

Neutrophil Extracellular DNA Traps as a Clinical Biomarker and Target for
Improved Outcomes Following Radical Cystectomy

BY

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Abstract

Introduction

Neutrophil extracellular DNA traps (NETs) are DNA scaffolding structures released by neutrophils in response to injury, stress and infection and have been implicated in cancer metastasis. We sought to determine if NETs are detectable in the plasma of patients with muscle-invasive bladder cancer before, during, and after radical cystectomy (RC). We also explored whether higher NET levels are associated with infection and early recurrence, and if specialized immunonutrition (SIM) supplementation of L-arginine reduces NET formation.

Methods

In a pilot randomized clinical trial, 29 men consumed nutrition supplementation 5 days before and 5 days after radical cystectomy at Kansas Health System. The intervention group (n=14) took a SIM beverage containing L-arginine (Nestlé, Impact Advanced Recovery®). The control group (n=15) also received oral nutrition support (Nestlé, Boost Plus®). Blood samples were collected at baseline, intra-operatively, and post-operative days 2, 14, and 30. Neutrophil elastase was measured using sandwich ELISA analysis. Granulocyte-colony stimulating factor (G-CSF) and plasma IL-6 were measured using MILLIPLEX® map human cytokine kit 1. High sensitivity C-reactive protein (hs-CRP) was measured at Quest Labs. Results were analyzed using generalized linear models with repeated measures and Spearman's correlations.

Results

Twenty-six of the 29 patients had enough plasma to evaluate for NETs. All samples analyzed had detectable levels of NETs. There were no significant differences between NET levels in those with clinically identified infection or 3-year cancer recurrence. G-CSF was significantly different between the intervention and the control groups from baseline to POD2 and surgery to POD2. There were no significant correlations between NETs and IL-6 or NETs and hs-CRP. We did not detect a significant difference in NETs between the intervention and the control groups.

Conclusions

In our study, NETs were detectable in patients with bladder cancer. NET levels were highly variable across participants at all time points. G-CSF was significantly higher in the intervention group intraoperatively. NET levels did not differ between patients receiving the SIM drink as compared to those receiving the control nutrition supplement. Future research should evaluate if NET formation is a risk factor for early recurrence in a larger, multi-site sample.

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Table of Contents

Abstract.....	iii
Acknowledgements.....	v
Chapter 1- Introduction.....	1
Chapter 2- Review of Literature.....	3
Introduction to Bladder Cancer.....	3
Population affected by bladder cancer and Treatment through RC.....	4
Complications in Treatment.....	5
Introduction to neutrophil extracellular DNA traps (NETs).....	6
NET-Associated Cytokines and Biomarkers.....	7
Granulocyte-Colony Stimulating Factor.....	7
Interleukin-6.....	7
High Sensitivity C-Reactive Protein.....	8
Mechanism of NETs in Cancer Recurrence.....	8
Measuring neutrophils as a prognostic tool.....	9
Summary.....	10
Chapter 3- Methods.....	11
Overview and Design.....	11
Population.....	11
Ethical Approval.....	11
Procedures.....	12
Statistical Analysis.....	13
Chapter 4- Results.....	13
Chapter 5- Discussion.....	22
Limitations, Strengths, and Future Research.....	26
Limitations.....	26
Strengths.....	27
Future Research.....	27
Conclusion.....	28
References.....	29

CHAPTER 1: Introduction

Bladder cancer accounts for 7% of all new cancer cases and approximately 12,520 deaths in the United States annually making it the fourth most common cancer diagnosis in men in 2018 (1). The American Society of Clinical Oncology (ASCO) treatment guideline identifies radical cystectomy (RC) as the gold standard treatment for muscle-invasive bladder cancer (2). Following RC, approximately 34% of patients experience cancer recurrence and metastasis (3). The gold standard treatment for this cancer type is the most efficient at sparing lives, but has a 25% post-operative infection rate (4) that is associated with the high rate of cancer recurrence. While there is a hypothesized connection between post-operative infection following RC and cancer recurrence, the mechanisms are not fully understood. Neutrophil extracellular DNA traps (NETs) are DNA scaffolding structures released by neutrophils in response to injury, stress and infection and have been found to promote cancer metastasis in cancers of the liver, pancreas, and lung (5-7). While NETs have been implicated in cancer metastasis, it is unknown whether NETs are induced by RC.

NETs are formed by neutrophils via the innate immune system to trap microbes that cause infection. Recent research has found this ‘trapping’ promotes tumor cell invasion into healthy tissue when NETs form following surgical stress (8). Immune cells and trapped cancer cells secrete inflammatory cytokines that mobilize neutrophils and stimulate NET formation, thus a higher number of measured NETs is hypothesized to be associated with cancer metastasis (7). If NETs are detectable in human plasma, measuring the number of NETs perioperatively may be a prognostic tool for immune defense and/or cancer recurrence. Though it is unknown whether NETs are induced by RC, the high rate of post-operative infection in this population

provides an opportunity to investigate the potential for utilizing NETs as a prognostic tool and target for reducing RC complications.

