The characterisation of systemic neutrophil function in patients undergoing colorectal cancer resection and the impact of HMG-CoA reductase inhibitors on host inflammation

by

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Abstract

Cancer-associated inflammation, both in the systemic circulation and in the tumour microenvironment, is widely recognised to influence disease progression and survival. Surgical resection is fundamental in achieving cure in patients with colorectal cancer. The generation and maintenance of a systemic inflammatory response, with subsequent compromise of the anti-tumour immune response, has been associated with poor outcome. Neutrophils may facilitate metastatic progression in the context of systemic inflammation and therefore implementing a therapeutic strategy in the peri-operative period to reduce the systemic inflammation associated with surgery may be beneficial, particularly therapeutic strategies aimed at modifying tumour-neutrophil interactions.

HMG-CoA Reductase Inhibitors (statins) were developed as lipid-lowering agents. In addition, they have been implicated in the modulation of the immune system and have demonstrable anti-inflammatory effects. It has been proposed that they could be utilised in the peri-operative period to modulate the systemic inflammatory response and to preserve the anti-tumour immune competency of the host.

This study was conducted to serially characterise neutrophil function in patients undergoing colorectal cancer resection over the peri-operative period and to explore the impact of HMG-CoA Reductase Inhibitors on neutrophil function.

The experiments conducted in this thesis demonstrate a distinct change in neutrophil phenotype in response to surgery which exhibits reduced NET formation, reduced apoptosis and increased phagocytotic activity. The immune-modulatory capacity of HMG-CoA Reductase Inhibitors was investigated and the neutrophil functional changes were

attenuated with in-vitro treatment with simvastatin in patients with colorectal cancer. Post-operatively, a reduction in NET production was identified with in-vitro treatment with simvastatin which is considered advantageous as NETs have been strongly implicated in cancer progression and dissemination.

Dedication

I dedicate this thesis to my wife Olivia and our children, Florence, Tobias,

Barnaby, Arlo and Ottilie

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Glossary of Terms

Abdomino-Perineal Excision of the Rectum (APER) - An operation to remove the entire rectum and anal canal.

Anastomotic Leak – A breakdown of an anastomosis (the join between two ends of bowel) allowing bowel fluid to leak out into the abdominal cavity.

Anterior Resection – An operation to remove part of or the entire rectum.

Adjuvant Treatment – An additional therapy (e.g. chemotherapy or radiotherapy) provided to improve the effectiveness of the primary treatment (e.g. surgery).

Bowel Cancer Screening – The NHS Bowel Cancer Screening Programme offers screening every two years to all men and women aged 60 to 74. Those over 74 can request a screening kit from the Programme.

Chemotherapy – Drug therapy used to treat cancer. It may be used alone, or in conjunction with other types of treatment (e.g. surgery or radiotherapy).

Colostomy – A stoma (surgical opening) constructed by bringing the large bowel (colon) out onto the

surface of the skin.

Enhanced Recovery Programme – An evidence-based perioperative care approach that facilitates recovery after major surgery.

Hartmann's Procedure – An operation to remove the left colon and part of the rectum and involves the formation of a colostomy.

Ileostomy – A stoma (surgical opening) constructed by bringing the end or loop of small intestine (ileum) out onto the surface of the skin.

Laparoscopic – Also called minimally invasive surgery or keyhole surgery, is a type of surgical procedure performed through small incisions in the skin instead of the larger incisions used in open surgery.

Lymph Nodes – Also referred to as lymph 'glands', which form part of the immune system. They are distributed throughout the body and can be one of the first place to which cancers spread.

Multi-disciplinary Team (MDT) – A group of professionals, from diverse specialties that work to optimise the diagnosis and treatment throughout the patient pathway.

Metastases – Deposits of cancer that occur when the cancer has spread from the place in which it started to other parts of the body. These are commonly called secondary cancers. Disease in which this has occurred is known as metastatic disease.

Neo-adjuvant Therapy – Therapy (radiotherapy, chemotherapy or chemo-radiotherapy) given before another treatment, usually surgery. This is usually given to reduce the size, grade or stage of the cancer and therefore improve the effectiveness of the surgery performed.

Segmental resection - An operation to remove part of, or the entire colon, but not the rectum.

List of Abbreviations

7-AAD	7-Amino-Actinomycin
AFU	Arbitrary Fluorescent Units
APC	Adenomatous Polyposis Coli (tumour suppressor gene)
ARDS	Acute Respiratory Distress Syndrome
AU	Arbitrary Units
AUC	Area Under Curve
BMI	Body Mass Index
CR-POSSUM	Colorectal Physiological and Operative Severity Score
CRP	C-reactive Protein
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
E.Coli	*Escherichia Coli*
fMLP	N-formylmethionyl-leucyl-phenylalanine
G-CSF	Granulocyte Colony Stimulating Growth Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GTPase (Rho / Rac)	Guanosine Triphosphate Hydrolase (Rho / Rac)
HAT	Histone Acetyltransferase
HBSS	Hanks Balanced Salt Solution
HDAC	Histone Deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperasineethanesulfonic Acid
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
ICAM	Intracellular Adhesion Molecules
ICU	Intensive Care Unit

IL	Interleukin
IFN-β	Interferon-β
IQR	Inter-quartile Range
KRAS	V-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene
LOS	Length of Stay
LPS	Lipopolysaccharide
mGPS	Modified Glasgow Prognostic Score
MMP	Matrix Metalloproteinases
Mnase	Micrococcal nuclease
MPO	Myleoperxoidase
mTOR	Mammalian Target of Rapamycin
NADPH	Nicotinamide Adenine Dinucleotide Phosphateoxidase
NETs	Neutrophil Extracellular Traps
NHS	National Health Service
NLR	Neutrophil-Lymphocyte Ratio
NO	Nitric Oxide
p21	Cyclin-dependent Kinase Inhibitor 1
p53	Tumour Protein p53 (tumour suppressor gene)
PBS	Phosphate Buffered Saline
pHrodo	pH-sensitive pHrodo™ Dyes
PE-Annexin-V	Phycocerythrin Annexin V
PI3Kase	Phosphatidylinositol-3-kinase
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

RPMI	Roswell Park Memorial Institute
S.Aureus	*Staphylococcus Aureus*
SEM	Standard Error of the Mean
SIR	Systemic Inflammatory Response
SIRS	Systemic Inflammatory Response Syndrome
SLE	Systemic Lupus Erythematosus
TGF-β	Tumour Growth Factor-β
TNF-α	Tumour Necrosis Factor-α
TRIS-HCl	Tris Hydrochloride
UK	United Kingdom
X^2 Test	Chi-squared Test

Chapter 1

INTRODUCTION

1.1 The Origins of Immunotherapy

Immunotherapy is centred on the concept that a patient's immune system can be stimulated, or augmented, to attack a malignant tumour. The first systematic study of immunotherapy was conducted in 1891 by William B. Coley, an American Bone Sarcoma Surgeon, from New York Cancer Hospital (which later became Memorial Sloan-Kettering Cancer Centre). Coley injected streptococcal organisms into patients with inoperable cancers. He hypothesised that the infection would stimulate the patient's immune system and this would initiate tumour regression. He successfully demonstrated his hypothesis and this highlighted the potential of immunotherapy in the treatment of cancer [1].

Because of the dangers associated with the transfer of live micro-organisms, Coley utilised heat-killed streptococcal and serratia organisms, which he named Coley's Toxin, to initiate an immune response against malignant tumours. Throughout his career, over 1,000 patients received the Coley's Toxin and a near complete response was observed in approximately half [2].

The field of immunology has developed into a sophisticated speciality and there is increasing interest in the development of vaccines and in the therapeutic modulation of the immune system for the treatment of numerous cancer types to improve patient outcomes in the continued fight against cancer.

William Colley was the first to document that the stimulation of the immune system may be effective in treating cancer and for this reason he is known as the 'Father of Immunotherapy'.

1.2 Colorectal Cancer

Colorectal cancer constitutes a major health problem. It is the third most common cancer type in both men and women and the second most common cause of cancer death in the UK. Approximately 41,500 patients were diagnosed with and 16,000 died of colorectal cancer in the UK in 2012 [3].

The 5 year survival rate is currently in the region of 50% and has improved significantly since the 1970's when the 5 year survival was reported at approximately 20% [3]. This reflects improved diagnostic strategies, surgical techniques and neo-adjuvant and adjuvant therapies implemented in the management of colorectal cancer.

Colonic tumours are tumours of the large bowel. It is important to differentiate them from tumours of the rectum which are tumours arising within 15cm of the anal verge or from where the two anti-mesenteric taenia converge into an amorphous area [4].

These distinctions are important in planning treatment as rectal cancers should be considered for neo-adjuvant long-course chemo-radiotherapy or short-course radiotherapy in an attempt to downstage the disease and allow complete resection (and enable sphincter preserving surgery) in selected cases [5-7]. The differentiation also assists clinicians in predicting short-term and longer-term oncological outcomes as these differ between patients undergoing colonic (segmental excision) and rectal (including total meso-rectal excision) resections [5-7]. This results from different pre-operative treatments, intra-operative techniques, post-operative complications and recovery following surgery.

It is accepted that most colorectal cancers (adenocarcinomas) are sporadic and arise from pre-existing adenomatous polyps following a well-recognised adenoma-carcinoma sequence

[8]. This is a multi-step process and it implicates mutations in K-*ras* and p-*53* genes [8]. It has been suggested that different pathways to carcinogenesis exist as rarely do the mutations occur simultaneously [9]. Tumours with a K-*ras* mutation are associated with an advanced stage at presentation and a poor prognosis in node negative disease [10]. Therefore precise knowledge of the genetic abnormality that initiates carcinogenesis may have implications in diagnosis, treatment and prognosis.

The main environmental factors that predispose patients to colorectal cancer are obesity, lack of exercise, a low-fibre diet, consumption of red and processed meat, alcohol and tobacco smoking, long-standing inflammatory bowel disease and previous gastric surgery which induces altered bile acid metabolism [11].

Colorectal cancers can present in a symptomatic patient population with well recognised chronic symptoms or as an emergency. They can also present in an asymptomatic population through the NHS Bowel Cancer Screening Programme (BCSP). This is a screening programme gradually introduced nationally from 2006-2010, where men and women from 60-74 years are invited to conduct a faecal occult blood test every two years [12]. If the test is positive then individuals are invited to attend screening colonic investigation (usually colonoscopy). The purpose of the BCSP is to detect colorectal cancer at an early stage where treatment is more likely to be effective or to detect and remove adenomatous polyps and prevent their development into colorectal cancers [12]. Despite advances in the BCSP, in the UK, approximately 20% of patients present as an emergency to acute surgical care. The usual presentation is obstruction (16%) and less commonly bleeding or perforation [13].

Staging describes the severity of an individual's cancer with regard to the magnitude of the primary tumour and the extent that it has spread. Colorectal cancers are staged utilising the

TNM Classification. This is a staging system based on the extent of the tumour (T), the extent of the spread to the lymph nodes (N) and the presence of distant metastases (M). The TNM Classification reflects the American Joint Commission on Cancer Classification and Dukes' staging system as outlined below [14]. The systems are often used concomitantly.

Table 1.1 Tumour, Nodal, Metastatic (TNM) Staging System for Colorectal Cancer 5th Edition

T0	No evidence of primary tumour
T1	Tumour is confined to the submucosa
T2	Tumour has grown into (but not through) the muscularis propria
T3	Tumour has grown into (but not through) the serosa
T4	Tumour has penetrated through the serosa and the peritoneal surface
T4a	Tumour extends directly into other nearby structures
T4b	Tumour perforates the bowel
N0	No regional lymph node metastases
N1	1-3 regional lymph node metastases
N2	≥ 4 regional lymph node metastases
M0	No distant metastases
M1	Distant metastases

Colorectal cancers are staged utilising the TNM Classification. This is a staging system based on the extent of the tumour (T), the extent of the spread to the lymph nodes (N) and the presence of distant metastases (M). *Table adapted from: The diagnosis and management of colorectal cancer, National Institute for Health and Clinical Excellence, Clinical Guideline Number 131* [14].

Table 1.2 Comparisons between the Tumour, Nodal, Metastatic (TNM) Staging System for Colorectal Cancer, the American Joint Commission on Cancer (AJCC) Classification and Dukes' Stage for Colorectal Cancer

T	N	M	AJCC	Dukes'
T1	N0	M0	I	A
T2				
T3	N0	M0	II	B
T4				
Any T	N1	M0	III	C
	N2			
Any T	Any N	M1	IV	D

Table adapted from: The diagnosis and management of colorectal cancer, National Institute for Health and Clinical Excellence, Clinical Guideline Number 131 [14].

An accurate histopathological stage of the disease allows clinicians to accurately prognosticate and determine potential adjuvant treatment strategies that may be employed to improve patients overall survival. After curative resection the age-adjusted 5 year survival for Dukes' A, B and C cancer is 85%, 67% and 37% respectively [14]. Adjuvant therapy with 5-fluorouracil (5-FU) is offered to patients with Stage III or Dukes' C cancer where it confers a 5-10% improvement in absolute survival. 5-FU has more recently been combined with oxaplatinin and may be offered to patients with high-risk Stage II or Dukes' B cancer. This combination therapy is not employed routinely and is reserved for those patients who are deemed fit as it only provides a marginal benefit in overall survival [14].

1.3 Patient Outcomes following Colorectal Cancer Resection

The National Bowel Cancer Audit is an annual, national clinical audit commissioned by the Healthcare Quality Improvement Partnership (HQIP) as part of the National Clinical Audit and Patient Outcomes Programme (NCAPOP) delivered by the Health and Social Care Information Centre (HSCIC) [13]. The primary aim of the audit is to improve the quality of care and survival of patients with a diagnosis of Colorectal Cancer. All patients over the age of 18 with a diagnosis of Colorectal Cancer who are managed in the NHS are included in the audit. Data is collected about each patient, their cancer, treatment and outcomes. The performance of Strategic Clinical Networks and individual hospitals are compared. This enables identification of the most effective treatments which benefit patients most.

A description of the patients undergoing major resection for colorectal cancer from 1st April 2013 to 31st March 2014 in the United Kingdom is outlined below.

Table 1.3 A description of the patients undergoing major resection for colorectal cancer from 1st April 2013 to 31st March 2014 in the United Kingdom

		Number	%
Total Number of Patients		**19,445**	-
Gender	**Male**	11,087	57.1
	Female	8,340	42.9
Age	**≤ 65 years**	6,099	31.4
	65-74 years	6,218	32.0
	75-84 years	5,702	29.3
	≥85 years	1,426	7.3
Performance Status	**Normally Active**	6,417	47.5
	Walk and Light Work	4,684	34.7
	Walk and Self-care >50%	1,869	13.8
	Limited Self-care <50%	487	3.6
	Completely Disabled	59	0.4
Cancer Site	**Caecum / Ascending Colon**	5,477	28.2
	Hepatic Flexure	830	4.3
	Transverse Colon	1,266	6.5
	Splenic Flexure / Descending Colon	1,269	6.5
	Sigmoid Colon	4,573	23.5
	Recto-sigmoid	1,052	5.4
	Rectum	4,978	25.6
T-stage	**T0**	338	1.8
	T1	1,204	6.4
	T2	2,982	15.8
	T3	9,455	50.1
	T4	4,678	24.8
N-stage	**N0**	11,026	58.4
	N1	4,615	24.4
	N2	2,973	15.7
M-Stage	**M0**	14,708	77.9
	M1	1,672	8.9
Neo-Adjuvant Therapy	**Chemotherapy**	895	4.6
	Chemoradiotherapy	1,736	8.9
Adjuvant Therapy	**Chemotherapy**	4,951	25.5
	Chemoradiotherapy	455	2.3

Table adapted from: The National Bowel Cancer Audit, 2015 [13].

The use of laparoscopic surgery has increased from 30% in 2009-2010 to 48% in 2013-2014. The utilisation of this technique varies considerably between NHS Trusts. Less than 40% of patients underwent laparoscopic resections in 36 NHS Trusts compared with over 80% of patients in 23 NHS Trusts [13].

The surgery-associated mortality, length of stay and emergency readmissions from 1st April 2013 to 31st March 2014 in the United Kingdom are outlined below**.**

Table 1.4 Total number of patients with Colorectal Cancer and surgery-associated mortality from 1st April 2013 to 31st March 2014 in the United Kingdom

	Number	%
Total Patients	30,663	-
Patients Undergoing Major Surgical Resection	19,445	63.5
Mortality within 90-days	746	3.8
Mortality within 24-months	3,448	18.0

Table adapted from: The National Bowel Cancer Audit, 2015 [13].

Table 1.5 **Length of stay in days, percentage of patients with a length of stay greater than 5 days and percentage of patients with an unplanned emergency readmission for patients with Colorectal Cancer undergoing major surgical resection from 1st April 2013 to 31st March 2014 in the United Kingdom**

	Colon		Recto-sigmoid		Rectum	
Length of Stay in days (Median / IQR)	7	5-12	7	5-13	8	6-14
Length of Stay > 5 days (%)	65.0		67.8		79.0	
Emergency Readmission (%)	18.4		19.8		24.0	

Table adapted from: The National Bowel Cancer Audit, 2015 [13].

The 90 day mortality for patients undergoing surgical resection for Colorectal Cancer has fallen since 2008 and is currently at the lowest recorded rate with less than 4 patients out of every 100 dying. Interestingly there is a great variation in 90 day mortality depending upon presentation type. Patients presenting as an emergency have a 90 day mortality of 13.3% compared to only 2.2% of patients presenting electively. The 24 month mortality for patients undergoing surgical resection for Colorectal Cancer currently stands at 18%. In those patients with a diagnosis of Colorectal Cancer who do not undergo major resection the 24 month mortality is approximately 60%. This group comprises those patients who are too unwell or have cancer too advanced for surgical resection. Interestingly, and perhaps as a direct result of the Bowel Cancer Screening Programme and an earlier stage of disease at presentation, 744 patients underwent a local excisional procedure (Endoscopic polyp excision or Trans-anal Endoscopic Microsurgery) with a resultant 24 month mortality of less than 10% [13].

Discharge from hospital in 5 days or less is endorsed as a measure of 'good care'. Enhanced Recovery Programmes in Colorectal Surgery have been implemented in the UK since 2002. The Enhanced Recovery Programme is an evidence-based peri-operative care approach that promotes recovery after major surgery. It is used with laparoscopic or open surgery to optimise rehabilitation after surgery. The programme aims to modify many aspects of the surgical and anaesthetic intervention and recovery to facilitate optimal recovery [15]. Despite the compliance with multimodal rehabilitation, over two thirds of patients had a hospital length of stay greater than 5 days and this approached 80% in patients undergoing rectal cancer resection [13].

There are many reasons why patients have a prolonged hospital recovery and the length of hospital stay not only accounts for post-operative complications and a prolonged functional recovery but represents an additional patient need for assistance with stoma therapy, occupational therapy and physiotherapy in this specific patient group. As expected, increasing age has been shown to increase the length of hospital stay. Those patients over 85 years of age had a median length of stay of 10 days (IQR 7-18 days) compared to those less than 65 years of age who had a median length of stay of 7 days (IQR 4-10 days) [13]. Of the 10,919 patients undergoing surgery for rectal cancer (Anterior Resection, APER, Hartmann's Resection) over 80% of patients underwent stoma formation of which the majority were temporary ileostomies used to 'cover' the colorectal anastomosis of an Anterior Resection [13].

1.4 Cancer and Inflammation

It is increasingly appreciated that outcomes in patients with colorectal cancer are not determined by tumour characteristics alone [16]. Cancer-associated inflammation, both in the systemic circulation and in the tumour microenvironment, is now widely recognised to be a key determinant of disease progression and survival in colorectal cancer.

1.4.1 Systemic Inflammation and Cancer

The patients' Systemic Inflammatory Response (SIR) is recognised to influence patient outcomes. The patients' SIR can be assessed by examining changes in concentrations of circulating acute phase proteins, such as C-reactive protein (CRP), serum cytokines (TNF-α, IL-6, IL-8, IL-10) and low levels of circulating albumin [17-18]. Pre-operatively, these factors have been demonstrated to be stage-independent prognostic factors in many cancer types including colorectal cancer [19-24].

It has been demonstrated that a simple pre-operative objective scoring system, the modified Glasgow Prognostic Score (mGPS), which measures CRP and albumin, is effective in predicting overall and cancer-specific survival in a variety of solid organ malignancies including colorectal cancer [25]. A significant correlation exists between the mGPS and prognosis in colorectal cancer with higher scores indicating a poorer prognosis.

Table 1.6 Modified Glasgow Prognostic Score (McMillan, 2008)

Feature	Score
CRP ≤ 10 mg/L	**0**
CRP > 10 mg/L	**1**
CRP > 10 mg/L *AND* Albumin < 35 g/L	**2**

The Modified Glasgow Prognostic Score (mGPS) is a pre-operatively determined inflammation-based score. It is suggested that a significant correlation exists between the mGPS and prognosis in several cancer types. Higher scores indicate a poorer prognosis. *Table adapted from* [20].

Acute phase proteins are just one aspect of the SIR. The cellular components of the SIR, such a neutrophils, lymphocytes, monocytes and platelets have all been reported to have prognostic value in patients with colorectal cancer [26-30]. The SIR results in changes in circulating white blood cells and the neutrophil-lymphocyte ratio (NLR) has been used to predict overall and cancer specific survival in solid organ malignancies including colorectal cancer [31].

The systemic inflammation prognostic scores (mGPS and NLR) were compared in a large cross-sectional cohort study which revealed a reduced cancer specific survival independent of age, gender, deprivation and tumour site for both prognostic scores ($p<0.001$) [32]. The mGPS and NLR have also been compared longitudinally (not just pre-operatively) and it was demonstrated that post-operative assessment of the mGPS and NLR also had prognostic significance in patients with colorectal cancer [33]. This indicates that an elevated SIR, both

pre-operatively and post-operatively, is associated with poorer prognosis and there is undoubtedly a role for monitoring the SIR in patients who are undergoing colorectal cancer resection.

The mechanism of a persistently elevated SIR in patients with colorectal cancer who have undergone surgical resection is undetermined. It has been proposed that the pro-inflammatory cytokine interleukin-6 (IL-6), which is upregulated by tissue injury and inflammatory cells, has the ability to activate and maintain the SIR via trans-signalling pathways involving the soluble IL-6 receptor [34]. A chronic dysregulation of the immune system may also account for the persistently elevated SIR, either as a consequence of its activation by micro-metastases or as a result of disease which induces tissue injury [35,36], for example in the presence of septic complications.

It has been revealed that a post-operative anastomotic leak is associated with up to three times the risk of disease recurrence [37] and the development of any post-operative septic complication (surgical site infection, respiratory tract infection, urinary tract infection) further augments this risk [38]. Interestingly it has been found that pre-operative SIR is independently associated with the risk of developing infective post-operative complications in patients undergoing curative colorectal cancer resection [39].

1.4.2 Peri-tumoural Inflammation and The Tumour Microenvironment

The prognostic value of a patient's SIR to a tumour is well established as a negative prognostic factor in primary operable [40] and metastatic colorectal cancer [16, 41-44]. The peri-tumoural, or local, inflammatory response has also provoked considerable interest. An inflammatory reaction in and around tumours is thought to represent an in-situ immune

response to the tumour by the host [45]. In contrast to the SIR, local infiltration of inflammatory cells in the tumour microenvironment is associated with improved survival in patients with cancer [45, 46]. There is great interest in establishing the cellular composition of immune cells infiltrating colorectal cancers and determining their prognostic value.

Whilst the prognostic value of a generalised peri-tumoural response is associated with improved survival it is apparent that specific immune cell types relate more closely to survival, particularly cytotoxic T-cells (CD8+), memory T-cells (CD45RO+) and regulatory T-cells (FOXP3+) [47]. This indicates that the adaptive anti-tumour immune response plays a key role in determining cancer progression.

The type, density and location of the tumour inflammatory cell infiltrate appear especially relevant to patient outcomes, but as yet, there is uncertainty regarding which parameters of tumour inflammatory cell infiltrate are most closely related to survival. In patients with primary, operable colorectal cancer a strong infiltration of inflammatory cells in the invasive margin conferred a marked survival advantage [46]. Particular survival benefit has been attributed to high numbers of lymphocytes and plasma cells [46] and it has been suggested that a co-ordinated inflammatory response at a local level, in the tumour microenvironment, mediated primarily through cells of the adaptive immune system are responsible [46]. Interestingly, the presence of peri-tumoural lymphocytes has been associated with favourable tumour characteristics such as an expanding, rather than infiltrative, growth pattern [48] and lower levels of venous invasion [46, 49].

Evidence regarding the prognostic significance of the innate immune response in the tumour microenvironment is contradictory. Inflammatory cell types associated with the innate immune response, namely neutrophils, macrophages and dendritic cells appear to

have a role in predicting outcomes. Increased neutrophil infiltration has been associated with improved survival [50-52] and with reduced local recurrence without a survival advantage [53] along with other immune cells (lymphocytes, eosinophils, mast cells). It has been reported that increased neutrophils at the invasive margin of the tumour is an important prognostic factor, but the positive prognostic value of multiple other cell types was also concurrently described [51]. This suggests that the generalised, non-specific immune reaction is important [45].

Although a number of studies have described that a strong infiltration of neutrophils [52] and macrophages [54] confer a survival benefit in patients with colorectal cancer, other studies have demonstrated no such association [53, 55]. It has been proposed that tumours may even exploit the innate immune system to promote tumour proliferation and invasion [56].

1.4.3 Peri-operative Immune Insult in Cancer Surgery

High grade peri-tumoural infiltrates are composed of a co-ordinated adaptive and innate immune response [57]. The maintenance of such co-ordination in the tumour micro-environment is associated with improved outcomes [58]. Cancer surgery elicits a high-grade, non-specific SIR. This overwhelming, systemic inflammation suppresses systemic cell-mediated immunity and consequently compromises the tumour immunity in the host [58]. This has been described as the 'immune-hit' [58, 59].

The immune-hit can be exacerbated by pre-operative SIR, for example, an emergency presentation with colonic obstruction, bleeding or perforation, confers a higher risk of disease recurrence independent of the stage of disease [40]. Additionally, the development

of post-operative complications and the associated post-operative SIR increases the risk of disease recurrence [38]. It is therefore appreciated that major surgical resection for colorectal cancer presenting as an emergency or in the presence of a post-operative infective complication results in further compromise of the immune response to residual disease [40, 58]. This is consistent with the negative prognosis associated with the presence of SIR in patients affected by most tumour types at any stage of disease [40].

The intricate balance of anti-tumour and pro-tumour responses of tumour and systemic immunity during the peri-operative period is outlined below.

