cancer surgery cohort did not reveal any difference in NETosis degree when exposed general anaesthesia with sevoflurane compared to propofol/paravertebral blockade.

Introduction

Breast cancer is both the most common cancer in women and the most common cause of cancer death amongst women globally.[1,2] Mortality among women with a breast cancer diagnosis is usually from metastasis of the primary breast tumour[3].

At present, surgery of curative intent is the main treatment of breast cancer. It has been hypothesised that a number of factors relevant in the perioperative period, such as the surgical stress response, immune suppression, and direct effects of anaesthetic drugs might influence cancer progression to metastasis[4]. The surgical stress response releases pro-inflammatory cytokines and other molecules into the bloodstream that may affect the body's post-operative immune response and create conditions that could facilitate residual tumour cell survival. This may later present as a clinical recurrence or metastasis.[5]

One of the immunological responses to cancer antigens is Neutrophil Extracellular Trapping (NETosis). NETosis occurs from the degranulation of neutrophils, releasing its contents into the

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bloodstream, including proteins and chromatin which form an extracellular net 'trapping' the cancer cells. Release of cytotoxic enzymes within the NETosis net kills cancer cells and creates a physical barrier preventing the spread of disease[6]. Neutrophil myeloperoxidase (MPO) and citrullinated histone H3 (H3Cit) are specific proteins released during NETosis, which can be measured in serum. High serum NETosis levels are associated with cancer pathologies such as increased risk of recurrence, inflammation and thrombosis[7,8,9].

It has been hypothesised that the anaesthetic technique during cancer surgery can influence cancer outcome by positively or negatively affecting the function of the immune system and other perioperative factors[10]. Earlier studies have shown that, in the serum of women who were given regional anaesthesia-analgesia using paravertebral block and propofol during cancer surgery, helpful immune responses were promoted, while in those who received volatile anaesthetic sevoflurane and opioid analgesia, these immune responses were inhibited[11,12].

Whether anaesthetic technique influences NETosis expression, a potential marker of metastatic risk, is unknown. Therefore, in this substudy within a larger clinical trial, we tested the hypothesis that women undergoing primary breast tumour resection with regional anaesthesia and propofol general anaesthesia have reduced postoperative serum expression of NETosis compared with women receiving volatile general anaesthesia and opioid analgesia.

Methods

After obtaining Institutional Review Board (IRB) approval and informed, written consent, both for participation in the larger, long term oncologic outcome trial (NCT-00418457) and this substudy), n=40 women due to undergo breast cancer resection of curative intent were enrolled in this study. The larger trial (NCT00418457) randomised women undergoing primary breast tumour resection to receive either regional anaesthesia and propofol general anaesthesia or sevoflurane volatile general anaesthesia and opioid analgesia.[13]. This subset of forty women consented in addition to donate a sample of peripheral blood for serum analysis just before induction of anaesthesia and again on Day 1 postoperatively. The patients were approached and enrolled the morning of surgery by a clinical research nurse.

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The inclusion criteria were: cancer Stage 1-3, nodal involvement stage 0-2, patients who were scheduled for a mastectomy or wide local excision with or without sentinel node biopsy and aged between 18-85 years old. Patients were excluded from the study if they had previous breast cancer surgery or inflammatory breast cancer, were scheduled for free flap reconstruction, had an American society of Anaesthesia (ASA) grade of 4 or higher, or had any contraindication to any aspect of anaesthetic technique.

Patients were randomly assigned, in 1:1 allocation, to either of two cohorts which determined the type of anaesthetic used for their procedure. We used a table of random numbers to generate a sequence of digits 0 to 9. Even numbers were assigned to volatile GA, odd numbers to PPA, in blocks of 10 to ensure both study groups had similar numbers as the study progressed. Patients were assigned a study number. This was written on a single A4 page, together with the group assignment, and placed in a sequentially-numbered sealed envelope. The randomisation process was conducted just prior to induction of anaesthesia. The anaesthetists involved were obviously aware of the cohort allocations. Because the paravertebral anaesthesia was conducted awake, patients were also aware of their group allocation. However, investigators involved in the postoperative follow-up, which included blood sampling, follow up calls, data analysis and interpretation were masked to group allocation, therefore this was a single-blind trial.