Pilot studies have investigated specialized immunonutrition (SIM) aimed to fulfill nutrient insufficiencies prior to RC and were found to reduce post-operative urinary tract infection, paralytic ileum, and index scores according to field-wide accepted Clavien grades (9, 10). NETs are not well understood, but it is known that NETs form in the presence of inflammation and infection. If SIM drinks reduce post-operative infection, it is possible that their mechanism also includes a reduction of plasma NETs. A nutrition intervention aimed to prophylactically reduce postoperative infection rates may also reduce subsequent NET formation. It is important to identify if NETs are induced by RC and whether NETs are associated with infection and recurrence rates.

Statement of Purpose: To investigate whether NETs are associated with infection or cancer recurrence rates following RC and whether NET formation is modulated by SIM.

Research Questions:

1. Are NETs detectable in the plasma of surgical oncology cystectomy patients?
2. Are higher NET levels associated with infections or 3-year recurrence rate in patients following RC?
3. Does perioperative SIM influence the formation of NETs compared to a perioperative nutrition control group?

CHAPTER 2: Review of the Literature

Introduction to Bladder Cancer

Bladder cancer is the fourth most diagnosed form of cancer in men in the United States and is generally diagnosed in an early stage of disease. This cancer accounts for 7% of all new cancer cases for males in the US (1). Bladder cancer is broadly categorized by whether the tumor is non-muscle-invasive or muscle-invasive. Non-muscle-invasive bladder tumors are found in the shallow layers of epithelial bladder tissue whereas muscle-invasive bladder tumors also invade deeper layers of muscle tissue (1). Approximately half of newly diagnosed cases are stage 1, non-muscle-invasive (1). The five-year survival rate for Stage I bladder cancer is 88%, but drops to only 46% for those with a Stage III diagnosis (1). The five-year survival rate for metastasized bladder cancer, or Stage IV, is 15% (1). Although most cases are diagnosed in early stages, bladder cancer has a high recurrence rate. There is a need for investigation into how to improve these outcomes to improve survival rates of patients who have advanced bladder cancer.

The gold standard treatment for muscle-invasive bladder cancer or high-risk non-muscle-invasive forms is radical cystectomy (RC), removal of the bladder, urinary diversion, and resection of intestinal tissue for urinary tract reconstruction (2). A post-operative infection rate of 25% is observed for bladder cancer patients who have undergone RC (4). The highest rate of recurrence is found in those patients who undergo RC (3). While there is a hypothesized connection between post-operative infection following RC and cancer recurrence, the mechanisms are not yet fully understood. Researchers are looking to identify these mechanisms in an effort to prevent post-operative infection and lower recurrence rate following RC.

Population affected by bladder cancer and Treatment through RC

Bladder cancer affects older adults, 9 out of 10 diagnoses are adults aged 55 years or older (1). Men have a 4 to 1 higher likelihood of developing bladder cancer than women and white Americans are twice as likely to develop bladder cancer as African Americans or Hispanic Americans (1). The biggest risk factor for development of bladder cancer is tobacco use; half of all diagnosed individuals have used or currently use tobacco (1). Another risk factor associated with disease development is exposure to industrial chemicals that include aromatic amines (1). The most commonly identified professions of those diagnosed are hair dressers, truck drivers, machinists, painters, and those who work in the leather industry (1). An association between daily fluid intake and bladder cancer has been suggested, which is thought to dilute carcinogen exposure to the bladder tissue (11). It is hypothesized that voiding the bladder of urine more often reduces contact time with the accumulation of carcinogens (11).

Approximately 25% of patients diagnosed with bladder cancer are treated with RC (12). Due to the high rate of metastasis, surrounding organs are also typically removed during this surgery (12). RC for male patients includes removal of the bladder, prostate, surrounding lymph nodes, and seminal vesicles (12). RC for female patients includes removal of the bladder, fallopian tube(s), ovaries, uterus, vaginal tissue, and surrounding lymph nodes (12). Following the removal of these tissues, the urinary tract is rebuilt using segments of intestinal tissue. Intestinal tissue is rich in bacteria, which may play a role in the high rate of post-operative infection (4). Prophylactic antibiotics are used in an effort to stave off post-operative infection following RC (12).

RC is carried out using either an open surgical method or a laparoscopic method. Open surgery includes a broad incision from the naval to the pelvis while the laparoscopic surgery includes an average of six small incisions (12). Both surgeries take between 6-8 hours and data show a positive correlation between surgical procedure duration and post-operative infection in both regardless of surgical approach (4). Parker et al. reported a significant increase in post-operative infection in cystectomy surgeries lasting longer than 480 minutes or those individuals who require a peri-operative blood transfusion, which tends to be associated with a longer surgical duration (4).

Complications in treatment

Participants who receive RC are prone to many serious complications. Surgeons utilize portions of the intestine to reconnect the urinary tract, therefore connecting two sets of tissue that innately contain a large number of bacteria (4). Parker et al. found that approximately 25% of individuals who receive RC are diagnosed with an infection within 30 days (median = 13 days) (4). When Shabsigh et al. followed RC recipients for 90 days post-operatively, 64% experienced a post-surgical complication (3).

Recurrence within 5 years following RC is approximately 34% (3, 13). Between 35-50% of patients who undergo RC experience cancer recurrence at some point in life (3, 4, 6, 10, 11, 13). Identifying the mechanisms through which recurrence and metastasis occur in surgical cancer therapies is important to improve treatment outcomes.