Table 1.7 The balance of anti-tumour and pro-tumour responses of tumour and systemic immunity during the peri-operative period

Tumour Immunity	Systemic Immunity
Anti-tumour	**Pre-operative State**
High Density Adaptive Immune Responses	Inflammation Suppression
CD3+ / CD8+ T-cell infiltrates	Intact Cell-mediated Immunity
Pro-tumour	**Post-operative State**
Absent T-cell Responses	Systemic Inflammatory Response
Myeloid Derived Suppressor Cells	Innate Response Upregulation
T-cell Suppressors	Pro-inflammatory Cytokines
Chronic Inflammation	Humoral Changes

High grade peri-tumoural infiltrates are composed of a co-ordinated adaptive and innate immune response. The maintenance of such co-ordination in the tumour micro-environment is associated with improved outcomes. Cancer surgery elicits a high-grade, non-specific SIR. This overwhelming, systemic inflammation suppresses systemic cell-mediated immunity and consequently compromises the tumour immunity in the host. This has been described as the 'immune-hit'. *Table adapted from* [58].

As a consequence of this theory there has been an increased interest in the development of immune-modulatory therapies that might be implemented in the peri-operative period. The goal of such a therapy is to suppress non-specific systemic inflammation and maintain an effective anti-tumour, cell-mediated immunity of the host [58]. The implementation of effective peri-operative immune-modulatory therapy may assist the clearance of circulating tumour cells and the development of occult metastases. Accumulating evidence suggests that simple immune-modulatory strategies (anti-inflammatories or immune-modulatory therapies) could be safely implemented in the peri-operative period [60]. These strategies may be of particular benefit in those patients presenting as an emergency or those deemed high-risk of developing post-operative infective complications.

The interaction between tumour immunity and systemic immunity is likely to be complex and the delivery of appropriate immune-modulatory therapies to modify the peri-operative inflammatory response could be utilised to preserve immune competency in the host.

1.5 The Neutrophil

Neutrophils are the most abundant polymorphonuclear (PMN) cell and contribute up to 70% of the circulating leucocytes in health [61-63]. Neutrophils undergo six distinct morphological stages in their maturation. They ultimately differentiate into mature segmented neutrophils which have a characteristic multi-lobar nucleus. They are released from the bone marrow as terminally differentiated cells and have a short circulating half-life of approximately 6-8 hours [61].

Neutrophils are an essential part of the innate immune system. They function as the first-line of defence against infections and are responsible for the containment and elimination of pathogens. They are prevalent at sites of tissue trauma and are the hallmark of acute inflammation [61]. Neutrophils are also appreciated to have an important role in cancer progression [64].

Neutrophils have a variety of specific of functions. In health, circulating neutrophils adhere to the endothelium before transmigrating through it in a process termed chemotaxis. At the site of inflammation neutrophils kill invading pathogens by phagocytosis, extracellular ROS release and by producing neutrophil extracellular traps (NETs) [65].

Activated neutrophils produce cytokines which attract other immune cells and they are therefore responsible for modulating the inflammatory response. After they have performed their function they subsequently undergo apoptosis (programmed cell death), an important process that contributes to the resolution of the inflammation [66].

1.5.1 Neutrophil Functions

Neutrophils were discovered at the origin of the immunological sciences and therefore the elucidation of their role in the immune response has been an ongoing process for over a century [67]. Neutrophils are not only 'professional killers' of pathogens but are also instructors of the immune system in the context of infection and inflammation. They are of vital importance in the innate immune system and are fundamental in immune function. Despite their importance in immunity, neutrophil research has been hindered by their experimental intractability (short-life span, terminally differentiated) and consequently they have been overlooked by breakthroughs in the adaptive immune system [68].

Within the bone marrow, under the instruction of growth factors and cytokines, pluripotent haemopoietic cells differentiate into myeloblasts which are developmental cells committed to becoming granulocytes. They pass through six distinct morphological stages in their maturation into mature segmented neutrophils which are characterised by their multi-lobar nucleus; Myeloblast, Promyelocyte, Myelocyte, Metamyelocyte, Immature Band Cell and Mature Segmented Neutrophil [67].

As the precursor cells develop into neutrophils, they synthesise proteins that are sorted into different granules [69]. These granules are subdivided into three classes, azurophilic, specific and gelatinase granules. The subdivision is generally artificial as the neutrophil alters its transcriptional profile during its maturation and this changes the granule content resulting in a continuum of granule species [70].

Chemokines control the release of neutrophils into the circulation and cells are stored for release when required. Neutrophils patrol the systemic circulation and search for

indications of developing inflammation where they then perform a multitude of cellular tasks [67].

Recent evidence suggests a wider functional diversity of neutrophils than previously appreciated, expanding their role in adaptive immunity. The concept of neutrophil heterogeneity has emerged with accumulating evidence of neutrophil populations with distinct functions under both homeostatic and pathological conditions. Cell surface markers, cell maturity and cellular functions have been used to identify neutrophil 'subsets'. Novel neutrophil populations have been identified during infection, autoimmunity, cancer, cardiovascular disease and pregnancy [302]. It is not yet understood if distinct neutrophil subsets are derived from separate lineages or if they represent different activation or polarisation states from a common plastic neutrophil precursor.

1.5.1.1 Neutrophil Activation

At sites of inflammation, either bacterial-derived or host-produced, there is an abundance of inflammatory signals. The bacterial derived LPS and fMLP and classical chemoattractants and cytokines, such as TNF-α, IL-1β and IL-17 stimulate endothelial cells to produce adhesion molecules. The adhesion molecules include P-selectin, E-selectin and members of the integrin superfamily [69]. Circulating neutrophils encounter the stimulated endothelial cells and utilising two constitutively expressed proteins (P-selectin glycoprotein ligand-1 and L-selectin) they can engage with the adhesion molecules of the endothelial cells resulting in selectin-mediated tethering, or 'capture', to the vessel wall [71, 72].

The neutrophils then perform characteristic, selectin-mediated, 'rolling' along the endothelial surface. During this process the complex activation cascade begins involving Src

kinases, Syk kinases, phosphoinositide 3-kinase and p38 mitogen-activated protein kinase [73-75]. The neutrophil can then enter its 'firm adhesion' state which is mediated by the β2 integrin family of proteins (LAF-1 and Mac-1) [75, 76] which engage with endothelial ligands, namely the ICAM-1 immunoglobulin superfamily [77]. The neutrophil then 'transmigrates' through the endothelium utilising a complex interaction between neutrophil integrins, neutrophil surface proteins and endothelial junction molecules [75]. It is speculated that the protein mesh of the basement membrane is digested by neutrophil granule proteases [67].

When the neutrophil has traversed the endothelial barrier its behaviour is determined by the chemoattractants and inflammatory stimulants from both the pathogen (LPS, fMLP) and the host (IL-8). The neutrophil follows chemotactic gradients towards invading microbes, stimulates the oxidative burst and begins to implement its regime of pathogen elimination via degranulation, phagocytosis and NETosis (the process of NET formation) [67].

Neutrophil activation can occur as a consequence of the recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors, but also in response to self-derived molecules that are either altered or relocated from their normal cellular compartment. Self-derived molecules from damaged tissue can therefore initiate neutrophil activation and the innate immune response. Damage-associated molecular patterns (DAMPs) are usually hidden from the innate immune system, but they are actively or passively released following cell damage which initiates an inflammatory response. External cell components can also act as DAMPs following chemical or proteolytic modification of their structures allowing detection by inflammatory cells [303, 304]. The detection of DAMPs occurs via pattern-recognition receptors indicating similar functionality

of DAMPs and PAMPs. For example, Toll-like Receptor (TLR) 4 binds self-molecules high mobility group box-1 (HMGB1) and heat shock proteins, but also binds bacterial derived LPS [305]. Irrespective of the source of the ligand the pattern-recognition receptors initiate an equal response. Other DAMPs include; S100 proteins, endogenous nucleic acids (RNA and DNA), altered extracellular matrix components (hyaluronan), mitochondrial DNA, mitochondrial formylated peptides and extracellular ATP [303, 304]. DAMPs are implicated in 'sterile injury', either by directly activating neutrophils or by facilitating neutrophil recruitment by altering the microenvironment following their detection by resident cells. Parallel signalling pathways are stimulated by PAMPs and DAMPs and this explains the similarities between microbial sepsis and the SIR associated with traumatic (sterile) injury.

1.5.1.2 Elimination of Microbes

The neutrophil is equipped with an array of microbial elimination techniques which reflects the neutrophil's attempt to exploit the weakness of microbes during the course of infection.

Degranulation

The neutrophil has developed a specialty storage organelle designed to transport dangerous substances and deploy them at the appropriate time. This is called the neutrophil granule and it is active in almost all neutrophil activities. Granules are subdivided into three classes, azurophilic, specific and gelatinase granules.

1. Azurophilic granules are named for their ability to take-up the dye Azure A and contain numerous enzymes and proteins including myeloperoxidase (MPO), an enzyme which is critical for the oxidative burst [78, 79].

2. Specific granules are characterised by the presence of glycoprotein and lactoferrin and contain many anti-microbial compounds including, NGAL, hCAP-18 and lysozyme [79, 80].
3. Gelatinase granules serve as a storage location for metalloproteases, including gelatinase and leukolysin [69].

Neutrophil secretory vesicles are often thought of as granules, but they are formed by endocytosis rather than budding from the Golgi Apparatus as are the granules.

The granule subdivisions have differing propensities for mobilisation and are associated with a particular stage of neutrophil activation. The gelatinase granules are mobilised as the neutrophils move through the endothelium [81, 82]. At the site of inflammation the specific and azurophilic granules are mobilised and contribute to the oxidative burst reaction. Flavocytochrome b558, which is found in the specific granule, is a key component of the NADPH oxidase machinery and subsequently allows reactive oxygen species formation [83].

Degranulation of specific and azurophilic granules also contribute to the creation of an inhospitable environment to invading pathogens [67].

Anti-microbial Proteins

Neutrophils produce a vast array of anti-microbial proteins which can eliminate microbes directly or indirectly. They can be broadly grouped into cationic peptides, proteolytic enzymes and proteins that deprive microbes of essential nutrients.

The neutrophil cationic peptides include defensins and cathelicidins which are thought to have roles in inhibition of bacterial cell wall synthesis [84] and in the DNA activation of dendritic cells [85] respectively. They also include BPI which binds LPS resulting in increased

bacterial permeability, bacterial hydrolysis and cell death [86] and histones which perform antimicrobial role by an unknown mechanism.

The neutrophil proteolytic enzymes contribute to microbial elimination by various mechanisms. Lysozymes degrade bacterial cell walls [87], proteinase-3 and azurocidin bind to the bacterial membrane, neutrophil elastase and cathepsin G cleave bacterial virulence factors and outer membrane proteins [88].

Several proteins produced by neutrophils deprive microbes of essential metals and subsequently impact on bacterial growth. Lactoferrin binds preferentially to iron and calprotectin sequesters zinc and results in 'nutritional immunity' [89].

Reactive Oxygen Species

Neutrophils kill micro-organisms by ingesting them into phagosomes. Phagocytosis is accompanied by activation of the NADPH oxidase, an enzyme complex that assembles in the phagosomal membrane (internalised neutrophil membrane) and converts oxygen into the superoxide radical anion ($O_2^{\bullet -}$) [67, 90]. $O_2^{\bullet -}$ undergoes dismutation, catalysed by superoxide dismutase (SOD), inside the phagosome to produce hydrogen peroxide (H_2O_2). Simultaneously, myeloperoxidase (MPO) is released into the phagosome which catalyses the formation of hypochlorous acid (HOCl) from chloride and H_2O_2. HOCl is considered the major oxidative weapon of the neutrophil. The build-up of high concentrations of H_2O_2 may kill micro-organisms directly or by formation of hydroxyl radicals and singlet oxygen in secondary reactions of HOCl [67, 90].

Phagocytosis

Phagocytosis is an active, receptor-mediated process used to remove pathogens and cell debris. Particles are internalised by the cell membrane into a vacuole called the phagosome [67].

Phagocytosis can be direct, through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors or opsonin mediated. Opsonin mediated phagocytosis includes FcϒR mediated phagocytosis, which relies on pseudopod extensions for engulfment of IgG-opsonised particles and complement receptor mediated phagocytosis, which does not require pseudopod extensions [91].

Our understanding of neutrophil phagocytosis is principally based upon the study of macrophage phagocytosis, however crucial differences between macrophage and neutrophil phagocytosis exist. Macrophage phagocytosis follows an endocytic maturation pathway whereas neutrophil phagosome maturation occurs upon fusion of the granules to the phagosome when antimicrobial molecules are delivered into the phagosome. In the neutrophil the assembly of the NADPH oxidase on the phagosome membrane allows ROS production and this creates an antimicrobial environment within the phagosome. NADPH oxidase activity also maintains an alkaline pH which is essential for the function of the proteolytic enzymes, neutrophil elastase and cathepsin G [92].

Neutrophil Extracellular Traps

Activated neutrophils can undergo 'NETosis'. This is an active form of cell death that leads to the release of decondensed chromatin into the extracellular space [93, 94]. The fibrous structures called NETs constitute a DNA backbone containing histones and neutrophil

granule products (MPO) and cytoplasmic proteins (BPI, neutrophil elastase, cathepsin G and lactoferrin) [95]. NETs trap many types of microbes and their anti-microbial properties are thought to arise from exposing them to high local concentrations of anti-microbials [96].

The exact mechanism for NET formation is not yet completely understood. It is thought that the ROS pathway is involved as both NADPH oxidase and MPO are required for NET formation [94, 97, 98]. In this oxidant dependent pathway the formation of NETs follows the sequence of loss of nuclear segregation and the fragmentation of the lobar nucleus, disintegration of the nuclear membrane into vesicles and the disappearance of granules. This allows the nuclear chromatin to combine with the granule products and cytoplasmic proteins. The cell membrane eventually ruptures and the NETs are released. The process of oxidant dependent NET formation results in eventual cell death and is distinct from apoptosis and necrosis being termed NETosis [94, 99].

The Raf-MEK-ERK pathway has also been implicated in NET formation [100] which occurs upstream of NADPH oxidase. Neutrophil elastase translocates from the granules to the nucleus and degrades histones leading to chromatin decondensation and consequently has been implicated in NETosis [101]. It is thought that histone citrullination may also play a role in NET formation. This process is catalysed by the enzyme peptidylarginine deiminase 4 (PAD-4), which is abundant in neutrophils and converts histone arginine to citrulline. This is a vital step in the decondensation of DNA [102-104].

Other pathways of NET formation have been described. The oxidant independent pathway results in a rapid NET formation and does not result in cell death, allowing activated neutrophils to perform their numerous anti-microbial actions. Neutrophils incubated with *S.Aureus* were demonstrated to condense their chromatin into discrete vesicles, which then

passed through the cytoplasm and acquired granule products. The vesicles were then exocytosed and the chromatin released from the neutrophil in a NET. This process was not dependent upon ROS production [105].

Although controversial, the oxidant dependent release of mitochondrial DNA in response to granulocyte macrophage colony stimulating factor (GM-CSF) and Complement factor 5a (C5a) has been described. This process is dependent on ROS production. It does not induce cell death and thus the neutrophil is able to conduct its other anti-microbial actions [62]. This pathway remains contentious as the detection of mitochondrial NETs may actually be a consequence of cell lysis.

As yet the clinical relevance of NETs is undetermined. Bacteria that express DNases may disseminate more efficiently in the host and thereby entrapment by NETs should be avoided [106, 107]. Conversely, NETs may be particularly important in the entrapment of large pathogens that are not readily phagocytosed [67].

NETs may also have detrimental effects on the host by inducing autoimmune disease. This occurs by the exposure of 'self' molecules extracellularly leading to the formation of auto-antibodies against chromatin and neutrophil components [108]. Platelet-induced NETs have been linked with hepatotoxicity in sepsis [109] and platelet adherence to NETs implicates them in thromboembolic disease [110].

1.5.1.3 Communication with Other Immune Cells

Neutrophils are one of the first cell types to arrive at sites of infection or inflammation and they have a vital role in communicating with other immune cells. Neutrophils secrete cytokines and chemokines critical to the development of the inflammatory response and

thus establish the correct environmental conditions for the adaptive immune system. Neutrophils undergo a transcriptional burst which facilitates the synthesis of signalling molecules [111, 112].

The initial neutrophil cytokine response is for re-enforcement via the production of IL-8 [113]. Neutrophil derived IL-1β and TNF-α induce other cells to produce chemoattractants. Neutrophils also release other signalling mediators which include, granule products, lipids and ROS products, such as hydrogen peroxide [114-116] and they can also communicate by cell to cell contact [117].

Neutrophils have roles in recruiting monocytes and macrophages and can modulate monocyte and macrophage cytokine production [118]. Neutrophils also recruit and activate dendritic cells and induce their maturation [117]. It has also been proposed that neutrophils activate natural killer cells directly or in combination with dendritic cells in a positive feedback loop [119].

Amazingly, neutrophils have been found to extensively communicate with lymphocytes at opposite ends of the immune spectrum and it has been shown that neutrophils and T-cells impact upon each other's functions [120]. Neutrophils have suppressive effects on T-cells via L-arganine depletion by the release of arginase which inhibits T-cell responses [121] and by hydrogen peroxide mediated suppression, as proposed in a cancer model [116]. Neutrophil modulation of the adaptive immune system appears to be extremely complex and as yet relatively little is known in this field.

1.5.1.4 Resolution of Inflammation

Apoptosis is a key component of inflammation resolution. After conducting their anti-microbial activities, neutrophils die by the process of apoptosis, or programmed cell death. The process of neutrophil apoptosis also produces signals to abolish the recruitment of further neutrophils indicating a resolution of inflammation. Neutrophil survival is prolonged by inflammatory mediators (GM-CSF, LPS) and by environmental factors such as hypoxia. It is well established that signalling networks regulate cell survival and can be either anti-apoptotic which favours cell survival (Mcl-1 and A-10) or pro-apoptotic which favours cell death (Bad, Bax, Bak, Bid and caspases) [122].

Several proteins are proposed to have anti-apoptotic effects. Survivin is one such protein which is highly expressed in immature neutrophils, but its expression is restored in mature cells in the presence of inflammatory mediators (G-CSF, GM-CSF) and is subsequently identified at sites of inflammation [123]. Likewise, cyclin-dependent kinases function as anti-apoptotic factors in neutrophils and their inhibition induces caspase-dependent apoptosis [124].

In the resolution of inflammation it is vitally important that the apoptotic cells are appropriately cleared. Apoptotic neutrophils are ingested by macrophages which in turn drives the production of anti-inflammatory cytokines, tumour growth factor-β (TGF-β) and IL-10 [123]. If apoptotic cells are not cleared then the resultant secondary necrosis re-establishes pro-inflammatory signals.

In the resolution of inflammation a major lipid mediator class shift is also apparent. The progression of inflammation depends upon the composition of lipids secreted by the

neutrophil. At the commencement of inflammation the neutrophil produces pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes. Conversely, at the termination of inflammation, neutrophils interact with many cell types (epithelial cells, endothelial cells, fibroblasts, platelets and leukocytes) and together they synthesise anti-inflammatory lipid mediators, such as lipoxins, resolvins and protectins [115].

1.5.2 Distinct Functions of Neutrophils in Cancer

Chronic inflammation has been associated with an increased susceptibility to the development of cancer, for example, inflammatory bowel disease and colorectal cancer [125] and Hepatitis B and hepatocellular carcinoma [126]. Neutrophils have been implicated in inflammation driven tumourogenesis but have also been found to limit tumour growth and metastatic progression which suggests that neutrophils have both pro-tumour and anti-tumour properties [127].

It has been suggested that neutrophils are not a homogenous population of cells and may consist of pro-tumour and anti-tumour subpopulations. The 'polarisation' of neutrophils towards a pro-tumour or anti-tumour phenotype may be mediated by the 'chemokine landscape' in the tumour microenvironment [128].

In the context of cancer, interferon-β (INF-β) demonstrates anti-tumour properties and is responsible for the inhibition of tumour cell proliferation and the promotion of tumour cell apoptosis [129]. INF-β is also responsible for initiating an anti-tumour immune response through the activation of T-cells, natural killer cells and macrophages [130]. With regard to neutrophils, INF-β appears to suppress the expression of pro-angiogenic factors (VEGF,

MMP9). This results in the suppression of tumour vascularisation [131] and inhibition of the formation of a pre-metastatic niche [132].

Conversely, tumour growth factor-β (TGF-β) appears to promote an immunosuppressive tumour microenvironment with a particular role in the suppression of anti-tumour neutrophil cytotoxicity. It has been demonstrated that in the absence of TGF-β the cellular composition of the tumour changes and neutrophils recruited to tumours have an anti-tumour phenotype (N1) indicating that TGF-β polarises neutrophils towards a pro-tumour phenotype (N2) [133-135].

Anti-tumour (N1) Phenotype

Anti-tumour (N1) neutrophils act to limit tumour growth and metastatic progression [127] through various mechanisms.

1. Direct Cytotoxicity: Neutrophils generate a wide variety of antimicrobial proteins. Although these are mostly non-toxic to host cells they have been found to be directly involved in anti-tumour neutrophil cytotoxicity, in particular the superoxides associated with NADPH oxidase [136-138].
2. Antibody Dependent Cell-mediated Cytotoxicity: Neutrophils express numerous receptors that can target antibody labelled tumour cells and it has been established that they participate in antibody dependent cell-mediated cytotoxicity in various cancer types [127].
3. Stimulation of T-cells: Neutrophils are required to initiate adaptive anti-tumour immunity. They have a role in the recruitment, proliferation and activation of T-

cells. They have also been implicated in the presentation of antigens to directly stimulate T-cell responses [139].

4. Neutrophil Extracellular Traps (NETs): It has been proposed that NETs are involved in cancer immunoediting. Their exact role, however is yet to be determined. NETs have been implicated in T-cell priming [140] and in the propagation of anti-tumour immune responses [141]. Conversely, they have also been linked to cancer progression, development of metastases and cancer-associated thrombosis.

Pro-tumour (N2) Phenotype

In the context of cancer, neutrophil function can be modified to promote tumour growth and the formation of metastases. The mechanisms of pro-tumour (N2) neutrophils are outlined below.

1. Pro-tumour Cytokines: Depending upon the 'cytokine landscape' in the tumour microenvironment neutrophils are capable of producing multiple growth factors. One such growth factor is EGF and it, along with its receptor EGFR, has been implicated in the pathogenesis and progression of multiple cancer types, including colorectal cancer [142]. It has also been demonstrated that amplifications of the EGFR occur in patients with cancer and it is also associated with angiogenesis, increased tumour proliferation, anti-apoptosis and enhanced tumour cell motility [143]. Another important neutrophil derived growth factor is platelet derived growth factor (PDGF) which has been implicated in the growth and metastasis of epithelial tumours [142]. Furthermore, neutrophils secrete other cytokines which effect tumour development. Neutrophil derived TNF-α, IL-6 and IL-17 have been

implicated in the promotion of tumour growth by modifying peri-tumoural stromal cell function and are involved in angiogenesis and anti-apoptosis [144-149].

2. Angiogenesis: It has not yet been elucidated how tumour associated neutrophils modulate tumour angiogenesis, but it has been proposed that they are involved in the process by remodelling the extracellular matrix, a process crucial for angiogenesis, under the influence of VEGF. The activated neutrophils have been shown to highly express VEGF and MMP9 which are responsible for the initiation of the 'angiogenic switch' which is necessary to support angiogenesis in tumours [131].
3. Tumour Cell Dissemination: Neutrophils can secrete soluble proteases and cytokines that can activate endothelial and parenchymal cells and it has been suggested that this improves adhesion of circulating tumour cells to distant sites [150-153] and a pro-metastatic state. It is thought that neutrophils may also tether circulating tumour cells to distant endothelial sites (liver and lung) mediated by the β2 integrin family of proteins which engage with endothelial ligands, namely the ICAM-1, on the tumour cells [153-155].
4. Neutrophil Extracellular Traps (NETs): It has been demonstrated that NET formation occurs within primary tumours and is associated with adverse clinical outcomes [141]. It has been proposed that NETs may act within the primary tumour and promote tumour progression, although the antimicrobial proteins and peptides associated with NETs, including MMP9, neutrophil elastase and cathepsin G, have been implicated in tumour progression without specific reference to NETs [156]. Therefore it is theorised that NETs expose tumour cells to high concentrations of biologically active proteins which favour tumour proliferation, anti-apoptosis and support the dissemination from the primary tumour. Recent studies have also shown

that NETs can sequester tumour cells and promote early adhesion of the tumour to distant metastatic sites. Following the dissemination, tumour cells must be able to proliferate to form stable metastatic foci. NETs may play a direct proliferative role and may also inhibit tumour cell apoptosis [157]. NETs have also been implicated as potential facilitators of tumour progression in the context of post-operative sepsis. This is of particular interest as the majority of patients diagnosed with cancer undergo at least one surgical procedure and post-operative infections occur with an incidence approaching 40% in some series [158].

5. Pre-metastatic Niche: Neutrophils are mobilised and accumulate in 'future sites' of metastases where they partake in the formation of a supportive metastatic microenvironment which is called the pre-metastatic niche [159-161]. Here neutrophils produce molecules that are capable of influencing tumour cell proliferation, survival and migration [162-163].
6. Suppression of T-cells: As previously stated neutrophils can present antigens to T-cells to stimulate their anti-tumour immunity, conversely, it has been demonstrated that neutrophils can suppress antigen-nonspecific T-cell proliferation [164, 165]. This has been attributed to granulocytic myeloid derived suppressor cells and immature neutrophils [166-168].

Neutrophil heterogeneity is a consequence of the remarkable ability of the neutrophil to respond to changes in the chemokine landscape of the tumour microenvironment. The distinct neutrophil phenotypes described, anti-tumour (N1) and pro-tumour (N2), suggests that neutrophils differ in their contribution to the progression and dissemination of cancer

and are capable of phenotypic plasticity. This opens up the possibility of therapeutic intervention to enhance anti-tumour and suppress pro-tumour properties.

Table 1.8 Characteristics of Anti-tumour (N1) and Pro-tumour (N2) Neutrophil Phenotypes

Anti-tumour (N1) Phenotype	Pro-tumour (N2) Phenotype
Mature	Immature
Cytotoxic	Carcinogenic
Pro-apoptotic	Anti-apoptotic
Anti-angiogenic	Pro-angiogenic
Stimulatory for T-cells	Suppressive for T-cells
Immuno-active	Immuno-suppressive

The polarisation of neutrophils towards an anti-tumour (N1) phenotype or a pro-tumour (N2) phenotype may be mediated by the chemokine landscape in the tumour microenvironment and results in neutrophils with distinct characteristics. *Table adapted from* [127].

1.5.3 Neutrophil Extracellular Traps in Cancer Progression

Neutrophils have been increasingly recognised as important players in the tumour microenvironment and both anti-tumour and pro-tumour properties have been demonstrated. Neutrophils appear to adopt phenotypic plasticity and a heterogeneous population of neutrophils can exert contradictory roles in the context of cancer [127].

NETs have been recognised as an effective antimicrobial mechanism in response to various stimuli. They appear to have an extended role in tumour biology and have been implicated in tumour progression, tumour dissemination and in tumour-associated thrombosis [156].