Patients randomised to receive volatile general anaesthesia and opioid analgesia were anaesthetised with fentanyl 1–2 $\mu g.kg^{-1}$ and propofol 1.5 –2 $mg.kg^{-1}$. Anaesthesia was maintained with sevoflurane (end-tidal concentrations 1–3%) in oxygen/air mixture. Intraoperatively, morphine 0.1–0.15 $mg.kg^{-1}$ was given at the discretion of the anaesthetist. Patients received postoperative patient-controlled analgesia with morphine, bolus 1 mg, lockout 6 min, and 4 h dose limit 30 mg. Paracetamol 1 g i.v. was given to all patients during surgery.

Patients receiving propofol-paravertebral anaesthesia had a catheter inserted using a standard technique, into the ipsilateral paravertebral space at the level of the second thoracic vertebra. A 20 ml bolus of levobupivacaine 0.25% was administered before surgery. Total i.v. general anaesthesia was then commenced using a target-controlled infusion of propofol. At induction, a dose of fentanyl 1–3 $\mu g.kg^{-1}$ was administered. Laryngeal mask airway (LMA) was the default used to maintain airways, with patients breathing spontaneously. Postoperative analgesia was a

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continuous infusion of levobupivacaine 0.25% at 5–10 $ml.h^{-1}$ via paravertebral catheter. Paravertebral catheters were removed at 24 h. Rescue analgesia if needed was triggered by a visual analogue scale (VAS) pain score ≥3, consisting of morphine 0.1 $mg.kg^{-1}$ i.m. every 3–4 h as required.

Approximately 10 ml peripheral venous blood was taken from each participant at induction of general anaesthesia immediately preoperatively and on Day 1 (typically 20-24 hr postoperatively), into BD vaccutainer™ serum tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The sample was centrifuged for 15 minutes at a rate of 4,000 rotations per minute. The remaining 2-3 ml plasma sample was then stored in a freezer at -80 C for later analysis. Samples were thawed and analysed for the enzyme-linked immunosorbent assay (ELISA) technique.

The co-primary end-point of this trial was the two NETosis specific biomarkers, myeloperoxidase (MPO) and citrullinated histone H3 (CitH3). The measurements were made using commercially available ELISA kits for MPO and CitH3. MPO (Human MPO, R&D Systems, Inc. Minneapolis, MN 55413, USA, assay range: 1.56-10 $ng.ml^{-1}$, sensitivity: 0.014 $ng.ml^{-1}$), and CitH3 (CitH3 Clone 11D3, CaymanChem, Ann Arbor, MI, USA, assay range: 1.56 – 10 $ng.ml^{-1}$, sensitivity: 0.3 $ng.ml^{-1}$) in accordance with manufacturer's instructions.

For the determination of MPO concentration, a sandwich ELISA technique was used with the anti-MPO antibody pre-coated in the well of the plates. Each patient sample was divided into two duplicate samples which underwent the same testing procedures to enhance accuracy. The serum samples were diluted 1:50 with sample diluent. 50 µL of diluted sample were added to each well, followed by 100 µL of assay diluent. The wells were covered by an adhesive strip and incubated for 2 h at room temperature on a horizontal orbital microplate shaker set to 500 rpm.

Each well was aspirated and washed thoroughly 4 times using a wash buffer. The detection antibody used was an anti-MPO antibody conjugated with horseradish peroxidase (HRP). This conjugated antibody reacts strongly with hydrogen peroxide and TMB (3,3',5,5"-tetramethylbenzidine). 200 µL of anti-Human MPO conjugated antibody were added to each well. The plate was again covered with an adhesive tape and allowed to incubate for 2 hours at room

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temperature on an orbital shaker at 500 rpm. The plate was aspirated and washed as before. 200 µL of a 1:1 solution of hydrogen peroxide and TMB was added to each well and used as a substrate for HRP. The plate was incubated for 30 minutes at room temperature while protected from light. The HRP-TMB reduction reaction in each well was halted using a stop solution of sulphuric acid. This caused the colour of the solution to change from blue to yellow. The absorbance of light passed through the plate wells was immediately measured at 450 nm using a Molecular Devices microplate reader and SoftMax® Pro 5 software with wavelength correction set to 540 nm. For each plate, a standard curve was generated by measuring the optical densities at 450nm of seven MPO standards of known concentration. This standard curve was used as a reference with which to calculate the MPO concentration in the diluted serum samples. The intensity of the signal produced per sample was proportional to the concentration of MPO (NETosis marker) present.