Introduction to neutrophil extracellular DNA traps (NETs)

Neutrophils are the innate immune system's first line of defense and recent studies have identified a DNA scaffolding structure built by these leukocytes that may promote cancer metastasis, especially in the presence of postoperative inflammation and/or infection (5-8). These scaffolding structures, or NETs, are produced by neutrophils in the presence of stress (ischemia, surgical stress, infection, etc.) through the ejection of their own DNA-containing chromatin near or into impacted tissue sites (5).

The biochemical mechanism of forming NETs is commonly referred to as NETosis. This form of programmed cell death is vaguely understood and is at the forefront of immunological research. This cell death is characterized by decondensation of chromatin, the dissolving and disintegration of both the nuclear and cellular membranes, (14) and the ejection of chromatin into the extracellular matrix. NETosis is stimulated by a ligand binding on toll-like and cytokine receptors, initiating the release of calcium into the cytoplasm by the endoplasmic reticulum (14). The shift in cytoplasmic calcium concentration causes NADPH oxidase-complexes to generate reactive oxygen species (ROS) (15) which in turn causes granular and nuclear membranes to lyse (14). The cytoplasmic enzymes neutrophil elastase and myeloperoxidase enter the nuclear area and work to decondense chromatin and deiminate nuclear histones (14, 16). Peptidyl arginine deiminase 4 (PAD4) citrullinates arginine and removes the positive charge of nuclear proteins, allowing neutrophil elastase and myeloperoxidase to decondense chromatin and deiminate chromatin-bound histones (17). Once chromatin decondenses, it is expelled out of the nucleus, into the extracellular space, and forms a large web with antimicrobial properties. This web is the neutrophil extracellular DNA trap, NET.

NET-Associated Cytokines and Biomarkers

Granulocyte-Colony Stimulating Factor

Granulocyte-colony stimulating factor (G-CSF) is a glycoprotein that stimulates the production, proliferation, and differentiation of neutrophils and increases NETosis (18).

Circulating G-CSF increases during injury and infection as the body attempts to produce more antimicrobial and immune regulating neutrophils. Data show that circulating histone-complexed DNA and myeloperoxidase increase within one hour following injection of G-CSF, suggesting that increased NETosis occurs rapidly with G-CSF elevation (18). Demers et al. found that certain tumor types secrete G-CSF which ‘primes’ neutrophils to enter NETosis (19). In mouse models, this priming and subsequent increase in NETs was associated with accelerated tumor growth (19).

Interleukin-6

Interleukin-6 (IL-6) is both a pro-inflammatory and anti-inflammatory cytokine produced by T cells and macrophages and helps to activate the complement system of the innate immune response (20). The complement system works to enhance antibodies and immune cell function during infection or injury. IL-6 also helps to stimulate neutrophil production in bone marrow during the inflammatory cascade (21). Studies of lung tissue damage found that *in vitro* NET formation stimulated alveolar epithelial cells to secrete a significantly higher amount of IL-6 compared to neutrophils that had undergone forced necrosis, indicating that IL-6 is directly increased by NETosis (22).

High Sensitivity C-Reactive Protein

High sensitivity CRP (hs-CRP) is a measure of both chronic and acute phase inflammation and increases in response to an increase in IL-6. Due to the mechanistic relationship between hs-CRP and IL-6, hs-CRP is used as a risk indicator by proxy in many inflammatory conditions (20). This biomarker rapidly shifts in response to multiple forms of stress, so its clinical use is global rather than necessarily localized in nature.

Mechanism of NETs in Cancer Recurrence

Recent data associate NET formation in the presence of cancer with trapping circulating tumor cells leading to subsequent metastasis in nearby tissue (5, 8). Tumor resection and partial or whole organ removal are common cancer treatments, yet metastasis is observed in many patients with operation-induced inflammation or infection (3, 8). Neutrophils eject DNA webs into the areas surrounding inflamed and infected tissue in an effort to protect the body, but recent data suggest that this action of building NETs may be harmful in the presence of cancer (5, 7, 8). Cools-Lartigue et al. observed cancer cell ‘trapping’ in NET formations and found that this action contributed to liver metastases in the presence of infection (8). The NETs that ‘trap’ these circulating cancer cells are located in different areas of the body and therefore increase linkage of cancer cells to previously healthy tissues (8). Huh et al. observed that circulating melanoma cells become stuck in the deep capillaries of the lung and secrete inflammatory cytokines, which recruits neutrophils (7). These mobilized neutrophils build NETs that allow the melanoma cells to anchor themselves to lung tissue (7). NETs have multiple mechanisms through which they help cancer cells both invade healthy tissue and proliferate.

In addition to their contribution to metastasis, NETs have attached protein complexes that release protease enzymes that have been found to speed the rate of cancer cell proliferation in murine models (4, 5, 23). Li et al. found that when instigating NET formation in the lungs of mice, proliferation of injected cancer cells was increased and metastasis occurred at a significantly faster rate (23). In this same study, two NET enzymes were identified as being involved in this metastasis: neutrophil elastase and matrix metalloproteinase 9 (23). These proteases can be used as a plasma marker for NET presence and volume. Multiple studies have demonstrated the use of DNase, an enzyme that cleaves linkages in the DNA structure, for the inhibition of NET formation. These studies found a reduction of cancer proliferation and migration (5, 8, 23), directly implicating the protease activity and ‘trapping’ behavior of NETs with cancer invasion and metastasis. The use of DNase as a post-operative cancer therapy is an area that should be further researched due to limited understanding of how the DNase treatment would impact transcriptive processes long-term (23). Though this method of prevention is not directly related to this intervention, it does identify NETs as playing a role in cancer cell proliferation.