Exposure of primed neutrophils to bacterial products (LPS, fMLP, PMA) results in NET formation as well as physical interaction with activated platelets, which is of particular relevance in the context of cancer [93, 169-172]. Additionally, cytokines (TNF-α and IL-8) have been shown to facilitate NET formation and these cytokines are highly expressed by a number of tumour types [173-175].

Neutrophil Extracellular Traps and the Primary Tumour

The role of NETs in tumour progression is poorly understood but the evidence to date does propose an association between intra-tumoural NET deposition and tumour progression in both experimental models and in patients with cancer [157,176, 177].

The ability of tumours to predispose neutrophils to produce NETs has been demonstrated and various tumour types have predisposed circulating neutrophils to produce NETs [178]. The evidence supports the theory that primary tumours can facilitate NET production in circulating neutrophils. This effect has been attributed, in part, to G-CSF production by tumours [178].

In the setting of lymphoma, NET formation within the primary tumour has been shown to enhance B-cell proliferation. It has therefore been proposed that NETs directly influence tumour cell behaviour and promote tumour growth [157].

It is believed that NETs operate within the primary tumour to promote tumour progression, but no mechanism has been outlined for this observation.

Neutrophil Extracellular Traps and Metastatic Progression

Neutrophils play a vital role in circulating tumour cell metastases. This has been shown in-vitro and in-vivo where neutrophils facilitate circulating tumour cell adhesion to both pulmonary and hepatic endothelial surfaces [174, 176, 179-181]. The direct contact of circulating tumour cells and neutrophils is an important precursor to the development of metastatic disease [179, 180]

In the context of systemic sepsis, widespread NET deposition occurs within the microvasculature of a host organ (liver, lung) and promotes adhesion of circulating tumour cells. It has been revealed that these NETs can sequester circulating tumour cells and promote metastases [158]. Trapped circulating tumour cells can not only survive interactions with NETs but NETs may also have a direct proliferative role and anti-apoptotic role on the tumour cells and therefore may promote the development of macrometastases [157].

The literature supports a pro-metastatic role of NETs and it is suggested that they may promote early adhesive events, thus increasing sequestration of malignant cells in end organs. NETS may also be responsible for promoting a pro-tumour phenotype in neoplastic cells as a consequence of their interaction.

Neutrophil Extracellular Traps in the context of Cancer Surgery

Loco-regional control in the form of a complete oncological resection remains the mainstay of treatment for the majority of solid tumours and provides improved disease-free and overall survival [182]. Unfortunately, surgery can lead to increased number of circulating tumour cells [183] and it is associated with complications, particularly post-operative infectious complications which can approach 40% in some series [158]. It is well established that infectious complications in patients with cancer are associated with adverse oncological outcomes independent of the morbidity associated with the infectious insult [184-187] and they are associated with an increased mortality as a consequence of metastatic disease [187-191].

As a consequence of the association of severe infection and tumour progression experimental models (murine caecal ligation and puncture model) were undertaken to test the hypothesis that NETs could trap circulating tumour cells, promote early adhesion of tumour cells to distant sites and facilitate metastatic disease progression. It was demonstrated that sepsis induced by a caecal ligation and puncture model resulted in widespread deposition of NETs and that systemic sepsis promoted the development of gross metastases (by increased tumour cell adhesion to hepatic and pulmonary microvasculature). This was attenuated by the administration of inhibitors of NET formation (DNase, neutrophil elastase inhibitor). It was also revealed that tumour cells were trapped within NETs and this was associated with the development of metastases [158].

Interestingly, in this experimental model NET formation in-vivo was inhibited by the exogenous administration of DNase and neutrophil elastase inhibitor with favourable oncological outcomes (attenuation of liver metastatic disease burden) [158]. This raises the

possibility that mitigating the adverse oncological outcomes associated with severe infection in patients with cancer is theoretically possible.

Clinical and experimental evidence suggests that activated neutrophils may facilitate metastatic progression in the context of systemic infection [192-195]. Therapeutic strategies aimed at modifying tumour-neutrophil interactions may therefore maximise the therapeutic benefit of surgery in the context of cancer.

Neutrophil Extracellular Traps as Therapeutic Targets

It appears logical to attempt to suppress NET formation and prevent the mechanism that may predispose tumour progression and dissemination.

As previously mentioned, in an experimental model, NET formation is inhibited by the exogenous administration of DNase and neutrophil elastase inhibitor with favourable oncological outcomes.

DNase has been used in the treatment of empyema, cystic fibrosis and systemic lupus erythematous (SLE) in humans without adverse effects [196]. No human cancer trials investigating the effect of DNase have been performed to date, however, a number of animal studies have been conducted which fascinatingly demonstrate an anti-metastatic effect in a variety of tumour models [197, 198].

It is known that excessive neutrophil elastase function is associated with NET formation and that this is implicated in tumour progression and dissemination [156] and elevated levels of neutrophil elastase have been detected in a variety of cancer types [199-201]. Individuals who are α1-antitrypsin deficiency heterozygotes have increased frequency of many tumour types, including colon, lung and bladder. This has been attributed to a diminished level of a

negative regulator for neutrophil elastase which appears responsible for tumour development [202]. In bronchoalveolar adenocarcinoma elevated neutrophil elastase levels appear to enhance tumour cell shedding and possibly intra-pulmonary spread [200].

Elevated post-operative levels of neutrophil elastase have been identified following thoracic surgery which is associated with adverse pulmonary outcomes including Acute Respiratory Distress Syndrome (ARDS) [200, 202-205]. The neutrophil elastase inhibitor, Sivelastat, has been examined in a randomised controlled trial in the setting of video-assisted thoracic surgery for primary oesophageal cancer. This revealed that peri-operative Sivelestat was safe and associated with a significantly reduced SIR compared to placebo [205]. A further randomised control trial in the setting of video-assisted thoracoscopic oesophagectomy, revealed a reduction in duration of mechanical ventilation, reduced duration of SIR, a more rapid reduction in CRP and reduced length of ICU stay in patients treated with Sivelestat compared with placebo [203]. Peri-operative neutrophil elastase inhibition appears to decrease the SIR to surgery.

The evidence suggests that it is conceivable to implement a therapeutic strategy in the peri-operative period to reduce the systemic inflammation associated with surgery, minimise post-operative infective complications and ultimately improve oncological outcomes.

1.6 HMG-CoA Reductase Inhibitors

HMG-CoA Reductase Inhibitors (statins) inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase and were developed as lipid-lowering agents. They are one of the most commonly prescribed medications worldwide and it is estimated that they are prescribed to approximately 7 million people per year in the UK [206-210]. Six statins are currently available for use in the UK; atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, simvastatin. Simvastatin and atorvastatin are the most commonly prescribed statins and account for 85% of the total use. They are well tolerated medications with a low side effect profile, mainly hepatic dysfunction and muscle toxicity, for which approximately only 4% of patients discontinue the medication [208, 211].

Statins have been extensively studied and have proven efficacy in the primary and secondary prevention of cardiovascular morbidity and mortality in a variety of populations [212-217]. Their main mechanism of action is reduction of serum cholesterol by means of competitive inhibition of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is the rate limiting enzyme in the mevalonate synthesis pathway. Consequently, statins reduce the intermediate products of cholesterol synthesis, namely mevalonate and the down-stream isoprenoid intermediaries (isopentenyl-pyrophospate, farnesyl-pyrophosphate, geranylgeranyl-pyrophosphate). These molecules play a vital role in several intracellular signalling pathways which include protein prenylation via GTPases (Rho, Rac) [206,207, 218-221]. This leads to a reduction in endogenous cholesterol biosynthesis and a reduction in low-density lipoprotein which is a major risk factor for cardiovascular disease.

1.6.1 HMG-CoA Reductase Inhibitors and The Pleiotropic Effect

The cholesterol independent effects of HMG-CoA reductase inhibition are known as 'pleiotropic' effects. These pleiotropic effects have been shown to modulate the innate and adaptive immune systems and have anti-inflammatory effects which counteract the detrimental effects of inflammation. It has been proposed that statins may be novel therapeutic agents for the treatment and prevention of sepsis and that their use in the peri-operative period may reduce surgical complications by modulating the post-operative pro-inflammatory response. In fact, statins have been demonstrated to reduce the release of pro-inflammatory mediators (CRP, TNF-α, IL-6, IL-8) and evidence from randomised control trials has demonstrated improved outcomes in cardiovascular disease and bacterial pneumonia with statin induced CRP reduction [221-230].

1.6.2 HMG-CoA Reductase Inhibitors and Cancer

In view of their pleiotropic effects it has been suggested that statins may affect cancer risk by means of cancer chemoprevention. It has been demonstrated in pre-clinical studies that statins exert anti-neoplastic effects; pro-apoptotic, anti-angiogenic and immune-modulatory effects all of which may prevent cancer growth [231, 232]. Observational studies and meta-analyses have demonstrated a reduced risk of prostate, hepatocellular, gastric and oesophageal cancer associated with statin use [233-236]. Statins have also been investigated in the context of a potential role in the modification of cancer outcomes and mortality. It is has been demonstrated that patients who take statins prior to their cancer diagnosis are less likely to die from any cause or specifically from cancer [237].

The relationship between statins and colorectal cancer has been investigated, but the results are inconclusive both with regard to cancer risk, cancer outcomes and mortality. Epidemiology studies have examined the risk of colorectal cancer with conflicting results from very protective [238] to moderately harmful [239]. A recent meta-analysis of 40 published studies concluded that statins did not strongly reduce the overall risk of colorectal cancer in the general population at the low doses used for managing hypercholesterolaemia and cardiovascular disease. There was, however, evidence to suggest an overall risk reduction with statin use; Randomised Control Trials (RR=0.89, 95%CI=0.74-1.07, n=8), Cohort Studies (RR=0.92, 95%CI=0.83-1.00, n=13), Case-control Studies (RR=0.92, 95%CI=0.87-0.98, n=19) [240].

The effect of statins may differ according to cancer type, as cancer is not a homogenous entity, and patient population. It has been suggested that basic science research should be performed to ascertain which cancer types and patient populations are more likely to benefit from statins, either as a primary chemopreventative intervention or as an adjunct to other treatments [240].

1.6.3 HMG-CoA Reductase Inhibitors and Surgery

Following major cardio-vascular surgery a reduction in post-operative cardiac complications and a reduction in post-operative infective complications has been documented in patients taking statins pre-operatively [241-245]. In the context of general surgery, pre-operative statin use has been independently associated with a decreased risk of major, non-cardiac complications, respiratory complications, venous thrombo-embolic events and infective complications [246-248]. Overall, the pre-operative use of statins appears to be associated with a reduction in major complications [246, 247]. In abdominal surgery, statin use is

associated with a reduction in mortality after bacteraemia in transplant recipients, a reduction in candida infection in patients with diabetes and a decreased need for surgery in adhesive small bowel obstruction [247].

Specifically in the context of colorectal surgery, statin use is associated with a significantly lower incidence of SIRS and a lower incidence of surgical site infection [248]. There appears to be no difference however in overall mortality, total complications or median hospital length of stay between statin users and non-statin users undergoing major colorectal resection [246, 247].

Studies have demonstrated contradictory results regarding the association of peri-operative statin therapy and anastomotic leak and a beneficial effect of peri-operative statin therapy on the incidence of anastomotic leak cannot be ruled out [249, 250]. Patients on peri-operative statins appear to have a greater baseline peri-operative risk compared to non-statin users, but they achieve equivalent outcomes overall suggesting that peri-operative statin therapy may reduce morbidity after elective colorectal resection [248, 250].

It has been proposed that the use of statins in the peri-operative period may reduce surgical complications by modulating the post-operative pro-inflammatory response. It seems logical to think that the pleiotropic effects (anti-inflammatory, immune-modulatory, anti-platelet, anti-thrombotic, protective against oxidative stress, anti-microbial [221-230]) could be advantageous in the setting of surgery. In particular, the use of statins in the context of cancer surgery may reduce the systemic inflammation associated with surgery and minimise post-operative infective complications with a consequent improvement in oncological outcomes.

1.6.4 HMG-CoA Reductase Inhibitors and Neutrophil Function

Previous work conducted in our laboratory has examined neutrophil migration in a healthy elderly population (Greenwood, 2013). A generic, robust migratory phenotype, characterised by a maintained speed of migration but with reduced migratory accuracy was identified in an ageing population. This phenotype was demonstrated to be driven by dysregulated PI3Kinase activity and was proven to be amenable to correction with simvastatin through PI3Kinase inhibition [293]. It was concluded that aberrant migration may cause increased collateral damage to otherwise healthy tissue and manifest as delayed arrival to the site of infection and raised systemic inflammation, ultimately compromising host defences and contributing to the increased rates of morbidity and mortality observed in an ageing population [293].

Further work conducted in our laboratory has studied neutrophil function in the context of systemic sepsis (Patel, 2014). It has been demonstrated that neutrophils display a specific phenotype, characterised by a failure of migration, a propensity to degranulate and generate ROS with preserved phagocytic capacity while suppressing NET formation and suspending apoptosis. In-vitro treatment with simvastatin was observed to attenuate both ROS production and NET formation [294]. It was inferred that neutrophils contribute to the pathogenesis of organ dysfunction in sepsis secondary to a failure of appropriate migration. This enables pathogen dissemination and sepsis propagation in conjunction with increased systemic activation leading to collateral tissue damage. It was suggested that statins may be of benefit in the setting of early sepsis, particularly in the elderly, and further investigation was advocated [294].

In summary, work conducted in our laboratory to date has demonstrated modulation of neutrophil function with simvastatin in the following circumstances:

1. The reduction in NET formation observed in patients with sepsis (mild sepsis, but not severe sepsis) was attenuated by in-vitro treatment with simvastatin [294].
2. The increase in ROS production observed in patients with sepsis was attenuated by in-vitro treatment with simvastatin [294].
3. The age-related decline in neutrophil migration was corrected by in-vivo treatment with simvastatin in the healthy elderly [293].

1.7 Histone Acetylation and Deacetylation

Histone acetylation regulates inflammatory gene expression and is responsible for a number of diverse functions including DNA repair, cell proliferation and apoptosis [251, 252]. In the resting cell, DNA is tightly compacted around a core of histones. Specific residues in the N-terminal tails of histones can be post-translationally modified by acetylation leading to the release of the tightly wound DNA. Conversely, histone deacetylation is thought to re-establish the tight nucleosomal structure [251, 252]. Histone acetylation is regulated by a dynamic balance between histone acetyltransferase (HAT), which is associated with an open chromatin state and transcriptional activation, and histone deacetylase (HDAC), which is associated with a closed chromatin state and transcriptional repression [253].

Histone deacetylation in cancer

Changes in histone acetylation patterns have been described in the context of cancer. It has been demonstrated that HDACs regulate the expression of a large number of genes by directly interacting with transcription factors, for example E2f, Stat3, p53 [254]. HDACs have also been implicated in the deacetylation chromatin proteins, leading to altered gene transcription regulation and the deacetylation of non-histone proteins that regulate cellular homeostasis (cell-cycle progression and differentiation) [255]. HDAC inhibitors have subsequently been investigated for a potential anti-tumour effect. Interestingly, HDAC inhibitors have been found to induce apoptosis in a number of tumour types [256, 257].

Histone deacetylation in inflammation

Recent evidence from animal models (arthritis [258] and airway hyper-responsive [259] models) has suggested that HDAC inhibitors may have the potential to act as anti-

inflammatory agents. Although it was initially believed that HDAC inhibitors did not induce apoptosis in non-malignant cells [257, 258] this has been subsequently disproven. HDAC inhibitors have been found to enhance apoptosis in human neutrophils and eosinophils both in the presence and absence of survival-prolonging cytokines [253]. This suggests that HDAC inhibitors could potentially resolve neutrophilic and eosinophilic inflammation by inducing apoptosis.

Histone Deacetylase Enzymes

Eighteen HDAC enzymes have been identified and they can be subdivided into four different classes as outlined below.

Table 1.9 Histone Deacetylase Enzyme Classes

Class I	HDAC-1, HDAC-2, HDAC-3, HDAC-8
Class IIa **Class IIb**	HDAC-4, HDAC-5, HDAC-7, HDAC-9 HDAC-6, HDAC-10
Class III	SIRT-1, SIRT-2, SIRT-3, SIRT-4, SIRT-5, SIRT-6, SIRT-7
Class IV	HDAC-11

Eighteen HDAC enzymes have been identified and they can be subdivided into four different classes: Class I, Class II (Class IIa and Class IIb), Class III, Class IV. *Table adapted from [260].*

Class I HDACs have a nuclear location and are universally expressed in human cell lines and tissues. Class II HDACs exhibit tissue specific expression and can move between the nucleus and cytoplasm suggesting that they may be involved in deacetylation of non-histone proteins. Class III HDACs are referred to as Sirtuins and the subcellular distribution and

pattern of tissue specific expression is not yet known. HDAC-11 is the only member of Class IV and is homologous with Class I and Class II. Class I and Class II HDACs are sensitive to the classical HDAC inhibitor Trichostatin, while Class III HDACs require a co-factor (coenzyme NAD+) [260].

HDACs can induce aberrant transcription of key genes in the context of cancer and inflammation causing dysregulation of cellular functions. Consequently, HDACs are promising therapeutic target for cancer treatment and the resolution of inflammation.

Histone deacetylation and HMG-CoA Reductase Inhibitors

As previously documented, the rate limiting step in the melavonate pathway is catalysed by HMG-CoA reductase which converts HMG-CoA to mevalonate. This reaction is inhibited by statins (HMG-CoA reductase inhibitors) [206]. It has been demonstrated that statins target epigenetic mechanisms including histone deacetylation and epigenetic studies have shown simvastatin to be a direct inhibitor of HDAC-1 and HDAC-2. Simvastatin caused HDAC downregulation via the RAS/PI3Kinase/mTOR pathway [261] and by direct competitive inhibition [262]. A competitive inhibition of HDAC-2 has been associated with an increased histone-H3 acetylation and the subsequent expression of P21 which is responsible for cell cycle arrest. A statin induced cell cycle arrest with accumulation of P21 has been shown in lymphoma [263] and it has therefore been proposed that statins may act by this mechanism and inhibit epigenetically influenced diseases such as cancer [264].

The epigenetic consequences of HMG-CoA reductase inhibitors certainly warrant further investigation and the inhibition of histone deacetylases could prove to be a novel mechanism for the observed pleiotropic effects of statins.

1.8 Summary

Neutrophils are key mediators in the SIR and in the resolution of inflammation. It is appreciated that an uncontrolled inflammatory response should be avoided in an attempt to minimise damage to the host. In the context of cancer surgery, an overwhelming SIR may suppress the anti-tumour immunity and promote tumour progression and dissemination. It is also recognised that post-operative infective complications result in further compromise of the anti-tumour immune response to residual disease and are associated with a poor oncological outcome and increased mortality secondary to metastatic disease.

The interaction between tumour immunity and systemic immunity is likely to be complex. The delivery of appropriate immune-modulatory therapies to modify the peri-operative inflammatory response could be utilised to preserve anti-tumour immune competency in the host. It has been proposed that the pleiotropic effects of statins could be utilised in the peri-operative period in an attempt to modulate the SIR and prevent infective complications. The use of statins in the context of cancer surgery may therefore influence oncological outcomes.

In the context of cancer, neutrophils appear to have two distinct phenotypes, anti-tumour (N1) and pro-tumour (N2), suggesting that neutrophils differ in their contribution to the progression and dissemination of cancer and are capable of phenotypic plasticity. This opens up the possibility of therapeutic intervention to enhance anti-tumour and suppress pro-tumour functional properties. Modifications in neutrophil function and phenotype have been demonstrated with simvastatin treatment in-vitro and in-vivo, but not in the context of cancer or indeed cancer surgery where the maximum therapeutic benefit may be achieved.

1.9 Thesis Hypothesis

1. It was hypothesised that the impact of surgery would replicate the neutrophil functional changes observed in patients with sepsis:
    i. Reduced NET formation
    ii. Reduced apoptosis
    iii. Increased phagocytosis
2. It was hypothesised that HMG-CoA reductase inhibitors (statins) would restore neutrophil function, from dysregulated to normal function.

1.10 Thesis Objectives

This study was conducted to:

1. Characterise neutrophil function in the peri-operative period.
    i. NET Formation
    ii. Neutrophil Apoptosis
    iii. Neutrophil Phagocytosis
2. Investigate whether NET Formation is associated with patient characteristics, operative characteristics, patient outcomes and existing validated prognostic scores.
3. Evaluate the effect of HMG-CoA Reductase Inhibitors (statins) on neutrophil function in-vivo and in-vitro over the peri-operative period.
4. Explore the role of Histone Deacetylase as a potential epigenetic marker of neutrophil phenotype change.

Chapter 2

RETROSPECTIVE COHORT STUDY

Is HMG-CoA Reductase Inhibitor Use Associated with Improved Outcomes in Patients Undergoing Colorectal Cancer Resection?

2.1 Introduction

HMG-CoA Reductase Inhibitors

HMG-CoA reductase inhibitors (statins) have been extensively studied and have proven efficacy in the primary and secondary prevention of cardiovascular morbidity and mortality in a variety of populations [212-217]. Their main mechanism of action is reduction of serum cholesterol by means of competitive inhibition of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is the rate limiting enzyme in the mevalonate synthesis pathway. Consequently, statins reduce the intermediate products of cholesterol synthesis, namely mevalonate and the down-stream isoprenoid intermediaries, which play a vital role in several intracellular signalling pathways leading to a reduction in endogenous cholesterol biosynthesis and a reduction in low-density lipoprotein which is a major risk factor for cardiovascular disease [206,207, 218-221].

HMG-CoA Reductase Inhibitors and The Pleiotropic Effect

The cholesterol independent effects of HMG-CoA reductase inhibition are known as 'pleiotropic' effects. These pleiotropic effects (anti-inflammatory, immune-modulatory, anti-platelet, anti-thrombotic, protective against oxidative stress, anti-microbial [221-230]) are thought to counteract the detrimental effects of inflammation. In fact, statins have been demonstrated to reduce the release of pro-inflammatory mediators (CRP, TNF-α, IL-6, IL-8) and evidence from randomised control trials has demonstrated improved outcomes in cardiovascular disease and bacterial pneumonia with statin induced CRP reduction [221-230]. Interestingly a dose dependent anti-inflammatory response has been observed where patients taking high-dose statins were found to have reduced levels of CRP following Acute

Coronary Syndrome [265] and reduced periodontal and carotid inflammation in periodontal disease [266].

HMG-CoA Reductase Inhibitors and Cancer

It has been suggested that statins may influence cancer risk by means of cancer chemoprevention. Statins, in pre-clinical studies, have been demonstrated to exert anti-neoplastic effects (pro-apoptotic, anti-angiogenic, immune-modulatory) which may prevent cancer growth [231, 232]. Observational studies and meta-analyses have demonstrated a reduced risk of prostate, hepatocellular, gastric and oesophageal cancer associated with statin use [233-236]. Statins have also been investigated in the context of a potential role in the modification of cancer outcomes and mortality and it has been demonstrated that patients who take statins prior to their cancer diagnosis are less likely to die from any cause or specifically from cancer [237].

The relationship between statins and colorectal cancer has been investigated, but the results are inconclusive both with regard to cancer risk, cancer outcomes and mortality. Epidemiology studies have examined the risk of colorectal cancer with conflicting results from very protective [238] to moderately harmful [239]. A recent meta-analysis of 40 published studies concluded that statins did not strongly reduce the overall risk of colorectal cancer in the general population at the low doses used for managing hypercholesterolaemia and cardiovascular disease. There was, however, evidence to suggest an overall risk reduction with statin use although this was not significant [240].

HMG-CoA Reductase Inhibitors and Surgery

Following major cardio-vascular surgery a reduction in post-operative cardiac complications and a reduction in post-operative infective complications has been documented in patients taking statins pre-operatively [241-245]. Interestingly a dose dependent modulation of inflammation has been observed in coronary artery surgery where high-dose statins almost completely prevented the SIR associated with surgery and virtually entirely suppressed surgery related changes in plasma concentrations of TNF-α and IL-6 [267].

In the context of general surgery, pre-operative statin use has been independently associated with a decreased risk of major, non-cardiac complications, respiratory complications, venous thrombo-embolic events and infective complications [246-248]. Overall, the pre-operative use of statins appears to be associated with a reduction in major complications [246, 247].

Specifically in the context of colorectal surgery, statin use is associated with a significantly lower incidence of SIRS and a lower incidence of surgical site infection [248]. There appears to be no difference however in overall mortality, total complications or median hospital length of stay between statin users and non-statin users undergoing major colorectal resection [246, 247]. Studies have demonstrated contradictory results regarding the association of peri-operative statin therapy and anastomotic leak and a beneficial effect of peri-operative statin therapy on the incidence of anastomotic leak cannot be ruled out [249, 250]. Patients on peri-operative statins appear to have a greater baseline peri-operative risk compared to non-statin users, but they achieve equivalent outcomes overall suggesting that peri-operative statin therapy may indeed be of benefit and may reduce morbidity after elective colorectal resection [248, 250].

It is well established that infectious complications in patients with cancer are associated with adverse oncological outcomes independent of the morbidity associated with the infectious insult [184-187] and that they are associated with an increased mortality as a consequence of metastatic disease [187-191]. It has been proposed that the use of statins in the peri-operative period may reduce surgical complications by modulating the post-operative pro-inflammatory response.

2.2 Objective

It seems logical to think that statins could be advantageous in the setting of cancer surgery where they may reduce systemic inflammation and post-operative complications with a consequent improvement in oncological outcomes.

It was therefore investigated whether statin use is associated with reduced post-operative complications and improved clinical outcomes in patients undergoing elective colorectal cancer resection and whether a dose dependent effect on clinical outcomes could be demonstrated.

2.3 Methods

2.3.1 Acknowledgements

Miss Charlotte Robert-Rhodes (Intercalating Medical Student at the University of Birmingham, UK) assisted in database validation and statistical analysis as part of a BMedSc Research Project. The Research Project was designed by myself and offered to students to help develop skills in critical thinking and data analysis. Statistical support was provided by Dr. Peter Nightingale (Statistician, University Hospitals NHS Foundation Trust, Birmingham, UK). The results of this Chapter have been included in the BMed Sc Dissertation of Miss Charlotte Robert-Rhodes (Do HMG-CoA Reductase Inhibitors Influence Surgical Outcomes? BMedSc Dissertation, University of Birmingham, UK, 2015).

2.3.2 Study Methodology

A retrospective review of prospectively collected data was conducted for all elective colorectal cancer resections performed at a single institution (University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK) within an established enhanced recovery programme, between June 2008 and July 2013.

2.3.3 Data Acquisition

Data was extracted from existing robust electronic patient databases (Prescribing Information and Communication System and Clinical Portal) by the Bio-informatics Department at University Hospital Birmingham to produce a working dataset. All data was independently controlled for accuracy. Data was manually extracted from the electronic patient databases for 10% of patients which were randomly sampled from the working

dataset. Any data field from the sample that was less than 100% accurate was then manually extracted from the electronic databases for all patients in the working dataset.