Similarly to MPO, the determination of CitH3 concentration was carried out using a sandwich ELISA technique. Assay buffer was used to dilute the serum sample, forming a 1:3 dilution. 100 µL of standard and the diluted samples were placed in the wells (pre-coated with anti-CitH3 antibody), with the samples being placed as duplicates. The wells were covered and incubated for two hours at room temperature on an orbital shaker. Following this incubation period, the wells were washed 4 times with a wash buffer and then 100 µL of HRP Conjugate working solution was added to each plate. This was incubated for one hour. Again, following this incubation period, the plates were thoroughly washed 4 times with wash buffer. 100 µL of TMB Substrate Solution was added to each well of the plate and the plate was then covered and left to incubate at room temperature for 30 minutes in the dark on an orbital shaker. Immediately after this final incubation period, 100 µL of HRP Stop Solution was added to each well of the plate and a colour change of blue to yellow was seen (while colourless wells remained colourless). Finally, using a microplate reader, the plate was read at a wavelength of 450 nm. A standard curve was generated for each plate using seven CitH3 standards of known concentration. This was used as a reference for calculation of CitH3 concentrations in each serum sample. The coefficient of variation was obtained for both MPO and CitH3 concentrations.

Patient characteristics were recorded including: age, Body-Mass Index (BMI), ethnicity, their American Society of Anaesthesiology (ASA) grade and whether or not the patient had received

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previous chemotherapy or radiation therapy. Characteristics about the tumour were also recorded including: which hormone receptors were present, the TNM stage, the histology grade of the tumour and a Nottingham Hill Prognostic Index was then calculated. Surgical characteristics were recorded during surgery and immediately post-operatively including: the type of procedure, the dose of intraoperative opioids given, the highest heart rate (HR) and lowest mean arterial pressure (MAP) intraoperatively, the total blood lost during the surgery and the post-operative pain score on VRS and the subsequent dose of opioids if required.

Statistical Analysis

The data collected was placed onto an excel spreadsheet and then copied and transferred onto Graph Pad Prism v8™ for statistical analysis. Data was inspected for normal distribution, using the Kolmogorov-Smirnov test. If normal distribution was confirmed, differences between independent groups were compared using an unpaired t test, while pre and post-operative NETosis levels within groups were compared using a paired t test. $P< 0.05$ was deemed statistically significant.

Previous serum estimations of NETosis MPO values indicated typical values in the order of 10-12 $ng.ml^{-1}$ with standard deviation in the order of 3 $ng\ ml^{-1}$. Taking a 20% reduction of 2.0 $ng\ ml^{-1}$ as being scientifically significant, and assuming a Type I error of 0.05 and Type 2 error of 0.2, then n=18 patients would be required each group to have 80% power to detect this difference. We enrolled n=20 patients each group to allow for missing data.

Results

The serum samples of 40 women were analysed.

The study flow sheet, according to CONSORT guidelines, is shown in Figure 1.

The physical characteristics of the patients were similar between the two cohorts as seen in Table 1. There were no significant differences in surgical or anaesthesiology characteristics between the two groups, except for intraoperative opioid, one-hour post-operative pain and first morning opioid use, all of which were greater in the GA group, as expected. The Tumour Node Metastasis (TNM) classification, histology grade and tumour prognosis measured by the Nottingham

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Prognostic Index and other cancer characteristics also showed no significant difference between the two anaesthetic groups (Table 2).

Table 3 displays the biomarkers of MPO and H3Cit, which represent the expression of NETosis. Regarding MPO, there was no difference preoperatively between the GA and PPA cohorts (8.6 + 4.7 vs 10.4 + 6.3 respectively, p= 0.28). The groups were similar again post operatively (10.5 + 6.6 vs 11.5 + 4.7 respectively, p = 0.60). There was no difference within the PPA cohort post operatively (11.5 + 4.7 vs 10.4 + 6.3, p= 0.44). There was also no difference within the GA cohort post operatively (10.5 + 6.6 vs 8.6 + 4.7, p=0.22). The coefficient of variation between the 2 duplicates for the MPO testing, ranged from 0-12 for the GA cohort compared to 0-14 for the PPA.