Measuring neutrophils as a prognostic tool

Measurements of neutrophil volume are used as a prognostic tool, but it is unknown whether increased neutrophil volume is associated with NET levels. Kawahara et al. found that a neutrophil-to-lymphocyte ratio (NLR) measured through a simple and cost-effective complete blood count panel was a useful indicator of overall patient survival following RC (24). The cancer recurrence rate was significantly higher for those with a higher NLR >2.38 (24). This impact was even greater when hs-CRP measures were high, further building the relationship

between neutrophil mobilization and inflammation (24). Tao et al. utilized this type of NLR as a prognostic tool in pancreatic ductal adenocarcinoma and found it to be effective in predicting post-operative metastasis (6). Use of a NLR as an indicator of post-operative risk has been used with effectiveness (24, 25), which may also implicate NETs in the mechanism of metastasis. In a previous analysis of this data set, Hamilton-Reeves et al. found that individuals supplemented with the specialized immunonutrition (SIM) beverage had a lower postoperative NLR than those in the control (10). The possible association between NETs and inflammation not only identifies poor prognosis on overall patient health, but it may also indicate increased risk of metastasis and morbidity (8, 24). SIM has shown the potential to lower NLR, but the effect of SIM on plasma NETs is unknown.

Summary

Bladder cancer is a common cancer with a gold standard treatment that does not prevent recurrence (1). Through improved understanding of cancer proliferation and migration, survival rates can be significantly increased. The immune system may be used by cancer for its own advantage and NET formation may be a contributor to this process. Immune system response to infection and surgical stress causes acute inflammation and NET formation (5-8, 24), which may increase the risk of post-operative infection and cancer recurrence. Using the NLR and plasma NETs as biomarkers (8, 24) perioperatively may help to identify those at greatest risk and who may benefit from treatments such as DNase therapy, PAD4 inhibitors, or SIM therapy (5, 23). Optimizing nutrition interventions to improve the response to surgery may offer an affordable, complementary approach to cancer treatment in those receiving RC.

CHAPTER 3: Methods

I. Overview and Design

Data were obtained from a pilot randomized clinical trial designed to compare immune response and clinical outcomes after cystectomy between standard oral nutrition support (Nestlé Boost Plus©) and SIM (Nestlé Impact Advanced Recovery©) (10, 26). Twenty-nine men scheduled to undergo RC were recruited within the Kansas Health System (KHS). Permuted block randomization was used to assign study arm. All participants received standard of care and consumed nutrition supplementations three times a day for 5 days before and 5 days after RC surgery. Blood samples were collected at baseline before surgery or supplementation, 3 hours into surgery, and post-operative days 2, 14, and 30.

II. Population

The population included 29 men diagnosed with primary bladder cancer with no existing metastasis. Patients were referred from the KHS by multiple urologic oncologists. Exclusion criteria included patients with unintentional weight loss $\geq 10\%$ in the previous 6 months, a BMI $\leq 18.5 \text{ kg/m}^2$, bladder cancer that is not urothelial in nature, active infections, difficulty swallowing or those with potential for aspiration, those unable to tolerate oral intake, those with a history of gout and gout-related arthritis, those with a history of uric acid kidney stones, and patients with food allergies or sensitivities to soy, milk, or fish.

III. Ethical Approval

Participants voluntarily entered this study. The study was approved by the Human Subjects Committee and Institutional Review Board at the University of Kansas Medical Center.

All participants provided informed consent according to institutional guidelines and acknowledged that blood samples would be analyzed for future research questions. The database was stored within Research Electronic Data Capture (REDCap) accessible only through a secure server. The University of Kansas Medical Center is a Health Insurance Portability and Accountability Act (HIPAA)-covered entity and complies with all HIPAA regulations regarding data privacy and security. Access to study data is password protected; individual identifiers such as names and medical record numbers were hidden from study data files in the data processing steps.

IV. Procedures

Stored plasma samples collected from ONS and SIM participants at baseline, intra-operative, and POD 2, 14, and 30 were analyzed for NET formation (elevated >0.2 OD). A sandwich enzyme-linked immunosorbent assay (ELISA) developed and provided by the Egeblad laboratory was used to measure neutrophil elastase that is associated with NET formation (27). This method was developed and validated as a proxy for NETs in human samples from patients with chronic disease and healthy controls (28-31). This assay is specific to human neutrophil elastase through its use of a horseradish peroxidase-linked polyclonal antibody and this method is utilized to measure NETs in a variety of disease states (28-31).

We measured plasma cytokines that are associated with recruitment of neutrophils to inflammatory or infected tissues and subsequent NET formation. Granulocyte-colony stimulating factor (G-CSF) and plasma IL-6 were measured using MILLIPLEX® map human cytokine kit 1 (Millipore, Billerica, MA, USA) with a standard curve range of 3.2-10,000 pg/ml. High-sensitivity C-reactive protein (hs-CRP) was measured in serum by Quest Diagnostics Laboratories (Lenexa,

KS) with a detection range of 1.0-10.0 mg/L. All cytokines were measured at the University of Kansas Medical Center.