Patients were divided into non-statin and statin users. Statin users were defined as patients who were prescribed statins at admission *and* who were prescribed statins within a five day post-operative period. Statin dose was classified as low-dose, moderate-dose or high-dose and defined as; low-dose ≤ 20mg simvastatin, moderate-dose = 40mg simvastatin, high-dose > 40mg simvastatin (or equivalent doses of other statins). All statins currently available for use in the UK were included (atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, simvastatin).

Data extracted from the electronic patient databases is outlined below.

Table 2.1 Data Extracted from the Electronic Databases - Patient Demographics, Patient Co-morbidities, Tumour Characteristics and Operative Characteristics

Patient Demographics	Age BMI Gender Smoking Status
Patient Co-morbidities	Chronic Kidney Disease Diabetes Mellitus Heart Failure Hypertension Ischaemic Heart Disease Lung Disease
Tumour Characteristics	Cancer Location T-stage N-stage M-stage Neo-adjuvant Therapy
Operative Characteristics	Operation Type Operation Technique Stoma Formation

Table 2.2 Data Extracted from the Electronic Databases - Post-operative Complications, Re-operations, Post-operative Antibiotic Use, Length of Stay, Re-admissions and Mortality

Post-operative Complications	Abdominal Collection Acute Kidney Injury Anastomotic Dehiscence Bacteraemia Cardio-respiratory Event Intestinal Obstruction Lower Respiratory Tract Infection
Re-operations	Re-operation (any cause)
Post-operative Antibiotic Use	Co-amoxiclav Piperacillin-tazobactam Meropenem Vancomycin
Length of Stay (LOS)	Critical Care LOS Total LOS
Re-admissions	30-day readmission
Mortality	30-day mortality 90-day mortality 12-month mortality 24-month mortality 60-month mortality

Table 2.3 Data Extracted from the Electronic Databases – Pre-operative and Post-operative Inflammatory Markers

Pre-operative	Absolute White Cell Count Neutrophil Count Lymphocyte Count Platelet Count Neutrophil-Lymphocyte Ratio
Post-operative	Absolute White Cell Count Neutrophil Count Lymphocyte Count Platelet Count Neutrophil-Lymphocyte Ratio C-reactive Protein

The following definitions were applied to the extracted data.

- Post-operative complications ≤ 30 days after surgery were recorded for all patients and were defined as follows:
  - Abdominal Collection was defined as a radiologically diagnosed collection of the abdominal cavity or pelvis that required intervention or prolonged hospital stay.
  - Acute Kidney Injury was defined as a percentage increase in serum creatinine ≥ 50% from baseline.
  - Anastomotic Dehiscence was defined as surgically confirmed dehiscence and/or radiologically diagnosed dehiscence (fluid collection in close proximity to an anastomosis that was drained yielding purulent fluid or gas and/or evidence of contrast leak).
  - Bacteraemia was defined as the presence of a positive microbiology culture from a central or peripheral blood sample.
  - Cardio-respiratory Events included a confirmed diagnosis of myocardial infarction, pulmonary embolism and/or cardiac dysrhythmia (which was not as a consequence of infection) that required appropriate treatment.
  - Intestinal Obstruction was defined as a confirmed radiological diagnosis of mechanical or functional intestinal obstruction which resulted in a prolonged hospital stay.
  - Lower Respiratory Tract Infection was defined as a confirmed diagnosis of pneumonia that required antibiotic therapy.
- Re-operations were defined as a return to theatre ≤ 30 days of the index procedure.

- Re-admissions were defined as a return to hospital ≤ 30 days of the index procedure requiring a hospital stay ≥ 24 hours.
- Critical Care LOS was defined by the number of days on critical care requiring level-2 or level-3 care.
- Total LOS was defined by the number of days in hospital including the day of surgery.
- Post-operative Antibiotic Use was defined as a prescription for co-amoxiclav, piperacillin-tazobactam, meropenem or vancomycin ≤ 30 days after surgery and was used as a surrogate marker for post-operative infective complication.
- Mortality was recorded and categorised into 30-day, 90-day, 12-month, 24-month and 60-month mortality.

Pre-operative inflammatory markers included pre-operative values closest to the day of surgery and not including the day of surgery.

Post-operative inflammatory markers included the minimum, maximum and mean values within a 7 day post-operative period.

2.3.4 Statistical Analysis

Statistical comparisons between non-statin and statin groups were calculated using Mann-Whitney U test and χ^2 test or Fisher Exact test for continuous and categorical data respectively.

Univariate linear and logistic regression analyses were performed for continuous and categorical data respectively. Multivariate analysis was then performed including all variables from the univariate analyses with a p-value < 0.4 with 100% complete data.

Survival was evaluated using Kaplan-Meier graphs. Tarone-Ware p-values were used as a measure of significance. Multivariate survival analysis was performed using Cox Regression analysis.

Statistical analyses were performed by using SPSS version 17 (IBM SPSS Statistics, IBM Corporation, Armonk, NY).

Statistical significance was defined a priori as a p-value <0.05.

2.4 Results

A total of 703 patients undergoing elective colorectal cancer resection within an established enhanced recovery programme, between June 2008 and July 2013 were reviewed. 246 (35.0% [246/703]) patients were statin users, defined as patients who were prescribed statins at admission *and* who were prescribed statins within a five day post-operative period. The mean number of statin doses omitted during the index admission was 1.36doses (95%CI=1.04-1.67doses).

The majority of patients were on simvastatin (76.0% [186/246]) and the remainder were on atorvastatin (18.3% [45/246]), pravastatin (2.9% [7/246]), rosuvastatin (2.4% [6/246]) and fluvastatin (0.4% [1/246]). The majority of patients were on moderate-dose statin (64.6% [159/246]) and the remainder were on low-dose (26.0% [64/246]) and high-dose (9.4% [23/246) statins high-dose statins.

The baseline population characteristics are shown in Table 2.4.

Table 2.4 Population Characteristics and Data Completeness

Variable	*Total (n=703)*	*Data Completeness (%)*
Age, median (IQR)	69 (60-76)	100
Gender, n (%), M/F	410 / 293 (58.3 / 41.7)	100
BMI, median (IQR), kg/m^2	26.58 (23.7-30.4)	67
Smoking Status, n (%), 1/2/3[a]	88 / 238 / 322 (12.5 / 33.9 / 45.8)	92
Chronic Kidney Disease, n (%), y	27 (3.8)	100
Diabetes Mellitus, n (%), y	111 (15.8)	100
Heart Failure, n (%), y	14 (2.0)	100
Hypertension, n (%), y	316 (45.0)	100
Ischaemic Heart Disease, n (%) y	88 (12.5)	100
Lung Disease, n (%), y	106 (15.1)	100
Cancer Location, n (%), 1/2[b]	368 / 335 (52.3 / 47.6)	100
T-Stage, n (%), 0/1/2/3/4	10 / 57 / 86 / 365 / 174 (1.4 / 8.1 / 12.2 / 51.9 / 24.8)	99
N-Stage, n (%), 0/1/2	396 / 199 / 101 (56.3 / 28.3 / 14.4)	99
M-Stage, n (%), 0/1	587 / 111 (83.5 / 15.8)	99
Neo-adjuvant Therapy, n (%), y	140 (19.9)	100
Operation Type, n (%), 1/2[c]	329 / 374 (46.8 / 53.2)	100
Operation Technique, n (%), 1/2[d]	480 / 223 (68.3 / 31.7)	100

y = yes, 1/2/3[a] = active / former / never, 1/2[b] = colon / recto-sigmoid junction and rectum, 1/2[c] = segmental / rectal, 1/2[d] = open and laparoscopic converted to open / laparoscopic

The median age of the population was 69 years (IQR 60-76 years) with 58.3% of male gender. Fifty three percent of patients underwent a resection involving the rectum and 31.7% of procedures were completed entirely laparoscopically. Data completeness was generally high for the different variables.

The population outcomes are shown in Table 2.5.

Table 2.5 Population Outcomes and Data Completeness

Outcomes	*Total (n=703)*	*Data Completeness (%)*
Abdominal Collection, n (%), y	25 (3.6)	100
Acute Kidney Injury, n (%), y	14 (2.0)	100
Anastomotic Dehiscence, n (%), y	28 (4.0)	100
Bacteraemia, n (%), y	7 (1.0)	100
Cardio-respiratory Event, n (%), y	31 (4.4)	100
Intestinal Obstruction, n (%), y	43 (6.1)	100
Lower Respiratory Tract Infection, n (%), y	38 (5.4)	100
Re-operation, n (%), y	19 (2.7)	100
Stoma, n (%), y	186 (26.5)	100
Post-operative Antibiotic Use, n(%), y	424 (60.3)	100
Critical Care Admission, n (%), y	111 (15.8)	100
Critical Care LOS, median (range, IQR), days	0 (0-72, 0-0)	100
Total LOS, median (range, IQR), days	9 (1-277, 6-15)	100
Readmission, n (%), y	63 (9.0)	100
Mortality, n (%), 1/2/3/4/5[a]	8 / 16 / 54 / 82 / 145 (1.1 / 2.3 / 7.7 / 11.7 / 20.6)	100

y = yes, 1/2/3/4/5[a] = 30-day / 90-day / 12-month / 24-month / 60-month

15.8% of patients required admission to Critical Care following surgery. 60.3% of patients received post-operative antibiotic therapy. 4.0% of patients had a confirmed anastomotic dehiscence. Only 2.7% of patients required a re-operation. The median length of stay was 9 days (IQR 6-15 days) and 9.0% of patients were readmitted up to 30 days from the index procedure. 30-day, 90-day, 12-month, 24-month and 60-month mortality was 1.1%, 2.3%, 7.7%, 11.7%, 20.6% respectively with a median follow-up of 51 months (Range 22-82 months, IQR 35-66 months). Data completeness was 100% for all outcomes.

The population characteristics according to statin use are shown in Table 2.6.

Table 2.6 Population Characteristics According to Statin Use

Variable	*Statin User (n=246)*	*Non-statin User (n=457)*	*P-value*
Age, median (IQR)	71 (65-78)	67 (58-76)	**<0.0001**
Gender, n (%), M/F	146 / 100 (59.3 / 40.7)	264 / 193 (57.8 /42.2)	0.6894
BMI, median (IQR), kg/m^2	27.5 (24.8-31.7)	26.0 (22.9-29.6)	**0.0005**
Smoking Status, n (%), 1/2/3[a]	28 / 87 / 112 (11.4 / 35.4 / 45.5)	53 / 147 / 234 (11.6 / 32.2 / 51.2)	0.4882
Chronic Kidney Disease, n (%), y	14 (5.7)	13 (2.8)	0.2000
Diabetes Mellitus, n (%), y	76 (30.9)	35 (7.6)	**<0.0001**
Heart Failure, n (%), y	12 (4.8)	2 (0.4)	**<0.0001**
Hypertension, n (%), y	163 (66.3)	153 (33.5)	**<0.0001**
Ischaemic Heart Disease, n (%) y	71 (28.8)	17 (3.7)	**<0.0001**
Lung Disease, n (%), y	39 (15.9)	67 (14.7)	0.6734
Cancer Location, n (%), 1/2[b]	148 / 98 (60.2 / 39.8)	220 / 237 (48.1 / 51.9)	**0.0023**
T-Stage, n (%), 0/1/2/3/4	18 / 33 /136 / 56 (7.3 / 13.4 / 55.2 / 22.7)	37 / 54 / 229 / 117 (8.1 / 11.8 / 50.1 / 25.6)	0.6544
N-Stage, n (%), 0/1/2	145 / 60 / 39 (58.9 / 24.4 / 15.9)	252 / 139 / 62 (55.1 / 30.4 / 13.6)	0.2185
M-Stage, n (%), 0/1	210 / 35 (85.4 / 14.2)	377 / 76 (82.5 / 16.7)	0.4480
Neo-adjuvant Therapy, n (%), y	38 (15.4)	102 (22.3)	**0.0298**
Operation Type, n (%), 1/2[c]	142 / 104 (57.7 / 42.3)	224 / 233 (49.0 / 51.0)	**0.0275**
Operation Technique, n (%), 1/2[d]	163 / 83 (66.3 / 33.7)	317 / 140 (69.4 / 30.6)	0.3988

y = yes, 1/2/3[a] = active / former / never, 1/2[b] = colon / recto-sigmoid junction and rectum, 1/2[c] = segmental / rectal, 1/2[d] = open and laparoscopic converted to open / laparoscopic

Statin users were significantly older (71 vs. 67, p<0.0001), had a higher BMI (27.5 vs. 26.0, p=0.0005), had a higher incidence of medical co-morbidities including diabetes mellitus, heart failure, hypertension and ischaemic heart disease (All p<0.0001). Interestingly, statin users were significantly more likely to have a colonic cancer than a rectal cancer (60.2 vs. 48.1, p=0.0023) and this reflects significantly fewer rectal resections, a reduced requirement for neo-adjuvant therapy and fewer stomas formed.

The population outcomes according to statin use are shown in Table 2.7.

Table 2.7 Population Outcomes According to Statin Use

Outcomes	*Statin User (n=246)*	*Non-statin User (n=457)*	*P-value*
Abdominal Collection, n (%), y	7 (2.9)	18 (3.9)	0.3829
Acute Kidney Injury, n (%), y	7 (2.9)	7 (1.5)	0.5274
Anastomotic Dehiscence, n (%), y	13 (5.3)	15 (3.3)	0.7003
Bacteraemia, n (%), y	3 (1.2)	4 (0.2)	0.3273
Cardio-respiratory Event, n (%), y	11 (4.5)	20 (4.4)	1.0000
Intestinal Obstruction, n (%), y	18 (7.3)	25 (5.4)	0.2631
Lower Respiratory Tract Infection, n (%), y	16 (6.5)	22 (4.8)	0.2258
Re-operation, n (%), y	7 (2.9)	12 (2.6)	0.8640
Stoma, n (%), y	52 (21.1)	134 (29.3)	**0.0199**
Post-operative Antibiotic Use, n(%), y	156 (63.4)	268 (58.6)	0.2175
Critical Care Admission, n (%), y	51 (20.7)	60 (13.1)	**0.0094**
Critical Care LOS, median (range, IQR), days	0 (0-72, 0-0)	0 (0-35, 0-0)	**0.0049**
Total LOS, median (range, IQR), days	10 (1-277, 7-17)	9 (1-246, 6-13)	**0.0142**
Readmission, n (%), y	25 (10.2)	38 (8.3)	0.8414
Mortality, n (%), y			
30-day	5 (2.0)	3 (0.7)	0.1359
90-day	9 (3.6)	7 (1.5)	0.1081
12-month	32 (13.0)	22 (4.8)	**0.0002**
24-month	46 (18.7)	36 (7.8)	**<0.0001**
60-month	74 (30.0)	71 (15.5)	**<0.0001**

y = yes

No statistical differences were identified in post-operative complications, post-operative antibiotic use, re-operations and re-admissions. Without appropriate statistical adjustment statin users appeared to have an increased rate of Critical Care admission, Critical Care length of stay and total length of stay. It also appeared that statin users had a greater mortality at 12-months, 24-months and 60-months.

The pre-operative and post-operative inflammatory markers according to statin use are shown in Table 2.8.

Table 2.8 Pre-operative and Post-operative Inflammatory Markers According to Statin Use

Inflammatory Markers		*Statin User (n=246)*	*Non-statin User (n=457)*	*P-value*
Pre-operative				
Absolute White Cell Count [x10^9/L]		7.4 (5.8-8.9)	7.3 (6-9.3)	0.8657
Neutrophil Count [x10^9/L]		4.7 (3.6-6)	4.9 (3.5-6.1)	0.7339
Lymphocyte Count [x10^9/L]		1.5 (1-2.2)	1.5 (1-2)	0.7501
Platelet Count [x10^9/L]		259 (204-323.5)	280 (224-376)	0.0599
Neutrophil-Lymphocyte Ratio		3.2 (2.2-5.4)	3.0 (2.2-4.7)	0.7655
Post-operative				
Absolute White Cell Count [x10^9/L]				
	Min, median (IQR)	6.9 (5.4-8.7)	7.0 (5.5-8.7)	0.7364
	Max, median (IQR)	13.1 (10.5-16.6)	12.4 (10.0-15.6)	**0.0418**
	Mean, median (IQR)	9.8 (8.3-11.8)	9.6 (7.9-11.6)	0.2358
Neutrophil Count [x10^9/L]				
	Min, median (IQR)	4.7 (3.5-6.3)	5.0 (3.7-6.3)	0.4074
	Max, median (IQR)	10.8 (8.3-14.2)	10.3 (8.1-13.2)	0.1131
	Mean, median (IQR)	7.6 (6.2-9.4)	7.5 (6.0-9.3)	0.3971
Lymphocyte Count [x10^9/L]				
	Min, median (IQR)	0.7 (0.5-1.0)	0.7 (0.5-1.1)	0.9361
	Max, median (IQR)	1.6 (1.1-2.1)	1.5 (1.0-2.0)	0.0595
	Mean, median (IQR)	1.2 (0.8-1.5)	1.1 (0.8-1.5)	0.2077
Platelet Count [x10^9/L]				
	Min, median (IQR)	198.0 (149.0-241.3)	208.5 (169.0-254.0)	**0.0217**
	Max, median (IQR)	347.0 (265.3-514.3)	345.5 (262.0-507.8)	0.9745
	Mean, median (IQR)	269.6 (216.6-339.2)	273.2 (216.5-359.9)	0.4291
C-reactive Protein [mg/L]				
	Min, median (IQR)	32.0 (14.0-59.5)	42.0 (17.0-69.0)	**0.0188**
	Max, median (IQR)	168.0 (98.0-245.5)	162.0 (96.0-255.0)	0.9224
	Mean, median (IQR)	94.3 (61.3-133.6)	99.3 (64.0-140.7)	0.1397

Post-operative results are presented as minimum, maximum and mean values which accounts for different post-operative recovery time periods of the study participants (presented as median [IQR].

There were no statistically significant differences in pre-operative inflammatory markers between statin and non-statin users. In particular there was no difference in pre-operative NLR.

Post-operatively, statin users had a higher maximum absolute white cell count (13.1 vs. 12.4, p=0.0418) but a lower minimal platelet count (198.0 vs. 208.5, p=0.0217) and minimal CRP level (32.0 vs. 42.0, p=0.0188).

Univariate analysis was performed to identify differences between the outcomes of statin users and non-statin and the results are displayed in Table 2.9.

Table 2.9 Statin Use - Univariate Logistic Regression Analysis and Univariate Linear Regression Analysis (indicating 'Odds Ratio' and 'Change in Risk' for *statin use* respectively)

Outcomes	*Odds Ratio*	*95% Confidence Interval*	*P-value*
Univariate Logistic Regression			
Abdominal Collection	0.71	0.29 - 1.73	0.4570
Acute Kidney Injury	1.88	0.65 - 5.43	0.2420
Anastomotic Dehiscence **	1.64	0.77 - 3.51	0.1990
Bacteraemia	1.40	0.31 - 6.30	0.6630
Cardio-respiratory Event	1.02	0.48 - 2.17	0.9530
Intestinal Obstruction **	1.36	0.73 - 2.55	0.3310
Lower Respiratory Tract Infection **	1.38	0.71 - 2.67	0.3460
Re-operation	1.09	0.59 - 1.98	0.7910
Stoma **	0.65	0.45 - 0.93	**0.0190**
Post-operative Antibiotic Use **	1.17	0.84 - 1.64	0.3470
Critical Care Admission **	1.91	1.23 - 2.97	**0.0040**
Readmission	1.25	0.73 - 2.12	0.4140
Outcomes	*Change in Risk (%)*	*95% Confidence Interval*	*P-value*
Univariate Linear Regression			
Critical Care LOS **	43	0.67 - 7.31	0.1020
Total LOS **	14	0.012 - 0.10	**0.0130**

Univariate analysis demonstrated significant increases in the rate of admission to Critical Care (OR=1.91, 95%CI=1.23-2.97, p=0.0040) and in the total length of stay (14% increase in LOS, 95%CI=0.012-0.10, p=0.0130) in statin users. Statin users were significantly less likely to require a stoma after elective colorectal cancer resection (OR 0.65, 95%CI 0.45-0.93, p=0.0190).

Multivariate analysis was performed to identify differences between the outcomes of statin users and non-statin when appropriately adjusted for age and gender. Outcome measures from univariate analyses which are marked with **** underwent further multivariate analysis. The criteria for inclusion into multivariate analysis was p<0.4000, event incidence ≥28 and data completeness of 100%. The results of multivariate analysis are displayed in Table 2.10.

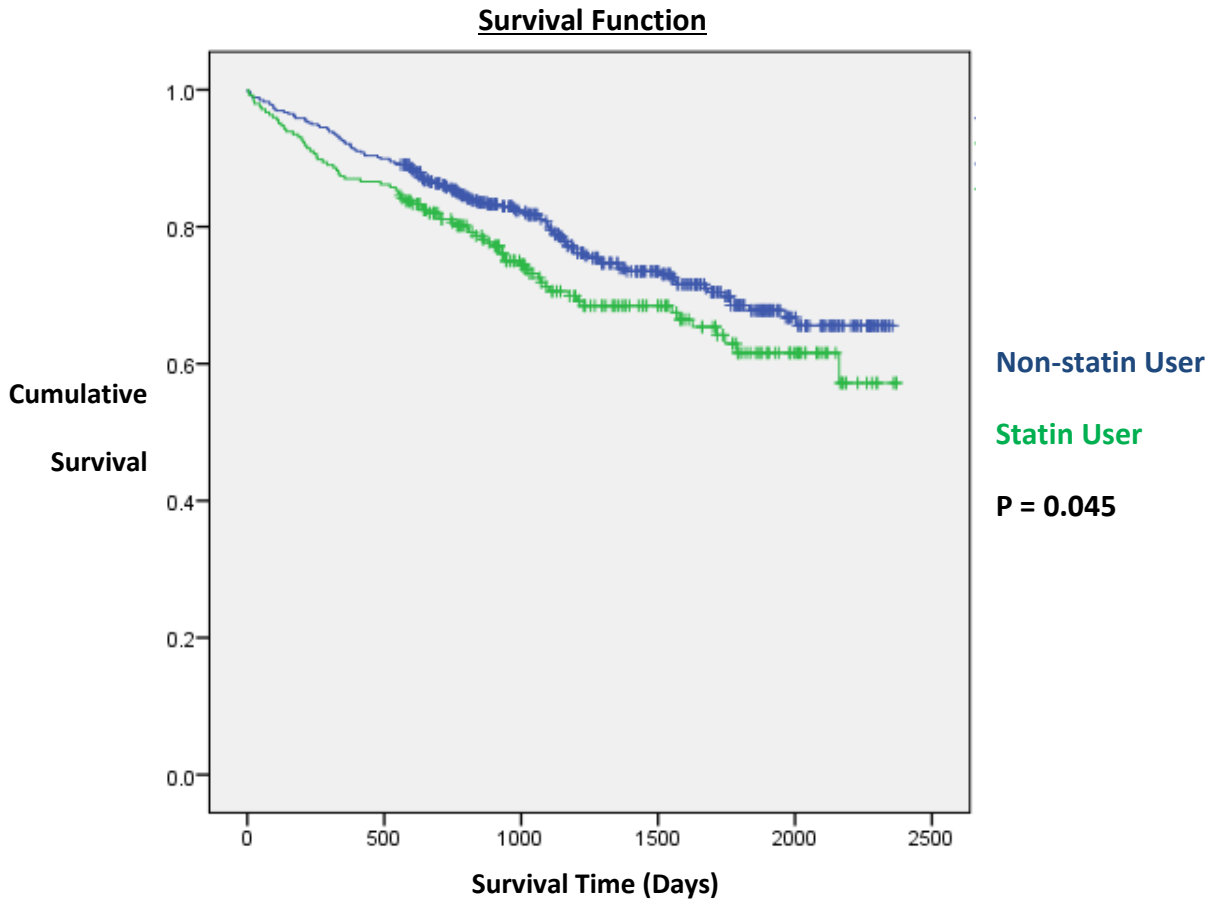
Table 2.10 Statin Use - Multivariate Logistic Regression Analysis and Multivariate Linear Regression Analysis (indicating 'Odds Ratio' and 'Change in Risk' for *statin use* respectively)

Outcomes	*Odds Ratio*	*95% Confidence Interval*	*P-value*
Multivariate Logistic Regression			
Anastomotic Dehiscence	1.40	0.65 - 3.05	0.3940
Intestinal Obstruction	1.50	0.78 - 2.89	0.2210
Lower Respiratory Tract Infection	1.24	0.63 - 2.43	0.5360
Stoma	0.65	0.45 - 0.95	**0.0280**
Post-operative Antibiotic Use	1.36	0.96 - 1.92	0.0870
Critical Care Admission	1.83	1.16 - 2.87	**0.0090**
Outcomes	*Change in Risk (%)*	*95% Confidence Interval*	*P-value*
Multivariate Linear Regression			
Critical Care LOS	36	-0.01 - 0.28	0.0650
Total LOS	12	0.002 - 0.10	**0.0420**

After adjustment for age and gender differences, multivariate analysis demonstrated significant increases in rate of admission to Critical Care (OR=1.83, 95%CI=1.16-2.87, p=0.0090) and in the total length of stay (12% increase in LOS, 95%CI=0.002-0.10, p=0.0420) in statin users. Statin users were significantly less likely to require a stoma after elective colorectal cancer resection (OR 0.65, 95%CI 0.45-0.95, p=0.0280) than non-statin users.

Survival analysis was conducted to identify any differences in overall mortality in statin users and non-statin users. Firstly, univariate survival analysis was conducted using Kaplan-Meier graphs and Tarone-Ware p-values were used as a measure of significance. Multivariate survival analysis was then performed using Cox Regression analysis which adjusted for age and gender. The Kaplan-Meier survival analysis is displayed in graphical form in Figure 2.1 and the Cox Regression analysis is shown in Table 2.11.

Figure 2.1 Statin Use - Kaplan-Meier Survival Analysis



Univariate survival analysis using Kaplan-Meier graphs and a Tarone-ware p-value indicates a statistically significant difference in survival with improved survival in Non-statin Users when compared with Statin Users (p=0.045)

Table 2.11 Statin Use – Cox Regression Survival Analysis

Outcomes	*Hazard Ratio*	*95% Confidence Interval*	*P-value*
Mortality	1.21	0.90 – 1.62	0.2120

Multivariate Cox Regression survival analysis revealed no significant difference in survival when comparing Non-statin Users and Statin Users (indicating 'Hazard Ratio' for **statin use**)

Univariate survival analysis revealed an increased mortality rate associated with statin use (Tarone-Ware p-value = 0.045). When adjusted for age and gender, multivariate Cox Regression analysis revealed no significant difference associated with statin use (HR=1.21, 95%CI=0.90-1.62, p=0.2120).