Regarding H3Cit, there was no difference preoperatively between GA and PPA cohorts (3.4 + 1.7 vs 3.1 + 2.1 respectively, p=0.62). The groups were similar again postoperatively (3.6 + 2.3 vs 4.0 + 5.9 respectively, p= 0.8). There was no difference within the PPA cohort post operatively (4.0 + 5.9 vs 3.1 + 2.1, p=0.43). There was also no difference post operatively in the GA cohort (3.4 + 1.7 vs 3.6 + 2.3, p= 0.70). The coefficient of variation for the H3Cit ranged from 0-15% for the GA cohort compared to 0-19% for the PPA.

Our study of n=40 women showed a breast cancer recurrence rate of 7.5% (n= 3 cases). The median post-operative value for MPO concentration in the n=3 patients with recurrence was 11 ng mL^{-1}, comparable to the mean values of the two groups shown in Table 3. The median post-operative value for CitH3 concentration in the n=3 patients with recurrence was 3.5 ng mL^{-1}, which is similar to the mean values obtained for the two groups shown in Table 3.

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Discussion

This pilot, prospective, randomised, single-blind, single-centre trial evaluated the effects of two different anaesthetic techniques on serum expression of NETosis markers (MPO and H3Cit). The results showed that there was no difference in NETosis expression post-operatively with the use of volatile general anaesthesia or propofol general anaesthesia combined with paravertebral regional anaesthesia.

During NETosis, DNA histones undergo citrullination which causes chromatin decondensation [8,9,15-17]. There are certain factors required for NET release, regardless of the stimulus that has initiated NET formation. These include myeloperoxidase (MPO) and neutrophil elastase (NE), which are found attached to the neutrophil extracellular traps that have been released during NETosis[15-17]. Therefore, high levels of CitH3 and MPO indicate high levels of NETosis which is associated with increased metastatic risk.[14-16] NETosis is involved in the inflammatory response and it has been observed that NETs also have a direct cytotoxic effect on the endothelium.[17] In breast cancer patients, NETosis has been associated with an increase in disease progression, metastasis and venous thromboembolism.[15] The exact mechanism by which it causes metastasis is still being determined.[8,9, 14-18] Understanding the role of neutrophils, and NETosis, in cancer is important due to the implications they have on cancer patients and the role they play in cancer progression.

Previous research shows that tumor-induced neutrophils are more prone to forming NETs than other neutrophils [16] This was observed using murine models of chronic myelogenous leukaemia, breast and lung cancer. Whether breast tumour is more likely to induce neutrophils is unknown. Previous studies also investigated whether NETosis could be a possible risk factor for cancer pathologies such as thrombosis and metastases [17, 18]. When human lung cancer cells were incubated with neutrophils and PMA to induce NET formation, there was a resultant increase in migration and invasion of the cells in the presence of intact NETs. This suggests that NETosis plays a role in metastasis of cancer cells[17, 19]. Another study, using caecal ligation and puncture (CLP) assay of sepsis in mice, showed that NETs can sequester tumour cells and can survive and progress to form gross metastatic nodules[17,18].

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Experimental and clinical retrospective studies have investigated the association between the type of anaesthetic used during cancer resection surgery and recurrence[11-14,19-21]. Propofol, a commonly used anaesthetic agent, has been shown to possess anti-inflammatory properties as well as stimulatory effects on immune function, which could have beneficial effects for cancer recurrence [19]. Volatile anaesthetics and opioids can impair immune function and reduce apoptotic effect in certain cell lines, such as in human colon cancer cells. A sevoflurane and opioid technique was shown to reduce apoptosis in breast cancer cells when compared to the use of a propofol and paravertebral anaesthetic technique.[11] Other studies have evaluated the effects of different anaesthetic drugs on the post-operative immune response either in a favourable or detrimental way in relation to cancer reoccurrence or metastases [12-14,20-21]. A recent, similarly-designed study showed that anaesthetic technique may have an effect on NETosis. This group observed that when lidocaine was added to either volatile sevoflurane or propofol-total intravenous anaesthesia (TIVA), there was a reduction in NETosis expression [14] among women undergoing surgery for breast cancer.