V. Statistical Analysis

Statistical analysis of differences between plasma markers in patients supplemented with SIM and ONS were calculated using the generalized linear mixed method of analysis using SAS. NET formation between groups and 30-day infection rate and 3-year cancer recurrence were calculated with generalized linear mixed models using GLIMMIX via SAS. A significance level of $p < 0.05$ was set as statistically significant. Spearman's correlation tests were used to investigate NET formation and IL-6 and G-CSF with R version 3.3.3 (32). Statistical analyses were run by Dr. Prahabakar Chalise at the University of Kansas Medical Center.

CHAPTER 4: Results

Twenty-nine study participants were randomized into the SIM (n=14) and ONS (n=15) groups. Group characteristics were similar for age, clinical staging, smoking status, Charlson Comorbidity Index score, urinary diversion type, PG-SGA score, and neoadjuvant chemotherapy treatment. Body mass index (BMI) differed between the groups (+3.6 kg/m² ONS, $p=0.004$). Complications with obtaining blood samples and participant inability to attend follow-up visits limits data at all time points. There were 7 (50%) participants from SIM and 2 (13.3%) from ONS that had enough plasma from all five time points for analyses. NETs were detectable in the plasma of all participant samples (Table 1).

Table 1- Minimum and maximum NET levels over time

	Minimum	Maximum
Baseline (n=29)	0.045	0.426
Surgery (n=28)	0.117	1.181
POD2 (n=23)	0.115	0.538
POD14 (n=13)	0.118	0.761
POD30 (n=19)	0.098	0.717

¹NET formation measured in optical density (OD)

²Seven SIM and 2 ONS participants were analyzed at all 5 points

There was no significant difference in NETs between those with clinically identified infection and those without infection ($p=0.647$). NETs trended higher at POD 30 compared to baseline when all participants were evaluated together, although this trend was not statistically significant ($p=0.115$, Figure 1).

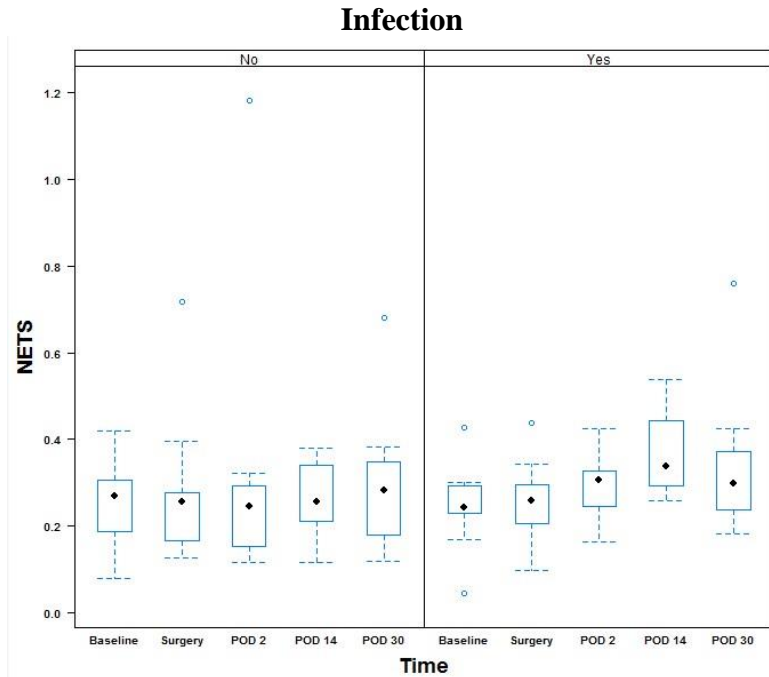


Figure 1- NETs (OD, left) and infection (No, Yes)

There was no significant difference in NETs between those who had cancer recurrence within three years following RC and those who did not recur ($p=0.258$). Regardless of participant recurrence, NETs trended higher at POD 30 on average ($p=0.118$, Figure 2).

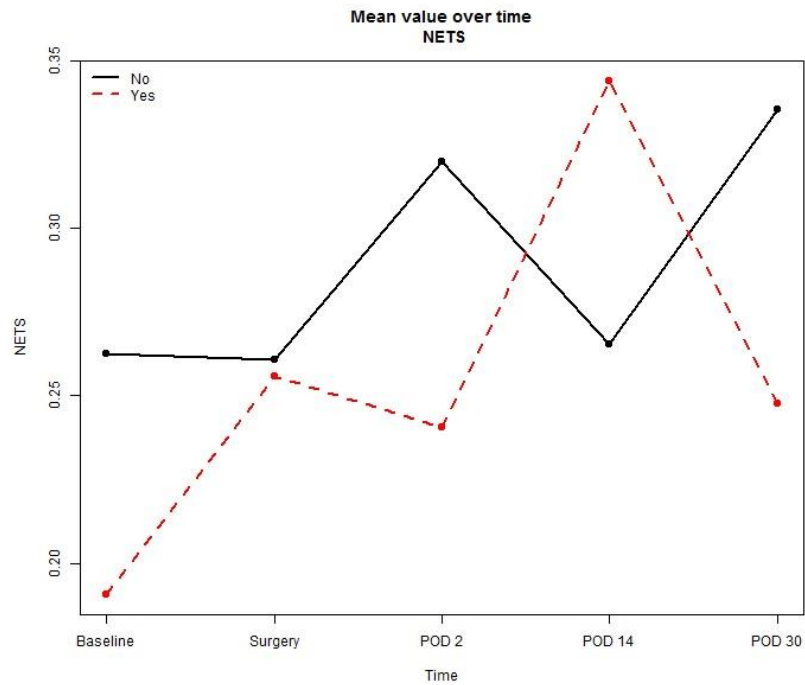


Figure 2- NETs (OD, left) and 3-year cancer recurrence (Yes, No)

NET formation was not statistically different between the SIM and ONS throughout the intervention. There was an observed shift of mean NET formation between groups from the intraoperative time point to POD2. At POD2, the largest difference in NET formation between groups was observed with SIM having higher plasma measures than ONS (+0.103, p=0.108, POD2).