Univariate and multivariate analyses were conducted to determine whether a dose dependent effect on clinical outcomes between statin users and non-statin users existed.

Statin dose was classified as low-dose, moderate-dose or high-dose and defined as; low-dose ≤ 20mg simvastatin, moderate-dose = 40mg simvastatin, high-dose > 40mg simvastatin (or equivalent doses of other statins).

The number of statin users on low-dose, moderate-dose and high-dose statins were 64 (26.0%), 159 (64.6%) and 23 (9.4%) respectively. There were no statistical differences in patient characteristics according to statin dose.

Univariate analysis was performed to identify any dose dependent differences in patient outcomes according to statin dose (low-dose, moderate-dose and high-dose) and is displayed in Table 2.12.

Table 2.12 Dose Response - Univariate Logistic Regression Analysis and Univariate Linear Regression Analysis (indicating 'Odds Ratio' and 'Change in Risk' for *increasing dose of statin* respectively)

Outcomes	*Odds Ratio*	*95% Confidence Interval*	*P-value*
Univariate Logistic Regression			
Abdominal Collection **	1.05	0.99 - 1.12	0.0810
Acute Kidney Injury **	1.01	0.88 – 1.16	0.0937
Anastomotic Dehiscence	0.98	0.90 – 1.06	0.5500
Bacteraemia	0.91	0.81 - 1.02	0.1150
Cardio-respiratory Event	0.98	0.91 – 1.06	0.6210
Intestinal Obstruction	0.62	0.91 - 1.06	0.6210
Lower Respiratory Tract Infection	0.99	0.97 – 1.05	0.7600
Re-operation **	0.95	0.89 - 1.00	0.0580
Stoma	0.99	0.97 – 1.02	0.4590
Post-operative Antibiotic Use	1.00	0.97 – 1.02	0.7120
Critical Care Admission	1.00	0.96 – 1.04	0.8960
Readmission	1.01	0.97 – 1.04	0.7500
Outcomes	*Change in Risk (%)*	*95% Confidence Interval*	*P-value*
Univariate Linear Regression			
Critical Care LOS	-10	-0.69 - 0.85	0.8360
Total LOS **	4	1.00 – 1.22	**<0.0001**

Univariate analysis demonstrated a significant increase in the total length of stay (4% increase in LOS, 95%CI=1.00-1.22, p.0.0001) with increasing dose of statin.

Multivariate analysis was performed to identify any dose dependent differences in patient outcomes according to statin dose (low-dose, moderate-dose and high-dose) when appropriately adjusted for age and gender. Outcome measures from univariate analyses which are marked with **** underwent further multivariate analysis. The criteria for inclusion into multivariate analysis was p<0.4000 and data completeness of 100%. The outcome, 'bacteraemia' was not included in the multivariate analysis as only three events occurred in all patients on statins. The results of multivariate analysis are displayed in Table 2.13.

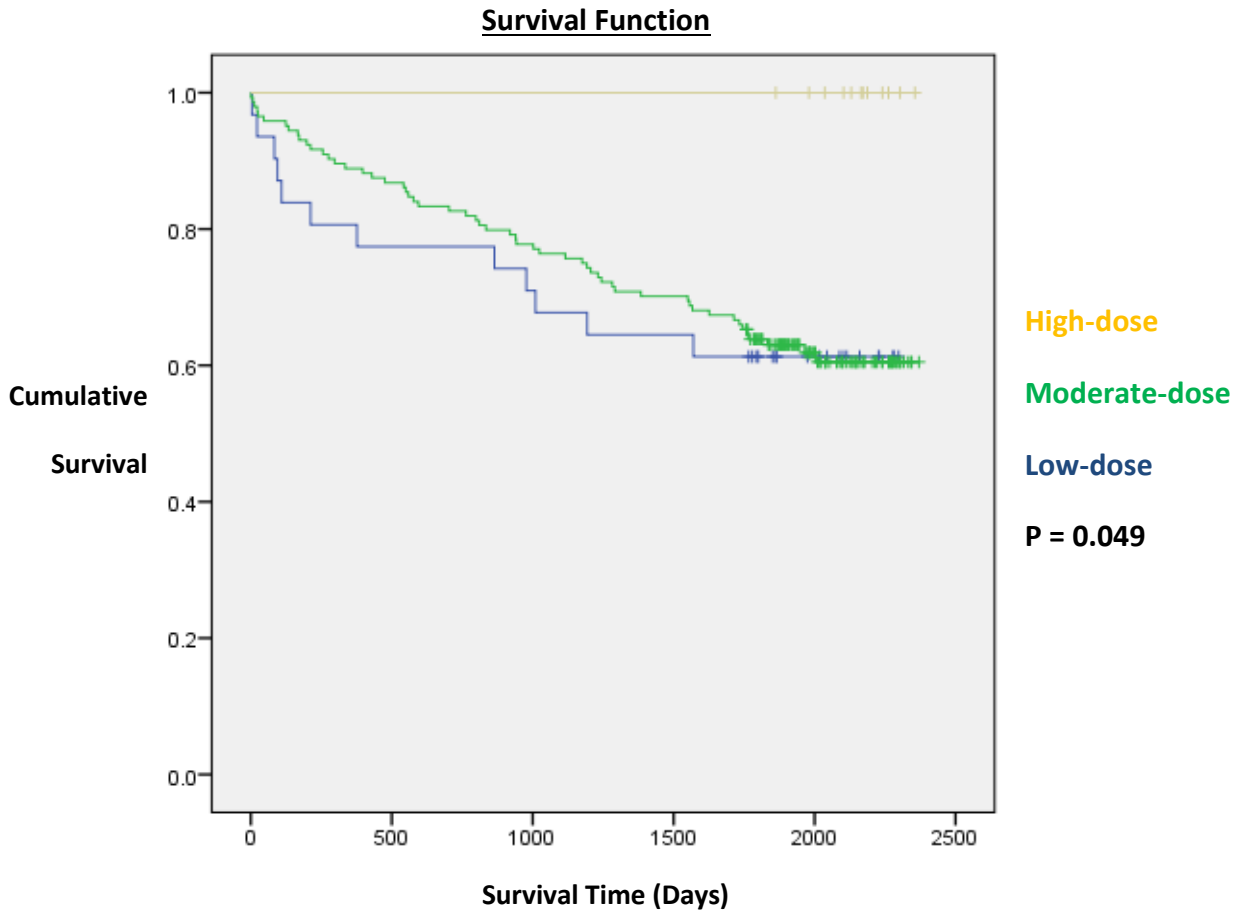
Table 2.13 Dose Response – Multivariate Logistic Regression Analysis and Multivariate Linear Regression Analysis (indicating 'Odds Ratio' and 'Change in Risk' for *increasing dose of statin* respectively)

Outcomes	*Odds Ratio*	*95% Confidence Interval*	*P-value*
Multivariate Logistic Regression			
Abdominal Collection	0.97	0.94 - 1.06	0.0960
Acute Kidney Injury	0.96	0.90 – 1.03	0.2500
Re-operation	0.95	0.90 – 1.06	0.5500
Outcomes	*Change in Risk (%)*	*95% Confidence Interval*	*P-value*
Multivariate Linear Regression			
Total LOS	269	-0.004-0.001	0.2700

After adjustment for age and gender multivariate analysis demonstrated no significant differences in rates of abdominal collection, acute kidney injury, re-operation or total length of hospital stay with increasing dose of statin.

Survival analysis was conducted to identify any dose dependent difference in overall mortality in statin users. Firstly, univariate survival analysis was conducted using Kaplan-Meier graphs and Tarone-Ware p-values were used as a measure of significance. Multivariate survival analysis was then performed using Cox Regression analysis which adjusted for age and gender. The Kaplan-Meier survival analysis is displayed in graphical form in Figure 2.2 and the Cox Regression analysis is shown in Table 2.14.

Figure 2.2 Dose Response - Kaplan-Meier Survival Analysis



Univariate survival analysis using Kaplan-Meier graphs and a Tarone-ware p-value indicates a statistically significant difference in survival with improved survival in patients receiving High-dose statin (p=0.049)

Table 2.14 Dose Response – Cox Regression Survival Analysis

Outcomes	*Hazard Ratio*	*95% Confidence Interval*	*P-value*
Mortality	0.67	0.41 – 1.09	0.1100

Multivariate Cox Regression survival analysis revealed no significant difference in survival when comparing High-dose, Moderate-dose and Low-dose statin use (indicating 'Hazard Ratio' for **High-dose statin use**)

Univariate survival analysis revealed a reduction in mortality associated with high-dose statin (Tarone-Ware p-value = 0.049). When adjusted for age and gender, multivariate Cox Regression analysis revealed no significant dose dependent difference in mortality (HR=0.67, 95%CI=0.41-1.09, p=0.1100).

2.5 Discussion

35.0% of patients undergoing elective colorectal cancer resection were on statins. The published literature documents the incidence of statin use in a general surgical population ranges from 10.5–32.0% [246,249,250]. The largest colorectal cancer patient cohort, form Denmark, revealed 18.8% (518/2755) of patients received statins in the peri-operative period [246]. This discrepancy may reflect differences in prescribing policy between different nations and/or true differences in levels of health in the patient populations as statins are principally used for primary and secondary prevention of cardiovascular morbidity and mortality in a variety of populations [212-217].

The mean percentage of statin doses omitted during the index admission was 8.5%, which equates to one missed dose per statin user, indicating that patients resumed statin therapy as they resumed enteral nutrition and their regular medications (median LOS in statin users = 10 days [IQR 7-17 days]).

In terms of population characteristics, statin users were significantly different from non-statin users. Those patients on statins were significantly older, had a higher BMI and were a more co-morbid population (diabetes mellitus, heart failure, hypertension and ischaemic heart disease). Despite these differences, statin users had equivalent outcomes in frequency of post-operative complications (abdominal collection, acute kidney injury, anastomotic dehiscence, bacteraemia, cardio-respiratory event, lower respiratory tract infection), post-operative antibiotic use (used as a surrogate to indicate post-operative infective complication), re-operations and re-admissions and there was no statistically significant difference in mortality after appropriate age and gender adjustments with a median follow-up of 51 months (Range 22-82 months, IQR 35-66 months).

It is known that patients with pre-existing co-morbidities are at an increased peri-operative risk of developing complications as the surgical insult increases the demand on organs with already compromised function [268]. It has also been demonstrated that elevated BMI, in conjunction with advanced age, is predictive of morbidity and mortality following general surgery [269]. Despite a greater base-line peri-operative risk, evidenced by an older more co-morbid population, statin users had analagous outcomes and complication profiles to non-statin users.

Univariate analysis, however, demonstrated significant increases in the rate of admission to Critical Care and in the total length of stay in statin users compared to non-statin-users and these findings remained statistically significant, after adjustment for age and gender differences, in multivariate analysis. Statin users were 1.83 times more likely to need Critical Care support (OR =1.83, 95%CI=1.16-2.87, p=0.0090) and had a hospital length of stay 12% longer than non-statin users (p=0.042). This is not only a reflection of an increased co-morbidity but a sign of a prolonged functional recovery and it may also represent an additional patient need for assistance with physiotherapy, occupational therapy and stoma therapy in this specific patient group.

There were no differences in pre-operative markers of inflammation between statin and non-statin users. Post-operatively differences were identified. Statin users had a higher maximum absolute white cell count than non-statin users, but conversely lower minimum platelet and CRP levels. It is known that IL-6 is the primary mediator of inflammation following surgery and is responsible for the production of CRP. IL-6 has been demonstrated to predict post-operative complications [270] and it has been shown that IL-6 is suppressed by statin therapy [271,272]. This would therefore explain the significant reduction in post-operative minimum CRP observed in statin users.

This is the first study to attempt to determine if a dose dependent effect of statin therapy on patient outcomes following elective colorectal cancer resection exists. There were no observed differences in post-operative complications, post-operative antibiotic use, re-operations, admission to Critical Care, Critical Care length of stay, total length of stay and re-admissions with increasing dose of statin. Univariate analysis demonstrated a significant increase in the total length of stay with increasing dose of statin but this finding did not remain statistically significant after adjustment for age and gender in multivariate analysis. Regarding survival, univariate survival analysis revealed a reduction in mortality associated with high-dose statin (p=0.049), but when adjusted for age and gender, multivariate Cox Regression analysis revealed no significant dose dependent difference in mortality although it is suggestive of an overall risk reduction with increasing dose of statin (HR=0.67, 95%CI=0.41-1.09, p=0.1100).

There were no observed differences in operative technique or tumour stage between statin and non-statin users. Interestingly, statin users were significantly more likely to have a colonic cancer (60.2% vs. 48.0%, p=0.0023) than a rectal cancer (39.8% vs. 51.9%, p=0.0023) and consequently significantly fewer rectal resections, a reduced requirement for neo-adjuvant therapy and fewer stomas formed. In multivariate analysis statin users were significantly less likely to require a stoma after elective colorectal cancer resection than non-statin users (OR=0.65, 95%CI=0.45-0.95, p=0.0280). It is known that of the 10,000 or so patients undergoing surgery for rectal cancer each year in the UK (Anterior Resection, Abdominoperineal Resection, Hartmann's Resection) over 80% undergo stoma formation of which the majority are temporary ileostomies used to 'cover' the colorectal anastomosis of

an Anterior Resection [13] and so this finding, in the context of fewer rectal cancer incidences, is not surprising.

The finding of relatively fewer rectal cancers compared to colon cancers has been reported in the published literature in cohorts from the USA and the Netherlands which describe a risk reduction for rectal cancer of 30-40% [273-275] in statin users. This finding has not been described in a patient cohort from the United Kingdom to date. It has been proposed that statins may affect cancer genotype [276] and they have been associated with a reduced incidence of KRAS-wild-type mutations which have a tendency to affect the rectum [277], perhaps by their role in prenylation of KRAS [278]. Statins have also been found to inhibit the production of cyclogenoxygenase-2 [279] which is also commonly found to be overexpressed in rectal cancer [280]. These molecular differences may, in part, explain the observed increased incidence of colonic versus rectal cancers in statin users.

This retrospective analysis has several limitations. As with any retrospective analysis, the study is limited by the patient groups not being matched at baseline and consequently this can introduce confounding with the potential for unmeasured factors influencing the observed results. Examples include the use of aspirin and metformin which, conceivably, would have been used more frequently in statin users and have been associated with reduced cancer related mortality [281-284]. Although peri-operative care was provided within the setting of an established enhanced recovery programme, which utilises a standardised surgical protocol, confounders in patient management cannot be excluded.

The definition of statin user was dependent upon the prescription of statins at admission and who were prescribed statins within a five day post-operative period. There was an assumption that patients prescribed statins at admission were taking statins regularly pre-operatively prior to their admission and were therefore within the window of potential

pleiotropy. Post-operatively only 8.5% of post-operative statin doses were omitted which equated to one dose per statin user (median=1, IQR 0-2 doses).

The strengths of this study include prospective data collection, high data completeness (associated with the use of robust electronic patient databases [Prescribing Information and Communication System and Clinical Portal)] and vigorous data validated) and the use of multivariate analysis in attempt to minimise confounding.

2.6 Conclusion

This study has explored the impact of peri-operative statin therapy on post-operative outcomes after elective colorectal cancer resection. Despite statin users having a higher peri-operative risk (significant increases in age, BMI and co-morbidity), they had equivalent outcomes in frequency of post-operative complications, post-operative antibiotic use, re-operations, re-admissions and there was no statistically significant difference in mortality after appropriate age and gender adjustments. Statin users were more likely to need Critical Care support and had an increased hospital length of stay than non-statin users. This may reflect a higher peri-operative risk but also an additional patient need for assistance with physiotherapy, occupational therapy and stoma therapy in this specific patient group. No statistically significant dose related differences were identified in patient outcomes although an overall risk reduction in mortality with increasing dose of statin remains possible. Statin users achieved equivalent short-term and long-term outcomes to non-statin users despite an increased operative risk and their use in the peri-operative period, particularly at high doses, merits further investigation.

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Chapter 3

MATERIALS AND METHODS

3.1 Study Methodology

3.1.1 Study Design

A favourable ethical opinion was awarded by the NRES Committee, West Midlands on 30th December 2013 (13/WM/0485).

A prospective, observational, cohort evaluation of consecutive consenting patients undergoing elective colorectal resection for cancer, within an established enhanced recovery programme, at the Heart of England NHS Foundation Trust, UK was conducted.

The cohorts evaluated were patients receiving peri-operative statin (statin users) and patients not receiving peri-operative statin (non-statin users). Statin users were defined as, 'patients were prescribed and had taken a statin ≤ 5 days before the index procedure *and* who were prescribed and received a statin ≤ 5 days after the index procedure'. All statins currently available for use in the UK were included (atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin).

Potential recruits were identified at the Colorectal Cancer Multi-disciplinary Team Meeting and patients were asked if they would consider taking part in research by members of the direct healthcare team and subsequently by a member of the study team. At all times patients were allowed as much time as they needed to read the Patient Information Sheet (Appendix 1) and were able to ask questions about the research study.

All patients over the age of 18 who were undergoing elective colorectal resection for cancer and were able to give written informed consent were eligible to be included in the study. Patients were excluded from the study if they were an acute presentation, pregnant or breast feeding or if consent was refused. All patients with a prior diagnosis of inflammatory

bowel disease (Ulcerative Colitis, Crohn's Disease), synchronous or metachronous malignancy or patents receiving any known immune-modulatory therapies (suppression immune-modulatory therapies; corticosteroids, monoclonal antibodies and activation immune-modulatory therapies; granulocyte-colony stimulating factor [G-CSF]) were also excluded from the study.

Written informed consent was obtained from patients prior to enrolment into the study. A suitably trained person, who had attended a UK regulations Good Clinical Practice training course, obtained written informed consent from each patient prior to participation in the study. This followed adequate explanation of the aims, methods, anticipated benefits and potential hazards of the study. This was recorded on a Patient Consent Form (Appendix 2).

The patient outcomes evaluated were:

1. Post-operative complications up to 30 days after surgery using pre-defined criteria and graded by the Clavien-Dindo classification system (Appendix 3).
2. Return to theatre within 28 days of the index procedure.
3. Admission to and length of stay on the Intensive Care Unit.
4. Total hospital length of stay.
5. Readmissions to hospital within 30 days of the index procedure requiring a hospital stay ≥ 24 hours.
6. 30 day and 90 day mortality.

Functional recovery and quality of life was assessed using the Surgical Recovery Scale. This is a validated, comprehensive multidimensional recovery questionnaire and was administered to patients pre-operatively and on post-operative day 3-5 (Appendix 4).

Pre-operative and post-operative haematological and biochemical parameters were collected prospectively from the hospital pathology system. These parameters, in conjunction with physiological indices, were used to determine a predicted mortality (CR-POSSUM [Appendix 5]) and a prognostic score (mGPS [Table 1.6]) for each patient.

Patient demographics, patient co-morbidities, pre-operative risk prediction, tumour characteristics and operative characteristics were collected by direct patient questioning and from the electronic patient record. This is summarised in Table 3.1 below.

Table 3.1 Patient Demographics, Patient Co-morbidities, Pre-operative Risk Prediction, Tumour Characteristics and Operative Characteristics recorded from study participants

Patient Demographics	Age BMI Gender Ethnicity Smoking Status ASA
Patient Co-morbidities	Cardiovascular Disease Respiratory Disease Chronic Kidney Disease Diabetes Mellitus
Pre-operative Risk Prediction	Colorectal-POSSUM Score
Tumour Characteristics	Cancer Location T-stage N-stage M-stage Dukes' Stage Tumour Differentiation Extra-mural Venous Invasion Resectional Completeness Total Mesorectal Excision Grade Neo-adjuvant Therapy
Operative Characteristics	Operation Type Operation Technique Stoma Formation Intra-operative Complication Intra-operative Blood Loss Operation Time Post-operative Level of Care

Patients underwent peripheral blood tests pre-operatively (Day-0) and post-operatively (Day-1 and Day-3) to assess neutrophil function at these specific time points. Blood tests were performed by an experienced medical practitioner by peripheral venepuncture or from a peripheral arterial cannula. A total of 24mls of blood was taken from each patient at each time point and deposited into 4x6ml lithium-heparin vacutainers (Becton-Dickinson, Oxford, UK). The samples were then placed on ice and transported to the laboratory for processing.

3.1.2 Data and Sample Management

Data was collected on Case Report Forms until the study participants were discharged from hospital. Data was anonymised and reviewed for completeness before entering onto a computer database which was stored securely against unauthorised access and accidental loss. All essential documents and study records were archived in conformance with the applicable regulatory requirements.

Samples were stored in a coded format in alarmed freezers at -80^{o}C located within the University of Birmingham Research Laboratories in accordance with the Human Tissue Act.

3.1.3 Study Regulations and Administration

The study complied with the principles of Good Clinical Practice, the requirements and standards set out by the EU Directive 2001/20/EC and the applicable regulatory requirements in the UK.

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki and was approved by the NRES Committee, West Midlands on 30th December 2013 (13/WM/0485).

The study was sponsored and indemnified by the Heart of England NHS Foundation Trust.

3.2 Neutrophil Isolation

Processing began within 45 minutes of obtaining the blood sample. Blood collected in lithium-heparin vacutainers (Becton-Dickinson) was transferred into a 50ml sterile Falcon-TM tube (Becton-Dickinson). 4mls of 2% Dextran (Sigma-Aldrich, Dorset, UK) was added to 24mls of blood (1ml per 6mls of blood) and gently mixed by inversion. The solution was incubated at room temperature for 30 minutes to sediment the erythrocytes.

The leucocyte-rich plasma was carefully layered on a Percoll (Sigma-Aldrich) density gradient consisting of 2.5mls of 80% Percoll and 5mls of 56% Percoll in a 15ml sterile Falcon-TM tube. Isolating neutrophils with Percoll has been demonstrated to induce minimal neutrophil activation [285] and was therefore utilised in the experiments conducted in this thesis. This was then centrifuged at 220G for 20 minutes at room temperature without acceleration or brake. The neutrophils were then isolated from the 80% and 56% gradient interface.

The neutrophils were suspended and subsequently washed in Phosphate Buffered Saline (PBS; Gibco Invitrogen, Paisley, UK) at 440G for 10minutes at room temperature. The resultant supernatant was removed and the neutrophils re-suspended in RPMI-1640 (Sigma-Aldrich) at a concentration of $1x10^6$/ml.

The purity and the viability of the neutrophil yield were checked using Giemsa stain (Diff-Quick, Gentaur Europe, Brussels, Belgium) and Tryptan-Blue exclusion respectively. A purity of ≥95% and a viability of ≥97% were routinely achieved.

3.3 Neutrophil Function Assays

3.3.1 Quantification of Neutrophil Extracellular Trap Formation

To ensure sterility the neutrophils were re-suspended in RPMI-1640 with 2nM L-Glutamine, 100U/ml Streptomycin and 100ug/ml Penicillin (all from Sigma-Aldrich) at a concentration of $1x10^6$/ml.

$6x10^6$ neutrophils were divided equally into two 15ml sterile Falcon-TM tubes (Becton-Dickinson). 333µl of RPMI-1640 with GPS was added to one 15ml sterile Falcon-TM tubes containing $3x10^6$ neutrophils (un-primed [1:10 dilution]) and 333µl of TNF-α (Sigma-Aldrich) was added to the other 15ml sterile Falcon-TM tubes containing $3x10^6$ neutrophils (primed [1:10 dilution]). These were gently mixed by inversion, incubated for 15 minutes at 37°C with 5% CO_2 and centrifuged at 400G for 10 minutes at room temperature. The resultant supernatant was removed and the neutrophils re-suspended in RPMI-1640 with GPS at a concentration of $1x10^6$/ml.

$1x10^5$ un-primed neutrophils, at a concentration of $1x10^6$/ml, were sited in 20 wells of a 96-well flat bottomed plate (Becton-Dickinson). An additional 75µl of RPMI-1640 with GPS was added to each well. 25µl of RPMI-1640 with GPS and 25µl Phorbol-12-myristate-13-acetate (PMA) (1mg/ml [1:800 then 1:10 dilution]) were each added to four wells as negative and positive controls respectively. Additionally, 25µl of the following stimulants (all from Sigma-Aldrich) were each added to four wells: interleukin-8 (IL-8) (6.25µM [1:625 dilution]), Lipopolysaccharide (LPS) (1mg/ml [1:1250 dilution]) and N-formylmethionyl-leucyl-phenylalanine (fMLP) (10mM [1:500 dilution]). The entire process was then repeated using primed neutrophils.

The wells of the 96-well flat bottomed plate were arranged as outlined in Figure 3.1 prior to incubation for 3 hours at 37°C with 5% CO_2.

	Un-primed Cells				*Primed Cells*			
A	Unstim	Unstim	Unstim	Unstim	Unstim	Unstim	Unstim	Unstim
B	PMA	PMA	PMA	PMA	PMA	PMA	PMA	PMA
C	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8
D	LPS	LPS	LPS	LPS	LPS	LPS	LPS	LPS
E	fMLP	fMLP	fMLP	fMLP	fMLP	fMLP	fMLP	fMLP

Figure 3.1 Arrangement of 96-well flat bottomed plate prior to incubation for Quantification of Neutrophil Extracellular Trap Formation Assay

Unstim = Unstimulated (RPMI-1640 with GPS), PMA = Phorbol-12-myristate-13-acetate, IL-8 = Interleukin-8, LPS = Lipopolysaccharide, fMLP = N-formylmethionyl-leucyl-phenylalanine

After the 3 hour incubation period, each well was then treated with 200units of Micrococcal Nuclease (MNase; Sigma-Aldrich) and 1µM of SYTOX-Green (Gibco Invitrogen) and incubated for 10 minutes at room temperature in the dark. This process stained and digested the extra-cellular DNA.

The contents of each of the wells were then transferred into individual 500µl eppendorfs and pelleted at 5000rpm for 10 minutes. 160µl of the DNA containing supernatant was transferred into a black 96-well flat bottomed plate (CoStar, Sigma-Aldrich) and fluorescence measured in a BioTek-Synergy-2 fluorometric plate reader (NorthStar Scientific

Ltd, Leeds, UK) with a filter setting of 485nm excitation and 530nm emission. NETs were recorded in arbitrary fluorescent units (AFU) and the mean was calculated from the 4 wells for each stimulant.

To account for the variation in number of circulating neutrophils between individuals the Absolute NET Production Potential (ANPP) was determined. This unique measure was calculated as follows:

ANPP (AFU / no. of neutrophils x10^5 / L) = NETs per 100,000 neutrophils (AFU) x Absolute Neutrophil Count (x10^9 / L) ÷ 10,000

3.3.2 Neutrophil Apoptosis

Neutrophil apoptosis was assessed using a commercially available assay and was carried out as per the manufacturer's instructions.

2x10^6 neutrophils suspended in RPMI-1640 (Sigma-Aldrich), at a concentration of 1x10^6/ml were divided equally into two cytometric tubes. One cytometric tube containing 1x10^6 neutrophils was incubated for 4 hours at 37°C with 5% CO_2 and the other was incubated in the same conditions for 24 hours.