The large clinical trial evaluating the effect of anaesthetic technique on breast cancer outcomes, which these patients participated in, had an overall recurrence rate of 10%, with no difference in oncologic outcomes between the anaesthetic techniques evaluated.[13] Our present study found similarities between the post-operative MPO and CitH3 concentrations for those with and without recurrence which suggests that NETosis may not be a discriminating factor for predicting metastatic disease. The results of this study suggest that NETosis expression is not affected by anaesthetic technique. However, our small population size could have played a role in these findings. Our study was designed to have 80% to detect a meaningful difference in NETosis expression, therefore it is possible that a Type II error occurred. Nonetheless, we believe this is a reasonable sample size for this pilot study to evaluate this novel hypothesis. Also, the use of ELISA for measuring MPO in serum was shown to be accurate and precise by intra- and inter-assay coefficients of variation of <10% for MPO concentrations ranging from 0.5 to 50 ng/ml and by the similarity between the standard curve and curves obtained with successive dilutions of MPO-rich serum samples [14-17,22]

Since the women were all breast cancer patients who were undergoing cancer resection surgery and were randomized into different anaesthetic groups, we theorised that any effect on expression

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of NETosis would be solely due to the type of anaesthetic technique that was used. However, as in the results, neither of the anaesthetic techniques used was shown to have any significant effects on the NETosis expression post operatively. The only difference seen post operatively between the cohorts was in the use of intra operative opioids and the post-operative pain reported on the visual analog scale. As expected, those who received GA combined with paravertebral regional anaesthesia reported less post-operative pain and required little to no intra operative opioids. Opioids themselves have been associated with an increase in tumour metastases, but the data is conflicting and further research and clinical trials are required [23].

A limitation of this study is the small sample size. A strength is its randomised double-blind design, thus reducing bias. The fact that two established sensitive tests for NETosis were used increases the overall sensitivity of the study as a true level of NETosis expression.

In conclusion, this pilot, prospective, randomised, double-blind, single-centre trial has shown that anaesthetic technique has no effect on the expression of NETosis as a marker of metastatic disease in breast cancer. This implies that NETosis is not influenced by these anaesthetic techniques and that it may not be a reliable marker in future trials evaluating the effect of these anaesthetic techniques on cancer recurrence.

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Table 1: Patient and Surgical Characteristics.

Data shown is mean (Standard deviation), median (25%-75% range) or n (%).

Parameter	**GA**	**PPA**	**P value**
Age (yr)	57 (10)	59 (9)	
BMI	29 (6)	29 (7)	
Caucasian Race	20 (100%)	20 (100%)	
ASA physical status			
1	11 (55%)	10 (59%)	
2	8 (40%)	5 (29%)	
3	1 (5%)	2 (12%)	
Duration of Anaesthesia (mins)	73 (16)	85 (45)	0.25
Wide Local Excision surgery	16 (80%)	18 (90%)	0.28
Full Mastectomy	4 (20%)	2 (10%)	0.28
Intraoperative Opioids (mg)	10 (10-10)	0 (0-0)	0.0001
Intraoperative Lowest MAP	59 (8)	65 (16)	0.09
Intraoperative Highest HR	78 (13)	78 (8)	0.99
Intraoperative Blood Loss (ml)	314 (166)	290 (129)	0.61
First Morning total Opioids (mg)	5 (0-10)	0 (0-0)	0.003
Postoperative Pain	4 (3-4)	0 (0-1)	<0.0001
Previous Chemotherapy	0	1 (5%)	0.30
Previous Radiation	0	0	

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Table 2: Cancer Characteristics.

Data shown is mean (Standard deviation), median (25%-75% range) or n (%).

Oestrogen Receptor positive	17 (85%)	19 (100%)	0.59
Progesterone Receptor positive	15 (79%)	13 (76%)	0.86
HER2 Receptor positive	5 (26%)	4 (22%)	0.78
Closest Tumour Margins	3.8 (2.9)	4.2 (3)	0.66
TNM Stage			0.25
0	1 (5%)	0	
1	6 (30%)	11 (55%)	
2A	8 (40%)	6 (30%)	
2B	3 (15%)	1 (5%)	
3A	1 (5%)	2 (10%)	
4	1 (5%)	0	
Histology Grade			0.09
1	1 (5%)	6 (30%)	
2	12 (60%)	8 (40%)	
3	7 (35%)	6 (30%)	
Nottingham Prognostic Index	4.3 (1)	3.8 (1.4)	0.22
Cancer Recurrence	2 (10%)	1 (5%)	0.64

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Table 3: NETosis serology values. All data shown is mean (standard deviation)