Table 2- Mean NET formation between groups over time

	SIM	ONS
Baseline	0.225	0.262
Intraoperative	0.246	0.272
POD2	0.345	0.242
POD14	0.298	0.290
POD30	0.312	0.313

¹OD

G-CSF was significantly different between SIM and ONS from baseline to POD2 ($p=0.0096$). G-CSF was significantly different between SIM and ONS from the intraoperative time point and POD 2 ($p=0.0148$)(Figure3).

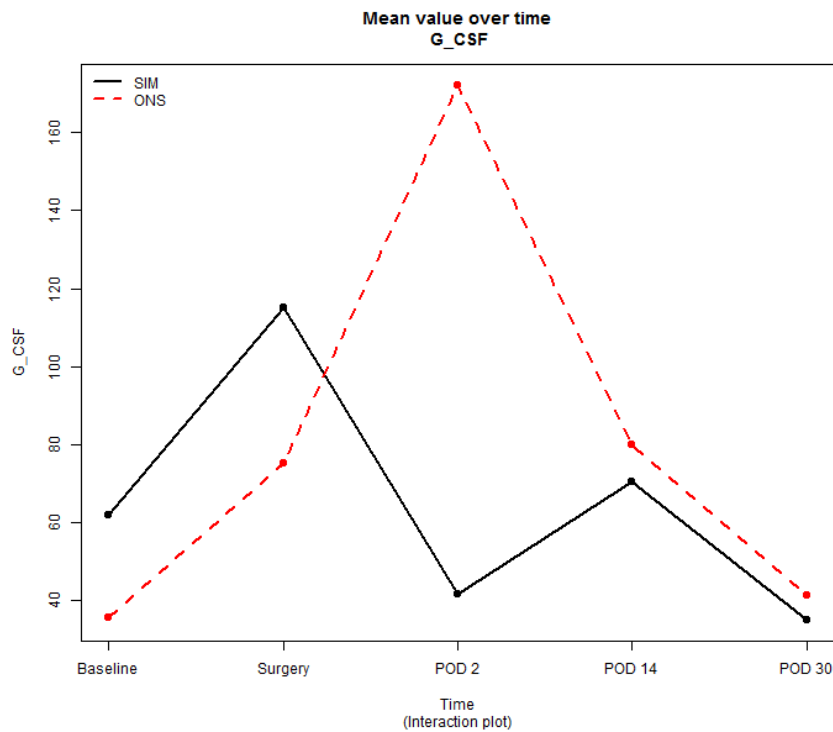


Figure 3- G-CSF (pg/ml) between SIM (black) and ONS (red)

G-CSF showed an inverse relationship with NET formation in both groups with lower plasma levels during periods of higher detectable NETs. There was a weak relationship between G-CSF and NETs at POD2 in the entire cohort ($r= 0.142$). However, there was no statistically significant difference between NETs and G-CSF in either group over time (Figure 4, Figure 5).

Correlational analyses identified a weak inverse relationship between IL-6 and NETs at the intraoperative time point ($r = -0.153$) and POD2 ($r = -0.129$). There was no significant relationship identified between IL-6 and NETs, though there appeared to be a tighter relationship in SIM versus ONS (Figure 6, Figure 7). There was no statistically significant correlation between NETs and hs-CRP (Figure 8, Figure 9).

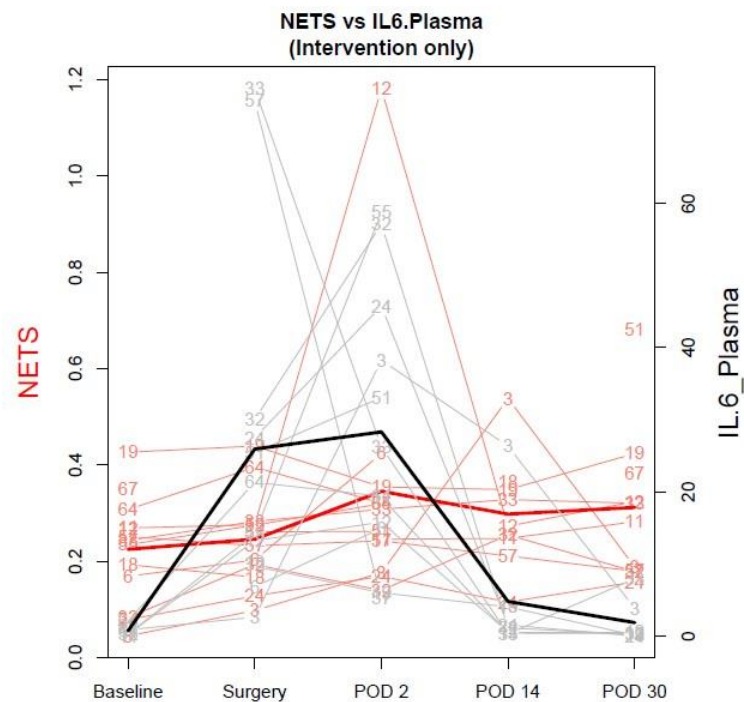


Figure 6- NETs (OD, left, red) and plasma IL-6 (pg/ml, right, black) over time in SIM group. Numbers represent participant study ID.