Following incubation the neutrophils were pelleted at 500G for 5 minutes at room temperature. The pellet was washed twice with PBS (Gibco Invitrogen) at 500G for 5minutes at room temperature and then re-suspended in Annexin Buffer (BD Biosciences) at a concentration of 1x10^6/ml.

1x10^5 neutrophils were transferred into four cytometric tubes. 5µl PE-Annexin-V, 5µl 7-AAD and 5µl PE-Annexin-V *and* 5µl of 7-AAD (both from BD Biosciences) were added to three of the cytometric tubes respectively. One cytometric tube remained unstained. The

neutrophils were gently vortexed and incubated for 15 minutes at 25°C in the dark. Then 400µl of Annexin Buffer was added to each cytometric tube prior to immediate flow cytometric analysis using the Accuri-C6 (BD Accuri™) flow cytometer.

Apoptosis is characterised by specific morphological features which include loss of plasma membrane, condensation of cytoplasm and nucleus, and inter-nucleosomal cleavage of DNA. Loss of plasma membrane is evident in early apoptosis. Phosphatidylserine, a membrane phospholipid, is translocated from the inner to the outer plasma membrane and is exposed to the external cellular environment. Annexin-V is a calcium dependent phospholipid-binding protein that has a high affinity for phosphatidylserine and binds to cells that expose it. Annexin-V is conjugated to the fluorochrome phycoerythrin (PE) and is a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis, even at an early stage. PE Annexin-V staining precedes loss of membrane integrity and cell death and therefore the vital dye, 7-Amino-Actinomycin (7-AAD), is used in combination with PE Annexin-V. Cells are permeable to 7-AAD when membrane integrity is lost. Viable cells with intact membranes exclude 7-AAD and dead or damaged cells are permeable to 7-AAD and can be detected by flow cytometric analysis. Therefore, neutrophils that stain negative for PE Annexin-V and 7-AAD are alive and not undergoing measurable apoptosis. Neutrophils that stain positive for PE Annexin-V and negative for 7-AAD are undergoing early apoptosis. Neutrophils that stain positive for both PE Annexin-V and 7-AAD are undergoing late apoptosis. Neutrophils that stain negative for PE Annexin-V and positive for 7-AAD are considered necrotic.

When conducting flow cytometry analysis cell debris was eliminated using appropriate side and forward scatter gating. Data was analysed using the Accuri-C6 integrated software and

is represented as the percentage of neutrophils in the different stages of apoptosis (alive, early apoptosis, late apoptosis and necrosis).

The entire process was then repeated for the remaining neutrophils after 24 hours incubation at 37°C with 5% CO_2.

An example of a flow cytometry plot is demonstrated in Figure 3.2 revealing cells in different stages of apoptosis in different quadrants of the plot (LL = alive, LR = early apoptosis, UR = late apoptosis, UL = necrosis) after determining PE Annexin-V and 7-AAD positivity.

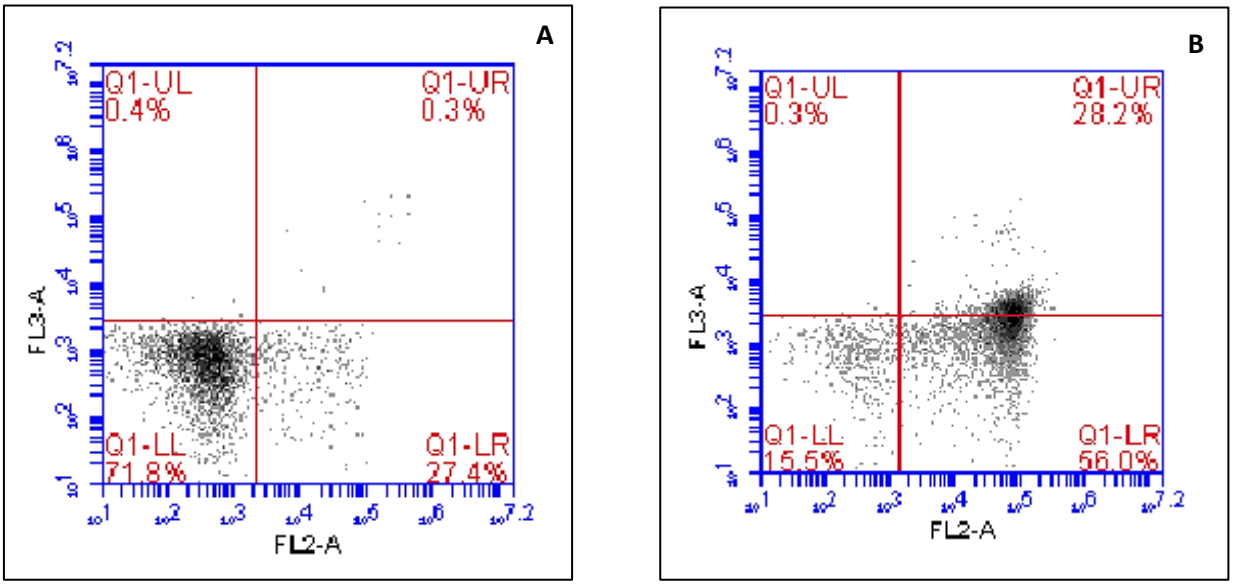


Figure 3.2 Flow Cytometry Plot for Neutrophil Apoptosis

Flow cytometry plot using Study Participant S37 on Day-0 at 4-hours [A] and 24-hours [B]. FL2-A (PE Annexin-V), FL3-A (7-AAD).

3.3.3 Neutrophil Phagocytosis

Neutrophil phagocytosis was assessed using a commercially available assay and was carried out as per the manufacturer's instructions.

The assay uses *Eschrichia coli* (*E.Coli)* and *Staphylococcus aureus* (*S.Aureus)* bio-particles conjugated to pHrodo (Gibco Invitrogen), a fluorescent dye that only fluoresces in the acidic environment of the phagolysosome.

2ml Hanks Balanced Salt Solution (HBSS) with 20mM HEPES (Sigma-Aldrich), was added to *E.Coli* and *S.Aureus* bio-particles conjugated to pHrodo and sonicated for 10 minutes.

$1x10^5$ neutrophils suspended in RPMI-1640 (Sigma-Aldrich) at a concentration of $1x10^6$/ml, were added to 2 eppendorfs on ice. 50µl of pHrodo *E.Coli* was added to one eppendorf and and 50µl of pHrodo *S.Aureus* was added to the other. These were the negative controls ('0-minute'). $1x10^5$ neutrophils, suspended in RPMI-1640 at a concentration of $1x10^6$/ml, were added to the wells of a 96-well 'U' bottomed plate (Becton-Dickinson) as shown in Figure 3.3.

		E.Coli	*S.Aureus*
Unstained	**X**		
IgG Alone	**X**		
CD16 Alone	**X**		
0-minute		**Added after 60 minutes on ice with pHrodo**	**Added after 60 minutes on ice with pHrodo**
30-minute		**X**	**X**
45-minute		**X**	**X**
60-minute		**X**	**X**
pHrodo Alone		**X**	**X**

Figure 3.3 Arrangement of 96-well 'U' bottomed plate for Neutrophil Phagocytosis Assay

50µl of pHrodo *E.Coli* and 50µl of pHrodo *S.Aureus* were added to the respective 'pHrodo Alone' and '60-minute' wells. The plate was then incubated at 37°C with 5% CO_2 in the dark. After 15 minutes and 30 minutes 50µl of pHrodo *E.Coli* and 50µl of pHrodo *S.Aureus* were added to the '45-minute' and '30-minute' wells respectively. After completing a further 30 minutes of incubation (60 minutes in total) the plate was removed from the incubator and placed on ice. The negative controls ('0-minutes') were then added to the plate. In addition, 50µl of 2% Bovine Serum Albumin in PBS (Gibco Invitrogen) was added to the 'Unstained', 'IgG Alone' and 'CD16 Alone' wells. The plate was centrifuged at 400G for 5

minutes at 4^{o}C and the supernatant was removed. The cells were re-suspended in 100µl of 2% Bovine Serum Albumin in PBS.

1µl of CD16 was added to the 'CD16 Alone', '0-minute', '30-minute', '45-minute' and '60-minute' wells and 1µl of IgG was added to the 'IgG Alone' wells. The plate was then kept on ice for 20 minutes. An additional 100µl of 2% Bovine Serum Albumin in PBS was added to each well and the plate was centrifuged at 400G for 5 minutes at 4^{o}C. The supernatant was removed and the cells were re-suspended in 200µl of 2% Bovine Serum Albumin in PBS and transferred to cytometric tubes for immediate flow cytometry analysis using the Accuri-C6 (BD Accuri™) flow cytometer.

When conducting flow cytometry analysis cell debris was eliminated using appropriate side and forward scatter gating. Data was analysed using the Accuri-C6 integrated software. Neutrophil phagocytotic function was represented by the Phagocytosis Index which provided a quantitative measure of neutrophil phagocytotic function. It was calculated as follows:

Phagocytosis Index = (Percentage of pHrodo 'bright' cells ÷ 100) x Median Fluorescent Intensity

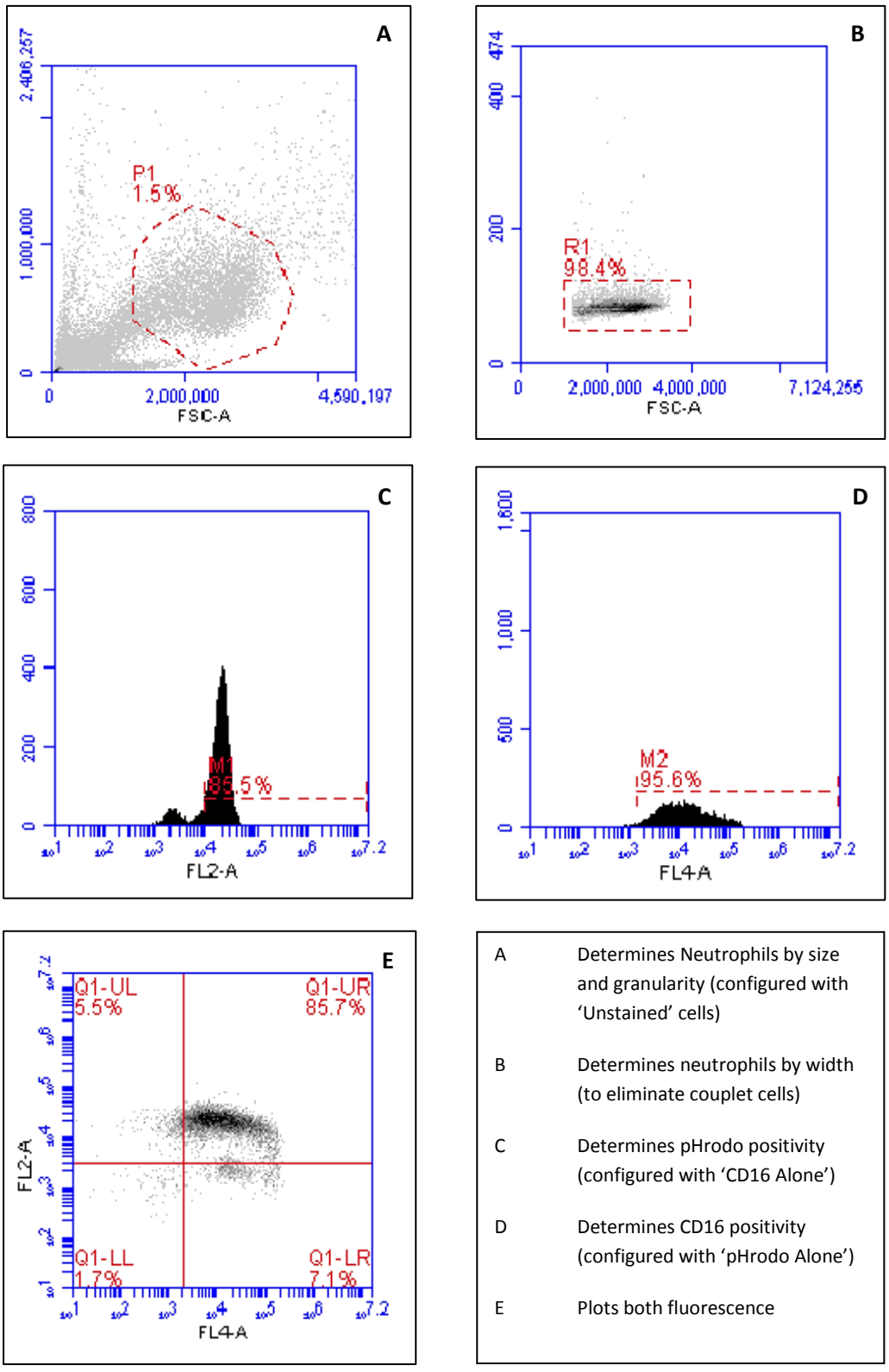


Figure 3.4 Flow Cytometry Plot for Neutrophil Phagocytosis

A flow cytometry plot using Study Participant S37 on Day-0 in response *to Staphylococcus aureus* bio-particles conjugated to pHrodo at '60-minutes' revealing the number of cells actively undertaking phagocytosis.

3.4 In-vitro Investigation with HMG-CoA Reductase Inhibitors

3.4.1 Dose Selection

The HMG-CoA reductase inhibitor used in the in-vitro experiments was simvastatin (Sigma-Aldrich).

The experiments previously conducted in our laboratory in establishing neutrophil function modulation with simvastatin suggest that the optimal in-vitro dose that can modify neutrophil function is 1μM (Greenwood, 2013 and Patel, 2014) [293, 294].

The dose used to investigate the in-vitro effects of simvastatin on neutrophil function was 1μM for the reasons outlined below:

- The 1μM dose of simvastatin was chosen as it represents the predicted plasma concentration following ingestion of 40mg simvastatin. This is calculated on the theory that simvastatin has high-first pass metabolism resulting in approximately 5% bio-availability. The concentration is calculated as follows:

(40mg x 0.05) / 5000ml [circulating volume] = 0.4μg/ml

Molecular Weight of simvastatin = 418μg/ml

This equates to an approximate 1μM concentration

In addition, the pleiotropic effects of simvastatin are thought to be dose-dependent with the greatest effects demonstrated at higher doses. Following ingestion of high-dose simvastatin (≥40mg) the plasma concentration is approximately 1μM (IQR 0.46-3.5μM), although great variability exists [286-288].

3.4.2 The Effect of Dimethyl Sulfoxide

The solvent Dimethyl Sulfoxide (DMSO; Sigma-Aldrich) is the vehicle control for simvastatin. The potential confounding effect of DMSO on NET formation was tested in ten separate experiments (Patel, 2014). No significant differences in NET formation were observed when testing unstimulated neutrophils with 1μM simvastatin or the equivalent dilution of DMSO ($p=0.436$) [294].

Quantification of Neutrophil Extracellular Trap Formation (3.3.1) and Neutrophil Apoptosis (3.3.2) assays were repeated after isolated neutrophils, suspended in RPMI-1640 (Sigma-Aldrich) at a concentration of $1x10^6$/ml, were incubated with 1μM simvastatin (Sigma-Aldrich) for 40 minutes at 37°C with 5% CO_2.

3.5 Histone Deacetylase Investigation

3.5.1 Histone Deacetylase Activity Assay

HDAC activity of neutrophils was evaluated using an HDAC Fluorometric Activity Assay Kit (Cayman Chemical) as per manufacturer's instructions.

The assay provides a fluorescent-based method of measuring HDAC activity. Firstly a lysine substrate is incubated with samples containing HDAC. Deacetylation sensitises the substrate such that treatment with the HDAC developer releases a fluorescent product that can be measured using a fluorescence plate reader.

Sample Preparation

$1x10^7$ neutrophils were suspended in 1ml of cold lysis buffer (10mM Tris-HCl, Gibco Invitrogen) in a sterile falcon-TM tube (Becton-Dickinson), then vortexed for 10 seconds and kept on ice for 15 minutes. 4mls of cold lysis buffer was then added and the neutrophils were then centrifuged at 1,300G for 10 minutes at 4^oC. The supernatant was removed and the resultant nuclei pellet was re-suspended in 1ml of cold lysis buffer which was centrifuged at 1,300G for 10minutes at 4^oC and the supernatant was discarded.

The nuclei pellet was suspended in 200µl of extraction buffer (50mM HEPES, Gibco Invitrogen) in an eppendorf, sonicated for 30 seconds and incubated on ice for 30 minutes. It was spun at 10,000G for 10 minutes at 4^oC in a micro-centifuge. The supernatant, containing the crude nuclear extract was stored at -80^oC until analysed.

Performing the Assay

The following were added to Deacetylated Standard Wells, HDAC-1 Positive Control Wells and Sample Wells:

- Deacetylated Standard Wells (used to prepare a standard curve for quantitative determination of HDAC activity).
  - 200µl of standard was diluted with 1.8ml of diluted assay buffer to obtain a stock solution of standard. The stock standard was then diluted as shown in Table 3.2.
  - 150µl of diluted assay buffer and 10µl of standard (A-F) were added to 12 wells (duplicate) of a black 96-well flat bottomed plate (CoStar; Sigma-Aldrich).
- HDAC-1 Positive Control Wells
  - 140µl of diluted assay buffer and 10µl of diluted HDAC-1 were added to four wells of the black 96-well flat bottomed plate.
- Sample Wells
  - 140µl of diluted assay buffer and 10µl of each sample were added to four wells of the black 96-well flat bottom plate (20 samples per plate).

Table 3.2 Standard Preparation for Histone Deacetylase Activity Assay

Tube	*Standard Stock (µl)*	*Assay Buffer (µl)*	*Standard Concentration (µM)*
A	0	1000	0
B	50	950	10.5
C	100	900	21
D	200	800	42
E	400	600	84
F	800	200	168

10µl of Trichostatin A was added to two of the positive control wells and to two of each of the sample wells. Trichostatin A eliminates all HDAC activity and is used as a control for generating sample background values. 10µl of diluted assay buffer were added to the remaining two wells of the positive controls and to the remaining two wells of each sample. The arrangement of the 96-well black plate is demonstrated in Figure 3.5.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	1	1	5	5	9	9	13	13	17	17
B	B	B	1T	1T	5T	5T	9T	9T	13T	13T	17T	17T
C	C	C	2	2	6	6	10	10	14	14	18	18
D	D	D	2T	2T	6T	6T	10T	10T	14T	14T	18T	18T
E	E	E	3	3	7	7	11	11	15	15	19	19
F	F	F	3T	3T	7T	7T	11T	11T	15T	15T	19T	19T
G	H	H	4	4	8	8	12	12	16	16	20	20
H	HT	HT	4T	4T	8T	8T	12T	12T	16T	16T	20T	20T

Figure 3.5 Arrangement of 96-well black plate for Histone Deacetylase Activity Assay

A – F = Standards A – F, H = HDAC-1 Positive Control, HT = Positive Control + Trichostatin A, 1 – 20 = Samples 1 – 20, 1T – 20T = Samples 1 -20 + Trichostatin A

10µl of HDAC substrate was then added to all of the wells and the plate was covered and incubated on a shaker for 30 minutes at 37°C. 40µl of Developer was then added to all of the wells and the plate was covered and incubated for 15 minutes at 25°C. Fluorescence was then measured in a BioTek-Synergy-2 fluorometric plate reader (NorthStar Scientific Ltd) with a filter setting of 360nm excitation and 465nm emission.

Calculations and Analysis

The average fluorescence of each standard, positive control, positive control + Trichostatin A, sample, and sample + Trichostatin A was calculated.

The average fluorescence of Standard A was subtracted from itself and all other standards and plotted as a function of the deactylated standard.

The average fluorescence of the Trichostatin A treated samples was subtracted from the fuorescence of its corresponding samples to give the corrected sample fluorescence (CSF)

The deacetylated concentration was then calculated as follows:

Deacetylated Compound (µM) = [(CSF – y-intercept)/slope]

The HDAC activity was calculated as follows:

HDAC Activity (nmol/min/ml) = [µM/30 minutes] x sample dilution

3.5.2 Quantitative Real-time PCR

Quantitative real-time PCR firstly requires RNA isolation and complementary DNA formation as outlined below.

RNA Isolation

Total RNA was extracted from neutrophils using NucleoSpin® RNA isolation kit (Macherney-Nagal) according to the manufacturer's protocol.

$1x10^7$ neutrophils were lysed by the addition of 350µl of Buffer RA1 and 3.5µl of β-mercaptoethanol and vortexed vigorously. The lysate was then cleared by filtration using the Nucleospin® Filter (Macherney-Nagal) and centrifuged at 11,000G for 1 minute. 350µl of ethanol (70%) was then added to the homogenised lysate and mixed by pipetting. The lysate was then transferred to a Nucleospin® RNA Column (Macherney-Nagal) and centrifuged at 11,000G for 30 seconds. 350µl of Membrane Desalting Buffer was then added and centrifuged at 11,000G for 1 minute.

10µl of reconstituted rDNase was added to 90µl of reaction buffer for rDNase and 95µl of DNase reaction mixture was added to the centre of the silica membrane of the Nucleospin® RNA Column and incubated for 15 minutes at 25 ^{o}C.

The Nucleospin® RNA Column was washed with 200µl Buffer RAW2 and then 600µl Buffer RA3 at 11,00G for 30 seconds. A subsequent wash was performed with 250µl Buffer RA3 at 11,00G for 2 minutes and the Nucleospin® RNA Column was placed into a nuclease-free collection tube.

The RNA was eluted in 60µl of RNase-free H_2O and centrifuged at 11,000G for 1 minute.

All RNA samples had high integrity and purity as assessed by NanoDrop ND1000 (NanoDrop Technologies).

Complementary DNA Formation

Complementary DNAs (cDNAs) were generated by reverse transcription using High Capacity RNA-to-cDNA Kit (Life Technologies).

20µl of the reverse transcriptase mix was added to each RNA sample (2µg total RNA per sample) of a 96-well plate. The plate was then loaded into a thermal cycler (37^{o}C for 60 minutes, 95^{o}C for 5 minutes then 4^{o}C thereafter) to begin the reverse transcription reaction and produce the cDNAs required for real-time PCR.

Quantitative Real-time PCR

Quantitative real-time PCR was performed under the expert guidance of Dr. Vijay D'Souza (Post-doctoral Research Scientist of the Institute of Inflammation and Ageing at the University of Birmingham, UK) using a Roche Light Cycler 480 (Roche; Life Science).

Quantitative real-time PCR was performed on the cDNA samples using the TaqMan® Gene Expression Assay and SYBR-green reagent (Applied Biosystems) as per manufacturer's instructions. SYBR-green dye is a double-stranded DNA binding dye that detects double-stranded DNA generated during PCR which negates the need to target specific probes.

GAPDH was used as the reference gene and the Class I (HDAC-1, HDAC-2, HADC-3) and Class II (HDAC-4, HDAC-7, HDAC-9) HDAC genes were the genes of interest.

The primer sequences and corresponding assay ID's used with the TaqMan® Gene Expression Assay are listed in Table 3.3.

Table 3.3 Primer Sequences and Assay IDs used with the TaqMan® Gene Expression Assay for Quantitative Real-time PCR

Genes		*Primer Sequence*		*Assay ID*
		Forward	*Reverse*	
Reference Gene	**GAPDH**	AGGGCTGCTTTTAACTCTGGT	CCCCACTTGATTTTGGAGGGA	HS99999905_m1
Genes of Interest	**HDAC-1**	ACCGGGCAACGTTACGAAT	CTATCAAAGGACACGCCAAGTG	HS00606262_g1
	HADC-2	TCATTGGAAAATTGACAGCATAGT	CATGGTGATGGTGTTGAAGAAG	HS00231032_m1
	HDAC-3	TTGAGTTCTGCTCGCGTTACA	CCCAGTTAATGGCAATATCACAGCT	HS00187320_m1
	HDAC-4	AATCTGAACCACTGCATTTCCA	GGTGGTTATAGGAGGTCGACACT	HS01041638_m1
	HDAC-7	CTGCATTGGAGGAATGAAGCT	CTGGCACAGCGGATGTTTG	HS00248789_m1
	HDAC-9	TCCCGATATGTCAGTATATATGA	GCTTCAATCAAAGAATGCACCAT	HS00206843_m1

Calculations and Analysis

Relative transcript abundance values of the genes of interest are expressed as ΔCt values (ΔCt = Ctreference – Cttarget) and they were used to calculate the statistical significance between groups.

The reciprocal values of the relative transcript abundance are expressed as 1/ΔCt and they were used to depict the data in graphical form.

3.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, California, USA).

Categorical data was analysed using Fisher's Exact tests for two variables or χ^2 tests for greater than two variables.

Continuous data was analysed using non-parametric statistical models. Mann-Whitney U tests were used for independent samples and Wilcoxon Signed Rank tests for related samples for two groups. Kruskal-Wallis tests were employed for independent samples and Freidmann's tests for related samples for greater than two groups.

Correlation was calculated using Spearman's Rank Correlation test to measure the association between two non-parametric variables. Results were represented as the correlation co-efficient and the 95% confidence interval.

All statistical tests performed were two-tailed and results were considered a significance when a p-value of less than 0.05 was calculated.

3.7 Sample Size Calculation

Sample size was based upon a power calculation related to NET formation. 42 patients were required to demonstrate a difference in NET production from Day-0 to Day-1 based upon a 2-tailed power calculation (80% power, p=0.05) using preliminary data (n=10) where a mean reduction of 3,045AFU (SD 4,929AFU) in NET formation in response to stimulation with fMLP was observed. To allow for experimental failure it was planned to recruit 45 patients to the study in total.

Chapter 4

NEUTROPHIL FUNCTION IN PATIENTS WITH COLORECTAL CANCER

4.1 Introduction

Neutrophils function as the first-line of defence against infections and are responsible for the containment and elimination of pathogens. They are prevalent at sites of tissue trauma and are the hallmark of acute inflammation [61]. Neutrophils are also appreciated to have an important role in cancer progression and dissemination [64]. It has been suggested that neutrophils are not a homogenous population of cells and may consist of pro-tumour and anti-tumour subpopulations. The polarisation of neutrophils towards a pro-tumour or anti-tumour phenotype may be mediated by the chemokine landscape in the tumour microenvironment [128]. The distinct anti-tumour and pro-tumour neutrophil phenotypes suggest that neutrophils differ in their contribution to the progression and dissemination of cancer and are capable of phenotypic plasticity. The characteristics of anti-tumour and pro-tumour neutrophil phenotypes are summarised in Table 1.8.