Parameter	**GA**	**PPA**	**P value**
Pre op MPO (ng ml^{-1})	8.6 (4.7)	10.4 (6.3)	0.28
Post op MPO (ng ml^{-1})	10.5 (6.6)	11.5 (4.7)	0.60
Pre op H3Cit (ng ml^{-1})	3.4 (1.7)	3.1 (2.1)	0.62
Post op H3Cit (ng ml^{-1})	3.6 (2.3)	4.0 (5.9)	0.80

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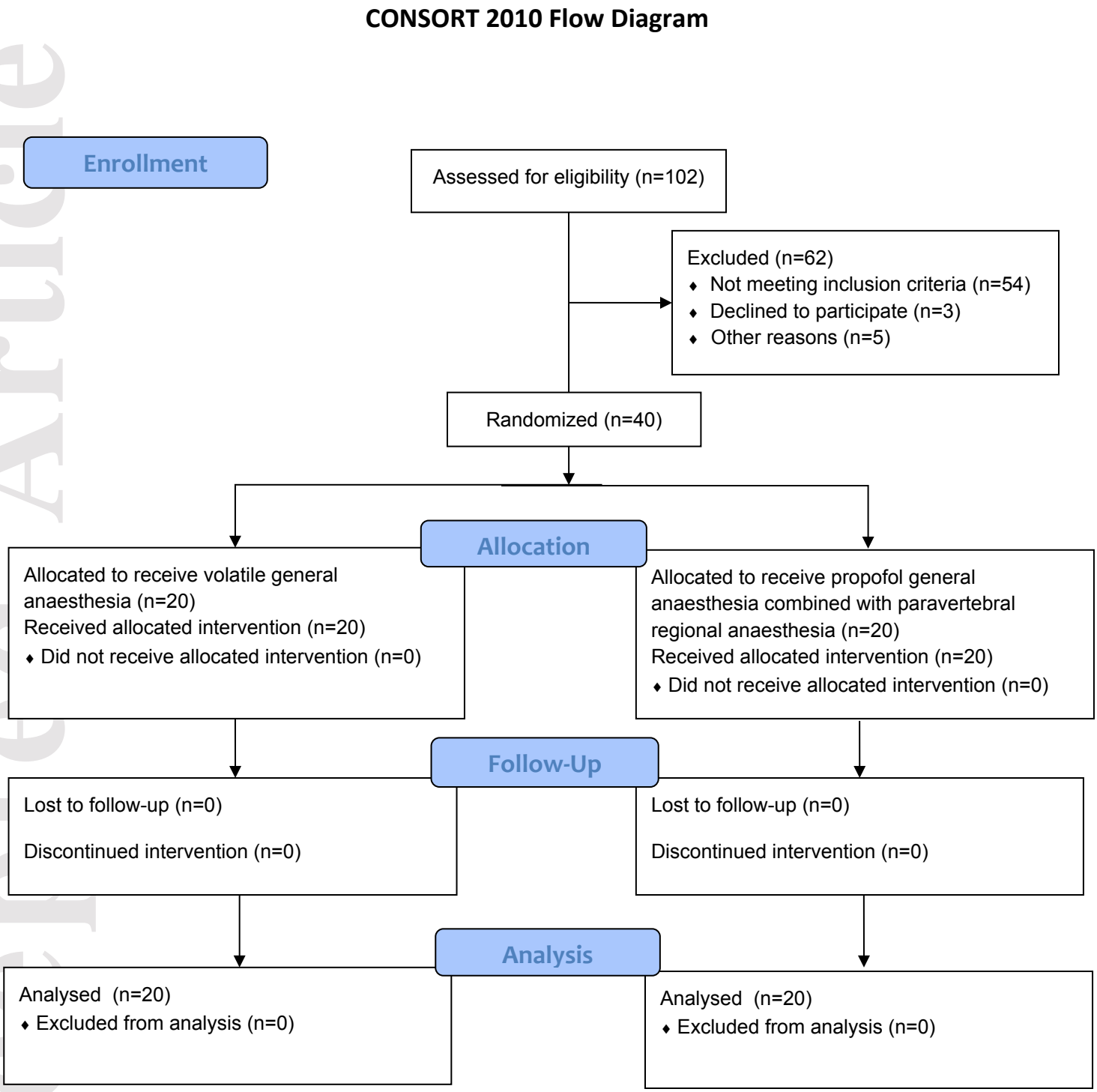

Figure 1: Trial profile