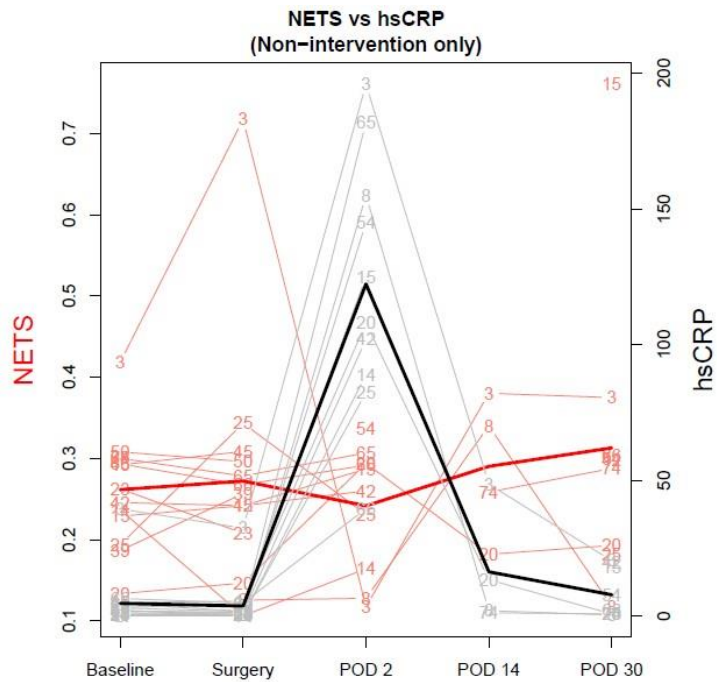


Figure 9- NETs (OD, left, red) and hs-CRP (mg/L, right, black) over time in the ONS group. Number represents participant study ID.

CHAPTER 5: Discussion

This study investigated whether NET measurement is feasible in individuals with muscle-invasive bladder cancer before, during, and after RC. We also examined to see if SIM modulated NET formation. NETs were present in all plasma samples provided. There were no significant differences between NET level by clinically identified infections or 3-year cancer recurrences. SIM did not modulate NET levels as mean NETs were not different between the two arms at any time point.

This is the first clinical investigation of NETs in bladder cancer patients undergoing RC. Our data suggest that NETs are detectable in the perioperative window of RC. NETs were

detectable in every participant sample at all time points. Difficulty with blood draws and participants' inability to attend follow-up visits account for the lower number of samples than patients at all time points. The process of NETosis takes approximately 4 to 6 hours and NETs have an observed lifespan of 6 to 8 hours in human blood (33). NETs can be observed in tissue samples for up to 7 days following infection or tissue injury (33). Our findings align with studies in other cancer types regarding the presence of NETs in patient plasma (5-7). Due to the novelty of NET research, there is no defined reference range and there is no established ceiling measure. Biomarkers and measures of NETs are highly variable across individuals and disease states, therefore it cannot be determined if our cohort was unique in NET levels. We observed multiple outliers that shifted mean values. Due to the lack of a reference range or ceiling measure, these outliers were not removed from statistical analyses and limit our interpretation in such a small sample size.

Individuals who undergo RC are at high risk of developing a post-operative infection (4) and cancer recurrence (3). Our data contribute to a large body of research aimed to improve post-operative outcomes by investigating whether NETs following surgical stress or infection promote recurrence. Previous research has shown that NETs trap circulating tumor cells (5, 8), promote metastasis (5, 8, 23), and increase proliferation of cancer cells (4, 5, 23), but we observed no difference in NET levels perioperatively in those who recurred and those who did not. There also was no statistically significant difference in NETs between those participants who developed an infection within 30 days post-operatively and those who did not. Therefore, the highly variable data from this small cohort did not establish a correlation between NETs, infection, and early recurrence.

Hamilton-Reeves et al. found that SIM had significantly lower IL-6 from intraoperative point to POD2 ($p= 0.022$) and that mean IL-6 was 42.8% lower ($p=0.020$) in the SIM group than ONS at POD2 (26). Overall, there was no significant relationship between IL-6 and NETs, suggesting that the reduction in IL-6 was related to other mechanisms. The hypothesized relationship between IL-6 and NET formation is better demonstrated in the SIM group, however data points in the ONS group are too variable to draw conclusions. A study with more frequent blood draws may better evaluate the associations between cytokine and NETs levels. Hamilton-Reeves et al. found no significant difference in hs-CRP between groups ($p= 0.908$) (26) and hs-CRP did not correlate with NET levels.