NETs are extra-cellular neutrophil derived DNA webs which have been implicated in cancer progression and in the development of metastases. NETs have been demonstrated to trap circulating tumour cells with a subsequent increase in the gross macro-metastatic disease burden, following tumour cell injection, in a murine model of infection of caecal ligation and puncture [158]. Activated neutrophils can undergo 'NETosis'. This is an active form of cell death that leads to the release of decondensed chromatin into the extracellular space [93, 94]. The process of oxidant dependent NET formation results in eventual cell death which is distinct from apoptosis and necrosis [94, 99]. NETs are recognised as an effective antimicrobial mechanism whereby microbes are trapped and exposed to high local concentrations of anti-microbials [96]. Conversely, NETs have been shown to have detrimental effects on the host with the formation of auto-antibodies against chromatin and

neutrophil components [108] and the adherence of platelets [109, 110] which has implicated them in autoimmune and thromboembolic diseases respectively.

NETs have been implicated in T-cell priming [140] and in the propagation of anti-tumour immune responses [141]. Conversely, and more frequently, they have been incriminated in tumour progression and tumour dissemination [64, 141, 157]. The role of NETs in tumour progression is poorly understood but the evidence to date proposes an association between intra-tumoural NET deposition and tumour progression in both experimental models and in patients with cancer [157,172,177]. The ability of tumours to predispose neutrophils to produce NETs has been demonstrated in murine models and various tumour types have been shown to predispose circulating neutrophils to produce NETs via NETosis [178]. The evidence supports the theory that primary tumours, through a systemic effect on the host, can induce an increase in peripheral blood neutrophils which are predisposed to NET formation.

It is anticipated that a greater understanding of neutrophil function in patients with colorectal cancer may help to elucidate their role and prognostic significance. It was therefore decided to investigate neutrophil function in patients with colorectal cancer.

4.2 Objectives

The objectives of this chapter were:

1. To define neutrophil function in patients with colorectal cancer with specific reference to:
    i. NET formation
    ii. Neutrophil apoptosis
    iii. Neutrophil phagocytosis
2. To determine the effect of priming neutrophils with TNF-α on NET formation.
3. To investigate the impact of cancer location and stage on NET formation.
4. To evaluate the differences in NET formation defined by patient outcomes with specific reference to:
    i. Post-operative Complications
    ii. Total Hospital Length of Hospital Stay
    iii. Mortality
5. To investigate the association between NET formation and existing validated prognostic scores:
    i. Neutrophil-Lymphocyte Ratio
    ii. Modified Glasgow Prognostic Score

4.3 Methods

4.3.1 Acknowledgements

Dr. Hongxia Mei (International Research Fellow from the Hospital of Wenzouh Medical University, China) assisted with the Neutrophil Phagocytosis experiments. The short life-span of neutrophils meant that neutrophil functional experiments were conducted simultaneously and it was impossible for all experiments to be performed by one individual in entirety.

4.3.2 Recruitment of Patients

Patients undergoing an elective colorectal resection for cancer, within an established enhanced recovery programme, who satisfied the specific inclusion and exclusion criteria, were recruited to the study as outlined in Section 3.1.1.

Patient Demographics, Patient Co-morbidities, Pre-operative Risk Prediction, Tumour Characteristics and Operative Characteristics were recorded from study participants as outlined in Table 3.1.

Patient outcomes were collected prospectively and included:

1. Post-operative complications up to 30 days after surgery using pre-defined criteria and graded by the Clavien-Dindo classification system (Appendix 3).
2. Return to theatre within 28 days of the index procedure.
3. Admission to and length of stay on the Intensive Care Unit.
4. Total hospital length of stay.

5. Readmissions to hospital within 30 days of the index procedure requiring a hospital stay ≥ 24 hours.
6. 30 day and 90 day mortality.

Functional and quality of life was assessed using the Surgical Recovery Scale (Appendix 4).

Haematological and biochemical parameters were collected prospectively from the hospital pathology system. These parameters, in conjunction with physiological indices, were used to determine a CR-POSSUM (Appendix 5), a NLR, and an mGPS (Table 1.6) for each patient.

4.3.3 Sample Collection

Patients underwent peripheral blood tests. Blood tests were performed by an experienced medical practitioner by peripheral venepuncture or from a peripheral arterial cannula. A total of 24mls of blood was taken from each patient and deposited into 4x6ml lithium-heparin vacutainers (Becton-Dickinson, Oxford, UK). The samples were thenne placed on ice and transported to the laboratory for processing.

4.3.4 Neutrophil Isolation

Processing began with 45 minutes of obtaining the blood sample. Neutrophils were isolated by following the method outlined in Section 3.2. A purity of ≥95% and a viability of ≥97% were routinely achieved.

4.3.5 Neutrophil Functional Assays

Quantification of Neutrophil Extracellular Trap Formation was conducted following the method outlined in Section 3.3.1. NETs were recorded in arbitrary fluorescent units (AFU).

To account for the variation in number of circulating neutrophils between individuals the Absolute NET Production Potential (ANPP) was determined. It was calculated as follows:

ANPP (AFU / no. of neutrophils x10^5 / L) = NETs per 100,000 neutrophils (AFU) x Absolute Neutrophil Count (x10^9 / L) ÷ 10,000

Neutrophil Apoptosis and Neutrophil Phagocytosis were assessed using commercially available assays which were carried out as per the manufacturer's instructions as outlined in Sections 3.3.2 and 3.3.3 respectively. Neutrophil Apoptosis was represented as the percentage of neutrophils in the different stages of apoptosis (alive, early apoptosis, late apoptosis and necrosis). Neutrophil phagocytotic function was represented by the Phagocytosis Index which provided a quantitative measure of neutrophil phagocytotic function. It was calculated as follows:

Phagocytosis Index = (Percentage of pHrodo 'bright' cells ÷ 100) x Median Fluorescent Intensity

4.3.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, California, USA).

Categorical data was analysed using Fisher's Exact tests for two variables or χ^2 tests for greater than two variables. Continuous data was analysed using non-parametric statistical models. Mann-Whitney U tests were used for independent samples and Wilcoxon Signed Rank tests for related samples for two groups. Receiver operator characteristic (ROC) curves were used to measure how well diagnostic tests distinguish between two diagnostic groups. Correlation was calculated using Spearman's Rank Correlation test and results were represented as the correlation co-efficient and the 95% confidence interval. All statistical tests performed were two-tailed and results were considered significant when $p<0.05$.

4.4 Results

4.4.1 Population Characteristics

55 patients were identified at the MDT meeting and approached for inclusion into the study. 45 patients (81.8%) were successfully recruited into the study, between March 2014 and March 2015, and were followed up for a median of 21.3 months (IQR 16.7-23.5 months). Consent was refused from the remaining 10 patients (18.2%). The baseline population characteristics are shown in Table 4.1.

Table 4.1 Population Characteristics

Patient Demographics	***N=45***
Age, median (IQR)	69.0 (63.0-75.0)
Gender, n (%), M/F	26 / 19 (57.8 / 42.2)
BMI, median (IQR), kg/m^2	26.8 (22.5-28.7)
Smoking Status, n (%), 1/2/3[a]	9 / 9 / 27 (20.0 / 20.0 / 40.0)
ASA, n (%), 1/2/3/4	4 / 25 / 14 / 2 (8.9 / 55.6 / 31.1 / 4.4)
Co-morbidity, n (%), 0/1/2/≥3	10 / 21 / 10 / 4 (22.2 / 46.7 / 22.2 / 8.9)
Medications, n, 1/2/3/4[b]	21 / 9 / 11 / 7
CR-POSSUM (Predicted Mortality [%]), median (IQR)	2.5 (1.3-3.9)
mGPS, n (%), 0/1/2	34 / 3 / 8 (75.6 / 6.7 / 17.8)
NLR, median (IQR)	3.2 (2.0-5.3)
Surgical Recovery Score (Pre-operative [%]), median (IQR)	75.3 (69.9-87.0)
Presentation	***N=45***
Presentation, n (%), 1/2[c]	39 / 6 (86.7 / 13.3)
Cancer Location, n (%), 1/2[d]	27 / 18 (60.0 / 40.0)
Tumour Characteristics	***N=44***
Neo-adjuvant Therapy, n (%), y	11 (24.4)
T-Stage, n (%), 0/1/2/3/4	2 / 2 / 9 / 22 / 9 (4.5 / 4.5 / 20.5 / 50.0 / 20.5)
N-Stage, n (%), 0/1/2	32 / 8 / 4 (72.7 / 18.2 / 9.1)
M-Stage, n (%), 0/1	42 / 2 (95.5 / 4.5)
Dukes' Stage, n(%), A/B/C/D	9 / 22 / 11 / 2 (20.5 / 50.0 / 25.0 / 4.5)
Differentiation, n (%), 1/2[e]	39 / 5 (88.6 / 11.4)
Extra-mural Venous Invasion, n(%), y	14 (32.6)

y = yes, 1/2/3[a] = active / former / never, 1/2/3/4[b] = antihypertensive / β-antagonist / anti-platelet / oral hypoglycaemic, 1/2[c] = symptomatic / screened, 1/2[d] = colon / recto-sigmoid junction and rectum, 1/2[e] = well and moderately differentiated / poorly differentiated

The median age of the population was 69 years (IQR 63-75 years) and 26 patients (57.8%) were male. The median BMI was 26.75kg/m^2 (IQR 22.5-28.7 kg/m^2). 14 patients (31.1%) had two or more significant co-morbidities and 16 patients (35.6%) had an ASA score of 3 or 4. The predicted mortality of the population, calculated by the CR-POSSUM, was 2.5% (IQR 1.3-3.9%).

39 patients (86.7%) were symptomatic and the remaining 6 patients (13.3%) were identified through the NHS Bowel Cancer Screening Programme. 27 patients (60.0%) had colonic cancer whereas 18 patients (40.0%) had rectal cancer or a cancer of the recto-sigmoid junction and 11 patients (24.4%) underwent neo-adjuvant therapy (10; long-course chemo-radiotherapy, 1; short-course radiotherapy) prior to surgery. 31 patients (70.5%) had early stage disease (Dukes' A or B) versus 13 patients (29.5%) who had late stage (node positive or metastatic) disease (Dukes' C or D).

44 patients (97.8%) underwent surgical resection. 1 patient was deemed unfit for surgery on the day of surgery and never progressed to surgical resection. The operative characteristics are outlined in Table 4.2.

Table 4.2 Operative Characteristics

Operative Characteristics	*N=44*
Operation Type, n (%), 1/2[a]	25 / 19 (56.8 / 43.2)
Operation technique, n (%), 1/2[b]	27 / 17 (61.4 / 38.6)
Stoma Formation, n (%), y	16 (36.4)
Length of Operation (minutes), median (IQR)	174 (129-240)
Post-operative Level of Care, n (%), 1/2[c]	27 / 17 (61.4 / 38.6)

y = yes, 1/2[a] = segmental / rectal, 1/2[b] = laparoscopic / open and laparoscopic converted to open, 1/2[c] = ward based care / Critical Care

27 procedures (61.4%) were completed entirely laparoscopically and the median operative time for all operations was 174 minutes (IQR 129-240 minutes). 16 patients (36.4%) underwent stoma formation, the majority of which were temporary ileostomies (10 patients [62.5%]) to cover a colorectal anastomosis. 17 patients (38.6%) required admission to Critical Care following surgery, the majority were planned admissions (12 patients [70.6%]) and a reflection of the patients' co-morbidity. The patient outcomes are shown in Table 4.3.

Table 4.3 Patient Outcomes

Patient Outcomes		*N=44*
Complication, n (%), y		22 (50.0)
Clavien-Dindo Classification, n (%), I/II/III/IV/V		12 / 4 / 2 / 2 / 2 (27.3 / 9.1 / 4.5 / 4.5 / 4.5)
Re-operation, n (%)		3 (6.8)
Critical Care Admission, n (%), y		19 (43.2)
Total LOS, median (range, IQR)		8.0 (4.0-67.0, 5.3-10.0)
Surgical Recovery Score (Post-operative [%]), median (IQR)		41.4 (34.6 – 53.4)
Readmission, n (%), y		3 (6.8)
Disease Recurrence, n (%)		4 (9.1)
Mortality, n (%), y		
	30-day	1 (2.3)
	90-day	2 (4.5)
	12-month	3 (6.8)
	24-month	5 (11.4)

Y = yes

22 patients (50.0%) developed a complication, although only 6 patients (13.6%) developed a significant complication (Clavien-Dindo ≥ 3). The median length of stay was 8 days (IQR 5.3-10.1 days) and only 3 patients (6.8%) were readmitted up to 30 days of the index procedure. 30-day, 90-day and 12-month mortality was 2.3%, 4.5% and 6.8% respectively with a median follow-up of 21.3 months (IQR 16.7-23.5 months). During the entire study period, 5 patients (11.4%) died and 4 patients (9.1%) developed recurrent disease, but were still alive.

Of the patients that developed complications the most frequent were cardio-resiratory events (14) which encompassed lower respiratory tract infection (4), ischaemic events (4), arrhythmias (4) and cardiac failure (2). This was followed by mechanical / functional bowel obstruction (7) and surgical site infection (7). 3 patients (6.8%) underwent re-operation; Anastomotic repair and defunctioning loop ileostomy (1), Evacuation of haematoma and defunctioning ileostomy (1), Re-suture of abdominal wall (1). The cumulative complication type is outlined in Table 4.4.

Table 4.4 Cumulative Complication Type

Complication	*Frequency*
Abdominal Collection	2
Acute Kidney Injury	6
Anastomotic Dehiscence	1
Bacteraemia	0
Blood Product Transfusion	4
Cardio-respiratory Event	10
Intestinal Obstruction	7
Lower Respiratory Tract Infection	4
Multi-organ Dysfunction Syndrome	4
Surgical Site Infection	7
Urinary Tract Infection	2

Functional and quality of life assessment was conducted pre-operatively and post-operatively (day 3-5) in the form of the Surgical Recovery Score. A median reduction of 33.9% (75.3% to 41.4%) function was identified which indicates the impact of resectional surgery on subjective functional capacity in this particular patient cohort.

4.4.2 Neutrophil Extracellular Trap Formation

Quantification of NET formation was performed in patients with colorectal cancer (n=45). The baseline population characteristics and NET production is summarised in Table 4.5. NET production is plotted graphically in Figure 4.1. The effect of priming neutrophils with TNF-α was assessed in patients with colorectal cancer. NET production is displayed in Table 4.6 and Figure 4.2.

Table 4.5 Age, Gender and NET Production in Patients with Colorectal Cancer

	Colorectal Cancer (n=45)
Age, median (IQR)	69.0 (63.0-75.0)
Gender, n (%), M/F	26 / 19 (57.8 / 42.2)
NET Production (AFU), median (IQR)	
Unstimulated	11347 (4927-8341)
PMA	39238 (29497-47988)
IL-8	11915 (9031-15358)
LPS	12473 (9381-16542)
fMLP	12194 (8602-15991)

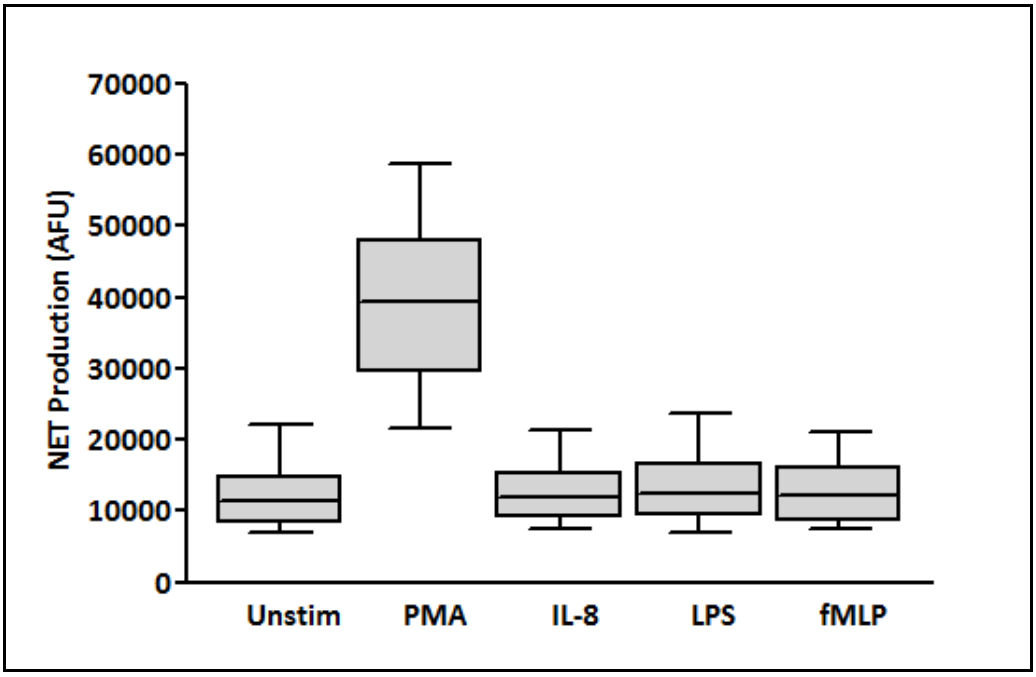


Figure 4.1 NET Production in Patients with Colorectal Cancer

Box and Whisker Plot (10-90 percentile) of NET production in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP in patients with a confirmed diagnosis of Colorectal Cancer (n=45).

Table 4.6 NET Production in Un-primed and Primed Neutrophils

	Un-primed (n=45)	*Primed (n=45)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	11347 (4927-8341)	12590 (11830-17020)	**0.0021**
PMA	39238 (29497-47988)	32180 (24970-40950)	**<0.0001**
IL-8	11915 (9031-15358)	14970 (11960-18080)	**0.0007**
LPS	12473 (9381-16542)	14480 (11940-18260)	**0.0233**
fMLP	12194 (8602-15991)	13720 (11560-18250)	**0.0002**

The effect of priming neutrophils with TNF-α on NET Production in patients with Colorectal Cancer (n=45). Statistical significance was measured by the Wilcoxon Signed Rank test.

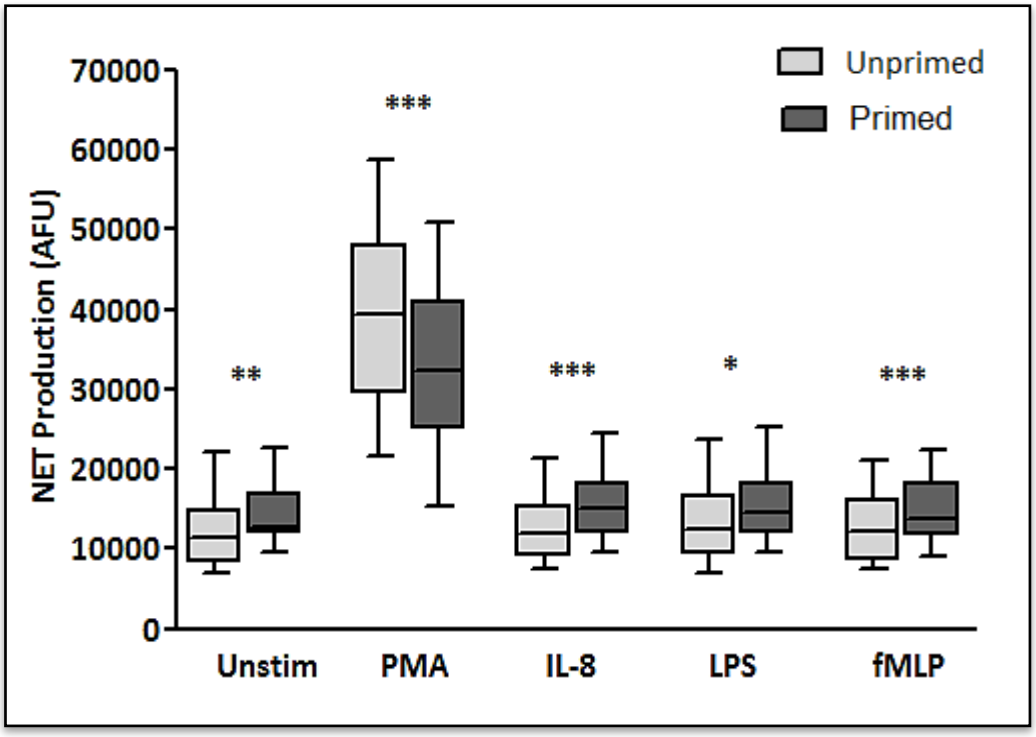


Figure 4.2 NET Production in Un-primed and Primed Neutrophils

Box and Whisker Plot (10-90 percentile) of NET production demonstrating the effect of priming neutrophils with TNF-α on NET production in patients with Colorectal Cancer (n=45) in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

When neutrophils from a colorectal cancer population were primed with TNF-α, NET production was significantly increased in response to No stimulant, IL-8, LPS and fMLP. Conversely, a significant reduction in NET production was demonstrated in response to PMA.

4.4.3 Neutrophil Apoptosis

Neutrophil apoptosis was assessed in patients with colorectal cancer (n=43). Neutrophil apoptosis was represented as the percentage of neutrophils in the different stages of apoptosis (alive, early apoptosis, late apoptosis and necrosis). The baseline population characteristics and stage of apoptosis is summarised in Table 4.7. Neutrophil apoptosis at 4-hours and 24-hours is plotted graphically in Figure 4.3.

Table 4.7 Age, Gender and Stage of Apoptosis at 4-hours and 24-hours in Patients with Colorectal Cancer

	Colorectal Cancer (n=43)
Age, median (IQR)	69.0 (63.0-74.0)
Gender, n (%), M/F	25 / 18 (58.1 / 41.9)
Stage of Apoptosis (%) 4-hours, median (IQR)	
Alive	76.90 (71.20-84.10)
Early Apoptosis	16.80 (11.50-25.90)
Late Apoptosis	3.10 (1.70-4.50)
Necrosis	1.40 (0.80-2.40)
Stage of Apoptosis (%) 24-hours, median (IQR)	
Alive	14.05 (10.23-21.30)
Early Apoptosis	71.85 (57.60-80.28)
Late Apoptosis	9.30 (4.90-22.05)
Necrosis	0.30 (0.10-0.60)

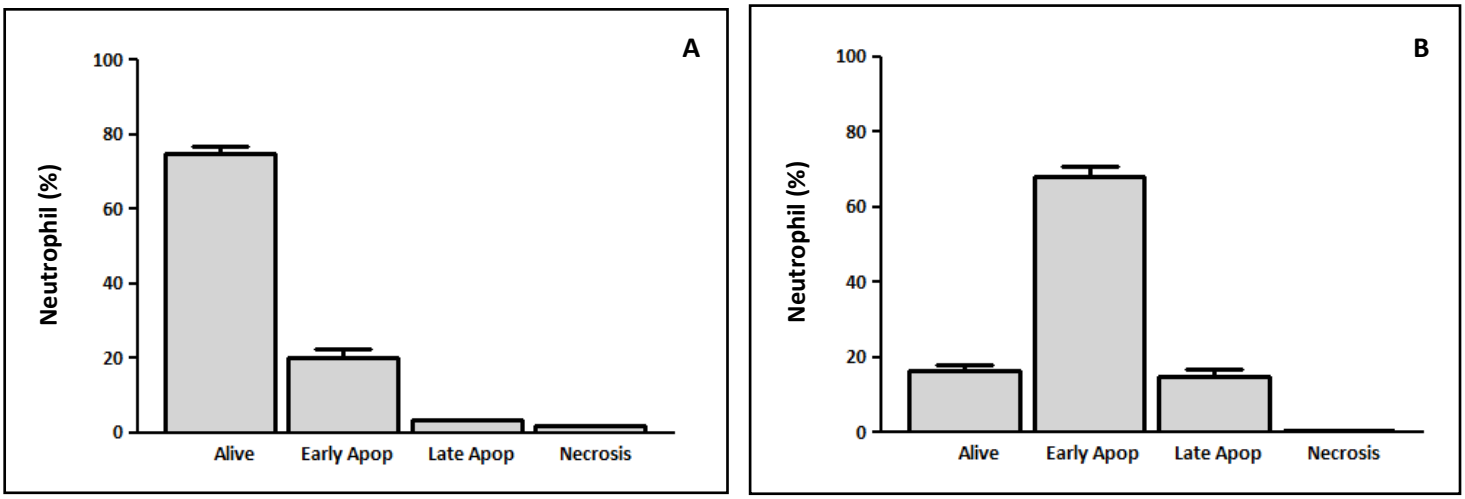


Figure 4.3 Stage of Apoptosis in Patients with Colorectal Cancer at 4-hours and 24-hours

Bar graphs depicting the Stage of Apoptosis (Alive, Early Apoptosis [Early Apop], Late Apoptosis [Late Apop], Necrosis) of Neutrophils (Mean [%] + SEM) at 4-hours (A) and 24-hours (B) incubation in patients with Colorectal Cancer (n=43).

4.4.4 Neutrophil Phagocytosis

Neutrophil phagocytosis was assessed in patients with colorectal cancer (n=27). Neutrophil phagocytotic function was represented by the Phagocytosis Index which provided a quantitative measure of neutrophil phagocytotic function. The baseline population characteristics and Phagocytosis Index in response to *E.Coli* and *S.Aureus* is shown in Table 4.8 and represented graphically in Figure 4.4.

Table 4.8 Age, Gender and Phagocytosis Index in response to *E.Coli* and *S.Aureus* in Patients with Colorectal Cancer

	Colorectal Cancer (n=27)
Age, median (IQR)	69.6 (64.0–76.0)
Gender, n (%), M/F	18 / 9 (66.7 / 33.3)
E.Coli (Phagocytosis Index), median (IQR)	
30 minutes	3446 (2300-7174)
45 minutes	6615 (4436-10700)
60 minutes	9777 (6463-14870)
E.Coli (Area Under Curve), median (IQR)	3255 (2220-5564)
S.Aureus (Phagocytosis Index), median (IQR)	
30 minutes	4630 (2194-8605)
45 minutes	8240 (6253-13370)
60 minutes	14760 (10030-18050)
S.Aureus (Area Under Curve), median (IQR)	4507 (3185-6602)

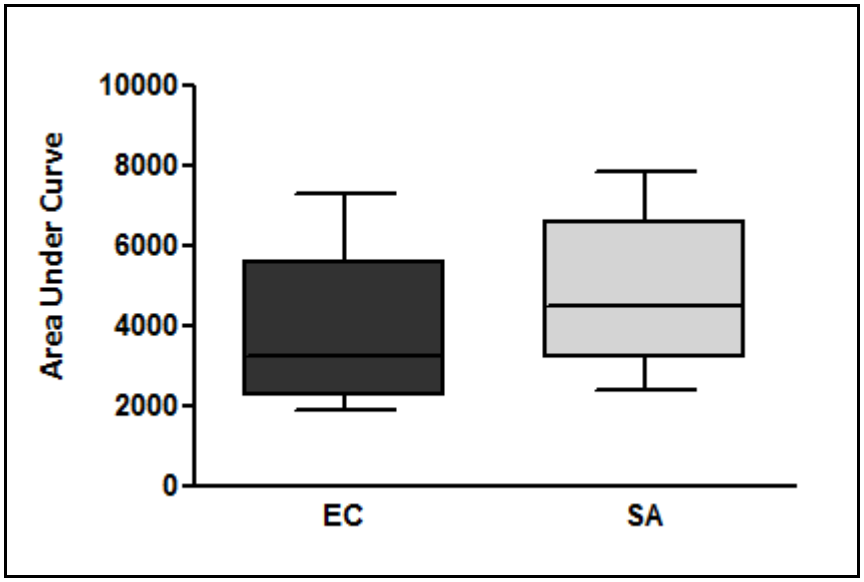


Figure 4.4 Phagocytosis Index in Patients with Colorectal Cancer in response to *E.Coli* and *S.Aureus*

Box and Whisker Plot (10-90 percentile) of Phagocytosis Index represented by Area Under Curve (considering 30, 45 and 60 minute time points) in response to *E.Coli* (EC) and *S.Aureus* (SA) in patients with a confirmed diagnosis of Colorectal Cancer (n=27).