There was a significant difference in G-CSF between SIM and ONS from baseline to POD2 and from surgery to POD2. G-CSF was only weakly correlated with NETs at POD2, suggesting that differences in G-CSF between groups may serve functions other than upregulating NETosis. G-CSF is provided as both a prophylactic and therapeutic treatment in immunosuppressed individuals (18) to stimulate leukocyte production and bolster immune function. Perioperative SIM reduced post-operative infection in this cohort (10, 26) and we observed a significantly higher amount of G-CSF in SIM at the intraoperative time point. ONS had significantly higher G-CSF at POD2. This suggests that the nutrients provided through our immunonutrition drink better prepared intervention participants to fight infection through a faster immune system response. Though G-CSF was not associated with NET levels, future studies should continue to use G-CSF as an immune system biomarker.

We observed no difference in NET levels between participants receiving SIM or ONS, suggesting that this specific SIM drink does not modulate NET formation. Previous analyses of these data found that individuals receiving SIM had fewer infections following RC than those receiving ONS (10, 26). It was previously inferred that arginine-enriched SIM modulates immune system function following surgery by preventing T cell suppression and reduced lymphocyte proliferation through arginine repletion (10, 26). A critical step in NETosis is the deamination of arginine into citrulline by PAD4, therefore it can be hypothesized that supplementation with arginine may promote NET formation. However, we observed no difference in NETs between those receiving arginine-enhanced SIM and ONS. Our findings therefore may indicate arginine is triaged and used for bolstered proliferation of macrophages, lymphocytes, granulocytes, and other immune cells (34) rather than directly promoting NETosis.

While NETs are detectable in the plasma of surgical oncology patients undergoing RC, our findings suggest NETs are quite variable and are not tightly associated with clinical outcomes. Further, NETs were not modified by a specialized immunonutrition intervention, therefore the improved outcomes observed in this cohort may be attributed to other mechanisms such as arginine repletion, anti-inflammatory omega-3 fatty acid supplementation, early post-operative feeding, and improved nutrition status which are all currently being explored in a large phase III randomized controlled trial

Limitations, Strengths, and Future Research

Limitations

We were the first to look at NET formation following RC and modulation through a specialized immunonutrition intervention, but this novel study has its limitations. Primarily, this was a small cohort treated at a single-site for a brief period of time. RC has a long-lasting impact on the body and immune system. SIM may have a greater impact on NETs over a longer period and modulation of this immune function could have benefits. Findings from other research that identified NETs as cancer-promoting were in other cancer types (5-8). Muscle-invasive bladder cancer is severe and has poor prognostic outcomes; therefore a study measuring one mechanism of the immune system following RC may be too narrow of a lens.

There was a lack of heterogeneity in this study cohort. All participants were male and predominantly white (93%). This cohort was not representative in that 85% of participants formerly or currently smoked. Of that 85%, 17% were current smokers. This is not representative of the national average and therefore may have skewed the results. Further, it is widely known that smoking tobacco is associated with primary lung cancer, secondary metastases, and other co-morbidities such as cardiovascular disease, COPD, and type II diabetes. It is difficult to limit confounding factors that may influence NET formation, immune system dysregulation, and the state of cancer disease.

Analyses of NETs was run on banked plasma samples through an off-site laboratory. The length of time in formation and limited lifespan of NETs make measures highly variable based on the time of the blood draw (33). NETs are fragile structures that are easily destroyed through

various methods of observation and handling of samples (35), therefore storage and shipment of plasma to an off-site, out-of-state laboratory may have affected our findings. Analyses on banked samples run the risk of cytologic degradation, which may also affect observed NET levels.

Strengths

This was the first randomized clinical trial to investigate the feasibility of detecting NETs before, during, and after RC, whether NETs are associated with infection and cancer recurrence, and whether SIM can modulate NETs. The gold standard treatment for this cancer does not prevent recurrence and has severe complications. Contributing novel data to the growing body of research aimed to improve outcomes is a primary strength of this study.

NETs were detectable in all obtained samples. Due to the limited research on NETs, there is no standardized methodology. We utilized a widely accepted method for measurement and were able to detect NETs in all samples. Larger samples in future studies will perhaps provide greater insight into best practices for measuring NET formation and contribute to standardization of methodology.

Future Research

Though our findings were null, these data provide a valuable contribution to research. We found that NET formation is detectable in the plasma of surgical oncology patients, but that measuring NETs was not a useful biomarker or target for improved outcomes. RC patients have a high rate of post-operative infection and cancer recurrence, but we did not find an association between NET formation on either outcome. NETosis and microbe trapping is a fundamental part

of the innate immune system that is poorly understood. Future research should be aimed at larger study samples with greater heterogeneity. As our understanding of NETs grows, specialized immunonutrition could be optimized to modulate identified pathways. A larger, multi-center study design investigating NETs as a primary outcome may yield different results.

Conclusion

Immune system regulation through improved perioperative nutrition was shown to benefit this population (10, 26). The SIM drink provided during this intervention improved post-operative infection through mechanisms other than modulating NETosis. NETs were similar between groups at all time points and were not implicated in post-operative infection or in those with cancer recurrence. Specialized immunonutrition has been identified as a low-cost, high-impact way of improving outcomes in surgical oncology patients. We will continue to investigate the mechanisms through which immunonutrition modulates inflammation and the immune system. Neutrophil extracellular DNA traps are at the forefront of immunological research in many disease states and our research provides further insight into whether NETs are feasible to evaluate for their role in prognostics or as a target for improved clinical outcomes.

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