4.4.5 The Impact of Cancer Location and Stage on NET Production

The impact of cancer location and stage of disease on NET formation was investigated. To account for the variation in number of circulating neutrophils between individuals the Absolute NET Production Potential (ANPP) was also determined. NET production and ANPP in patients with rectal cancer compared to colonic cancer is outlined in Table 4.9 and Figure 4.5. NET Production and ANPP in patients with early stage (Dukes' A/B) compared to late stage (Dukes' C/D) colorectal cancer is summarised in Table 4.10 and Figure 4.6.

The use of neo-adjuvant therapy, which is generally reserved for late stage rectal cancers, was also analysed to determine the effect on NET production and ANPP and is displayed in Table 4.11 and Figure 4.7.

Table 4.9 NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients with Rectal Cancer vs. Colonic Cancer

	Rectal Cancer (n=18)	*Colonic Cancer (n=27)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	10190 (8057-14580)	12140 (9980-1499)	0.2711
PMA	39490 (29050-49190)	38100 (29370-48840)	0.8078
IL-8	11080 (8245-15610)	11960 (9998-15350)	0.4655
LPS	11600 (8515-15580)	12730 (11210-18640)	0.2813
fMLP	10520 (8384-14910)	12440 (9203-16850)	0.4241
Absolute NET Production (AFU / no. neutrophils x 10^5/ L), median (IQR)			
Unstimulated	3.89 (2.58-5.67)	5.67 (4.54-9.58)	**0.0145**
PMA	12.47 (9.188-20.27)	19.36 (12.58-36.72)	**0.0500**
IL-8	3.98 (3.21-6.24)	6.35 (3.95-10.33)	**0.0165**
LPS	4.105 (2.93-6.19)	6.390 (5.36-10.68)	**0.0092**
fMLP	4.11 (2.48-6.10)	6.34 (4.160-10.98)	**0.0165**
Absolute Neutrophil Count (x10^9/L), median (IQR)	3.93 (2.73-4.44)	5.20 (4.19-6.50)	**0.0085**

A comparison of NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in patients with Rectal Cancer (n=18) versus Colonic Cancer (n=27). Statistical significance was measured by the Mann Whitney U test.

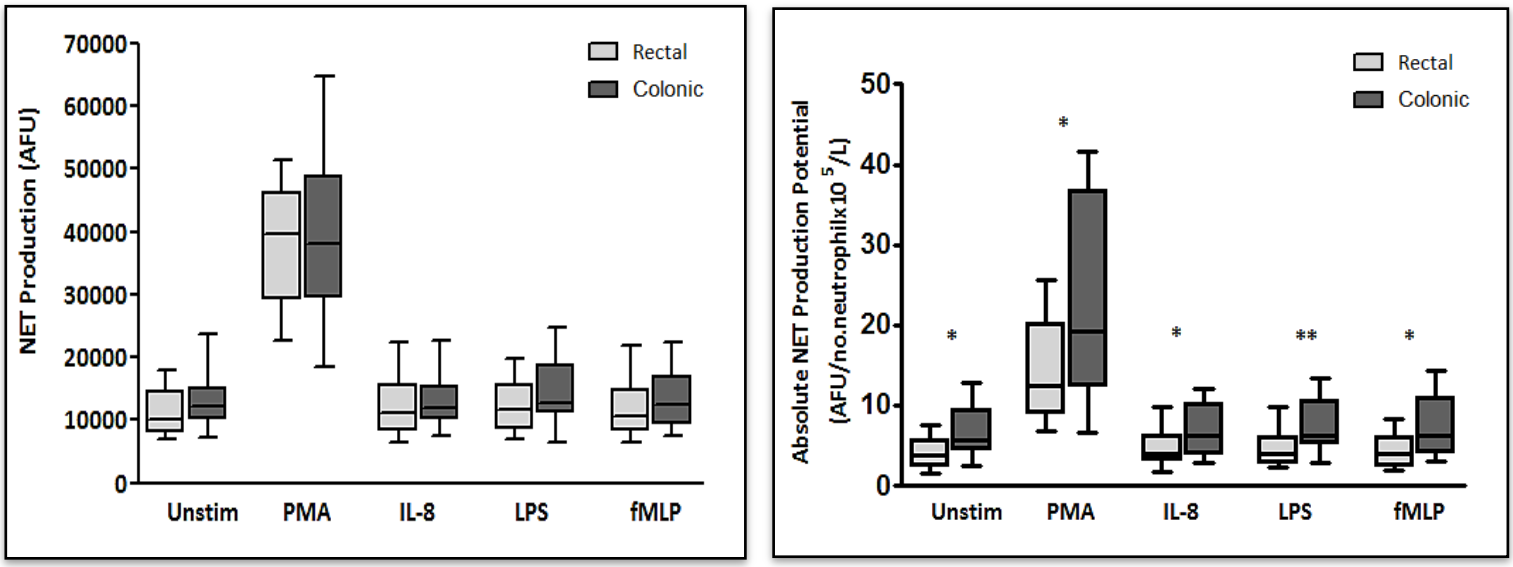


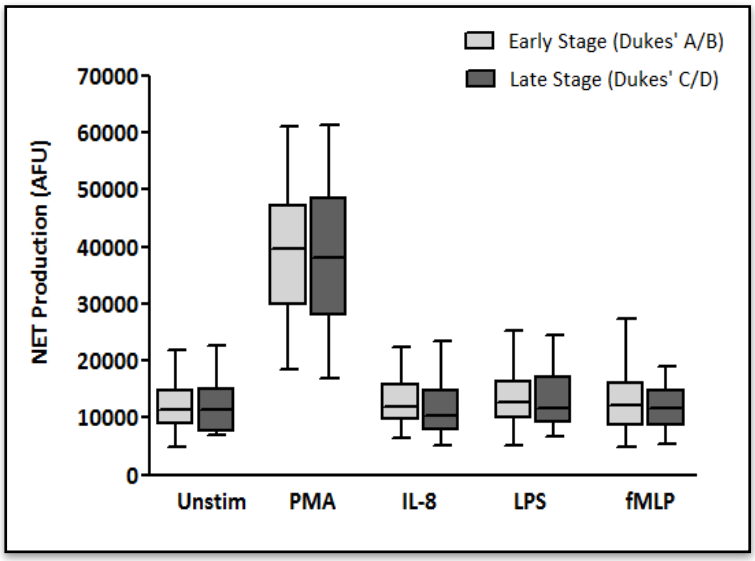
Figure 4.5 NET Production and Absolute NET Production Potential in Patients with Rectal vs. Colonic Cancer

Box and Whisker Plots (10-90 percentile) of NET production and Absolute NET Production Potential in patients with Rectal Cancer (n=18) versus Colonic Cancer (n=27) in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 4.10 NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients with Early Stage vs. Late Stage Colorectal Cancer

	Early Stage (Dukes' A/B) (n=32)	*Late Stage (Dukes' C/D) (n=13)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	11340 (8862-14880)	11350 (7476-15150)	0.7353
PMA	39490 (29640-47200)	38100 (27730-48530)	0.8314
IL-8	11990 (9574-15750)	10320 (7790-14820)	0.3105
LPS	12600 (9783-16280)	11540 (9106-17270)	0.6795
fMLP	12200 (8500-16050)	11780 (8624-14840)	0.6432
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	5.54 (3.76-8.98)	4.46 (2.55-7.34)	0.1464
PMA	17.76 (12.12-29.59)	12.01 (6.92-24.16)	0.3876
IL-8	6.11 (4.01-9.45)	3.60 (2.74-7.31)	**0.0438**
LPS	6.06 (3.95-9.65)	4.07 (2.94-9.50)	0.2392
fMLP	5.96 (3.98-8.28)	4.09 (2.90-8.19)	0.1571
Absolute Neutrophil Count (x10^9/L), median (IQR)	4.94 (3.87-5.97)	4.21 (2.93-6.14)	0.2733

A comparison of NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients with with Early Stage (n=32) vs. Late Stage (n=13) Colorectal Cancer**.** Statistical significance was measured by the Mann Whitney U test.



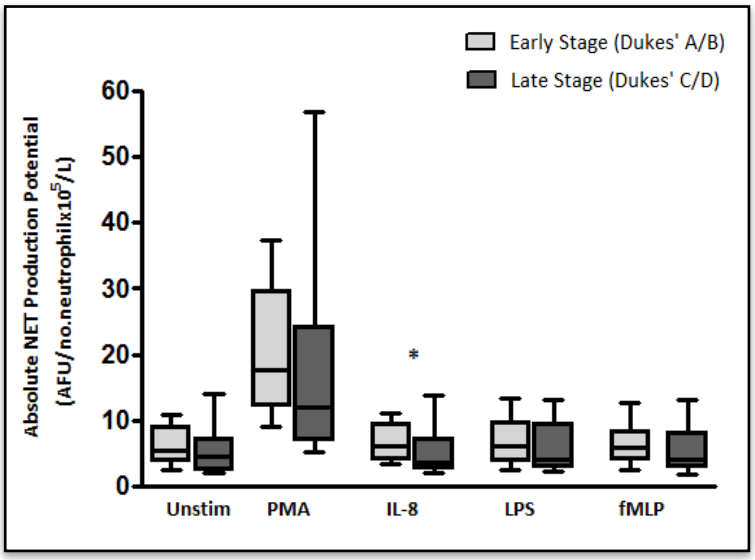


Figure 4.6 NET Production and Absolute NET Production Potential in Patients with Early Stage vs. Late Stage Colorectal Cancer

Box and Whisker Plots (10-90 percentile) of NET production and Absolute NET Production Potential in patients with Early Stage Colorectal Cancer (n=32) versus Late Stage Colorectal Cancer (n=13) in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 4.11 NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who were treated with Neo-adjuvant Therapy vs. Patients who went Straight to Surgery

	Neo-adjuvant Therapy (n=11)	*Straight to Surgery (n=34)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	9039 (7507-13230)	11780 (9031-15460)	0.1577
PMA	34830 (27140-41590)	40330 (29560-49070)	0.2002
IL-8	10320 (8306-15190)	12010 (9349-15980)	0.2295
LPS	13900 (8323-14930)	12380 (10070-17120)	0.4516
fMLP	11400 (8339-15870)	12330 (8704-17200)	0.4205
Absolute NET Production (AFU / no. neutrophils x 10^5/L), Median (IQR)			
Unstimulated	3.87 (2.47-5.66)	5.54 (3.75-5.54)	0.1161
PMA	11.97 (7.96-24.93)	19.08 (12.33-26.42)	0.2194
IL-8	5.14 (2.33-6.18)	5.87 (3.68-9.53)	0.1391
LPS	4.19 (2.36-9.34)	6.14 (3.93-9.65)	0.1357
fMLP	4.74 (2.23-6.31)	5.85 (3.70-8.77)	0.2097
Absolute Neutrophil Count (x10^9/L), median (IQR)	3.92 (2.49-6.26)	4.47 (3.66-5.77)	0.3834

A comparison of NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who were treated with Neo-adjuvant Therapy (n=11) vs. Patients who went Straight to Surgery (n=34). Statistical significance was measured by the Mann Whitney U test.

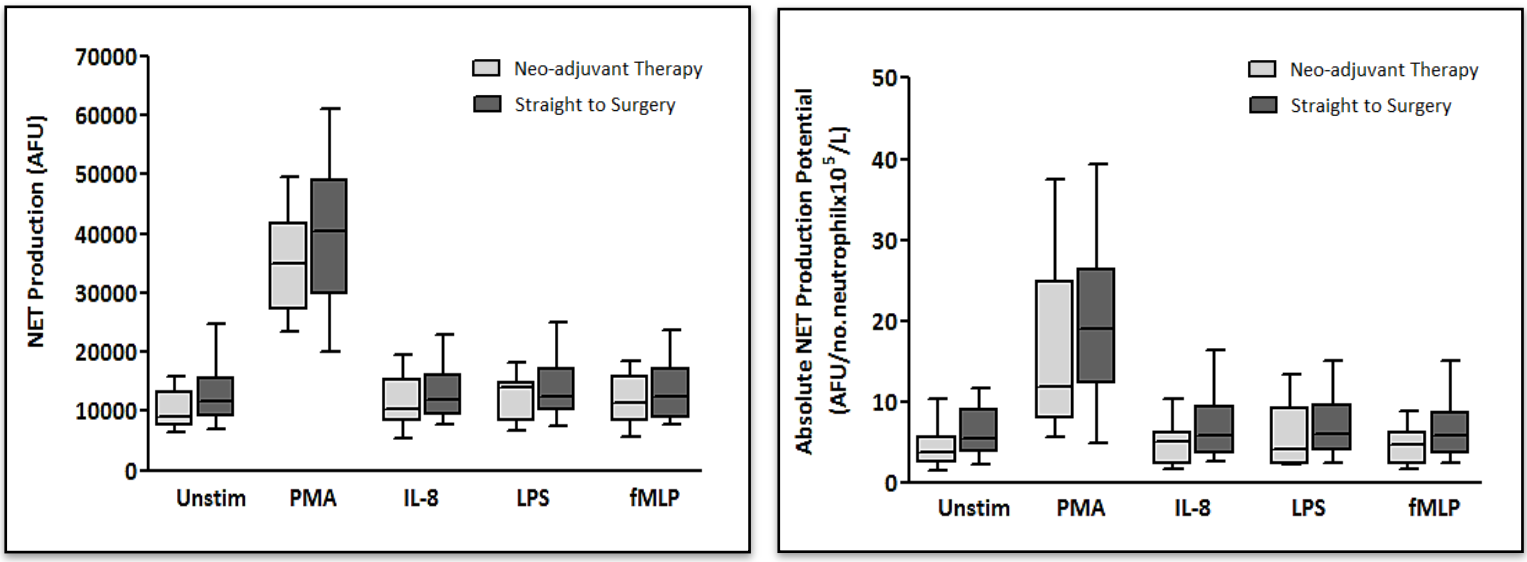


Figure 4.7 NET Production and Absolute NET Production Potential in Patients who were treated with Neo-adjuvant Therapy vs. Patients who went Straight to Surgery

Box and Whisker Plots (10-90 percentile) of NET production and Absolute NET Production Potential in patients who were treated with Neo-adjuvant Therapy (n= 11) versus Patients who went Straight to Surgery (n=34) in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

No significant differences were identified in NET production when comparing rectal and colonic cancers, however, when taking into account the variation in circulating neutrophils significant increases in ANPP were identified in patients with colonic cancer in response to No stimulant, PMA, IL-8, LPS and fMLP.

Similarly, no significant differences were identified in NET production when comparing early stage and late stage colorectal cancer. There was a notable reduction in ANPP in late stage cancer compared to early stage cancer but this only reached statistical significance in response to IL-8.

Interestingly, no significant differences in NET production or ANPP were identified when comparing patients who received neo-adjuvant therapy compared to those patients who went straight to surgery.

When analysing absolute neutrophil counts between groups a significant difference was identified when comparing patients with rectal cancer and colonic cancer which reached a greater level of significance than when analysing ANPP. No significant differences were identified in absolute neutrophil count when comparing early stage and late stage colorectal cancer and when comparing patients who received neo-adjuvant therapy compared to those patients who went straight to surgery.

4.4.6 Changes in NET Production Defined by Patient Outcome

An evaluation of NET formation according to patient outcome was performed. The patient outcomes investigated included; post-operative complications categorised into minor or no Complications (Clavien-Dindo <3) and significant complications (Clavien-Dindo ≥3), total hospital length of stay (LOS) categorised into LOS ≤ 5 days and LOS > 5 days, and mortality after a median follow-up of 21.3 months (IQR 16.7-23.5 months). To account for the variation in number of circulating neutrophils between individuals the ANPP was also determined.

NET production and ANPP in patients categorised by post-operative complication, LOS and mortality are outlined in Table 4.12 and Figure 4.8, Table 4.13 and Figure 4.9 and Table 4.14 and Figure 4.10 respectively.

Table 4.12 NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who developed Minor or No Complications (Clavien-Dindo <3) vs. Patients who developed Significant Complications (Clavien-Dindo ≥3)

	Clavien-Dindo <3 (n=38)	*Clavien-Dindo ≥3 (n=6)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	11090 (8240-14670)	14780 (12630-18330)	0.0639
PMA	38100 (29620-47760)	40330 (27690-60140)	0.6522
IL-8	11820 (8517-14300)	15840 (11300-19070)	0.0796
LPS	12290 (8858-15750)	15180 (11620-19710)	0.2359
fMLP	11760 (8339-14510)	18340 (12330-22400)	**0.0242**
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	5.15 (3.00-8.96)	6.79 (5.19-9.93)	0.0984
PMA	16.53 (9.98-34.83)	17.59 (13.43-37.13)	0.5368
IL-8	5.14 (3.52-8.170)	6.22 (5.48-10.62)	0.2045
LPS	5.603.08-9.63)	6.23 (5.43-10.70)	0.2853
fMLP	4.51 (3.40-8.17)	7.31 (5.99-12.05)	**0.0434**
Absolute Neutrophil Count (x10^9/L), median (IQR)	4.40 (2.96-6.14)	4.50 (3.93-5.65)	0.8674

A comparison of NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who developed Minor or No Complications (Clavien-Dindo <3) (n=38) versus Patients who developed Significant Complications (Clavien-Dindo ≥3) (n=6). Statistical significance was measured by the Mann Whitney U test.

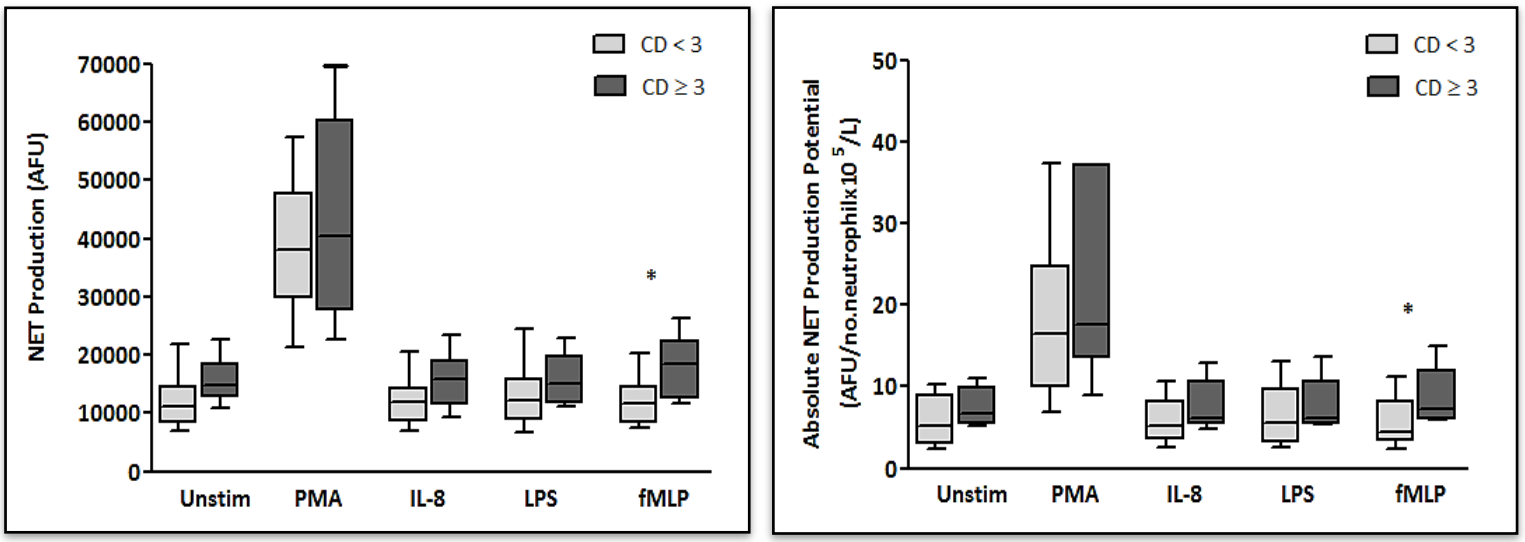


Figure 4.8 NET Production and Absolute NET Production Potential in Patients who developed Minor or No Complications (Clavien-Dindo <3) vs. Patients who developed Significant Complications (Clavien-Dindo ≥3)

Box and Whisker Plots (10-90 percentile) of NET production and Absolute NET Production Potential in Patients who developed Minor or No Complications (Clavien-Dindo <3) (n=38) versus Patients who developed Significant Complications (Clavien-Dindo ≥3) (n=6) in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 4.13 NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who had a Total Length of Stay ≤ 5 days vs. Patients who had a Total Length of Stay > 5 days

	LOS ≤ 5 days (n=15)	*LOS > 5 days (n=29)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	11090 (7507-14200)	12140 (8879-15260)	0.3220
PMA	36430 (27510-43650)	39740 (29500-50280)	0.2760
IL-8	11840 (7935-14200)	12250 (9261-6443)	0.4576
LPS	9408 (6915-15170)	13180 (11250-17270)	0.1812
fMLP	9008 (7599-13290)	12530 (10690-17940)	**0.0476**
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	4.54 (3.00-9.35)	5.66 (3.79-8.98)	0.2445
PMA	12.92 (9.98-21.44)	19.30 (10.92-28.69)	0.3596
IL-8	4.20 (3.60-10.33)	6.03 (3.86-8.80)	0.4282
LPS	4.02 (2.72-10.68)	6.29 (4.13-9.64)	0.1979
fMLP	3.93 (2.590-8.17)	6.03 (4.34-8.64)	**0.0500**
Absolute Neutrophil Count (x10^9/L), median (IQR)	3.70 (2.95-4.54)	4.94 (3.82-6.15)	0.1911

A comparison of NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who had a Total Length of Stay ≤ 5 days (n=15) vs. Patients who had a Total Length of Stay > 5 days (n=29). Statistical significance was measured by the Mann Whitney U test.

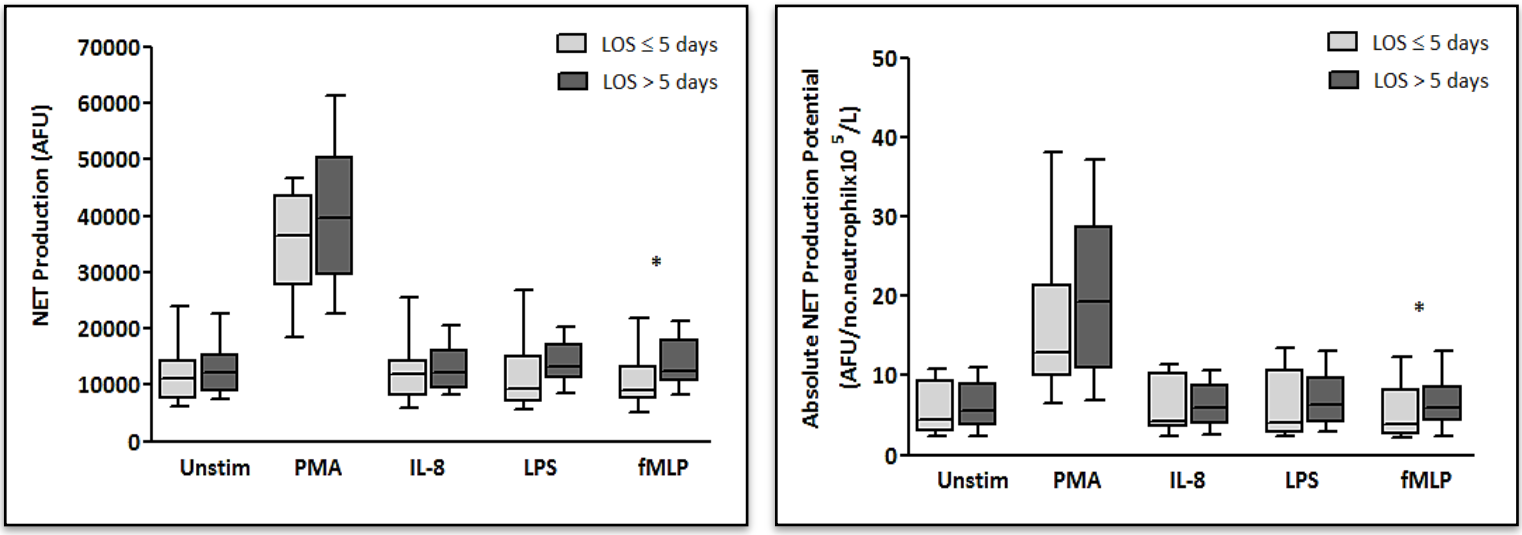


Figure 4.9 NET Production and Absolute NET Production Potential in Patients who had a Total Length of Stay ≤ 5 days vs. Patients who had a Total Length of Stay > 5 days

Box and Whisker Plots (10-90 percentile) of NET production and Absolute NET Production Potential in Patients who had a Total Length of Stay ≤ 5 days (n=15) vs. Patients who had a Total Length of Stay > 5 days (n=29) in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 4.14 NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who Survived vs. Patients who Died

	Survived (n=40)	*Died (n=5)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	11180 (8301-14640)	14760 (10340-18880)	0.2707
PMA	38670 (29640-45400)	50810 (25310-74680)	0.3033
IL-8	11830 (9004-14970)	15370 (10960-20510)	0.1644
LPS	12290 (8982-16280)	13900 (12360-20780)	0.2405
fMLP	11680 (8354-14530)	16110 (12310-23420	0.0533
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	5.24 (3.07-7.29)	9.58 (3.91-13.93)	0.1644
PMA	16.15 (11.49-22.93)	37.10 (9.38-53.62)	0.3206
IL-8	5.17 (3.60-8.05)	9.86 (4.37-14.62)	0.1486
LPS	5.79 (3.22-9.26)	9.65 (4.76-14.36)	0.1120
fMLP	4.685 (3.42-8.04)	11.05 (5.24-14.87)	**0.0346**
Absolute Neutrophil Count (x10^9/L), median (IQR)	4.41 (3.01-5.86)	4.21 (3.73-9.38)	0.5392

A comparison of NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Patients who Survived (n=40) vs. Patients who Died (n=5) following Colorectal Cancer Resection to a median follow-up of 21.3 months (IQR 16.7-23.5 months. Statistical significance was measured by the Mann Whitney U test.

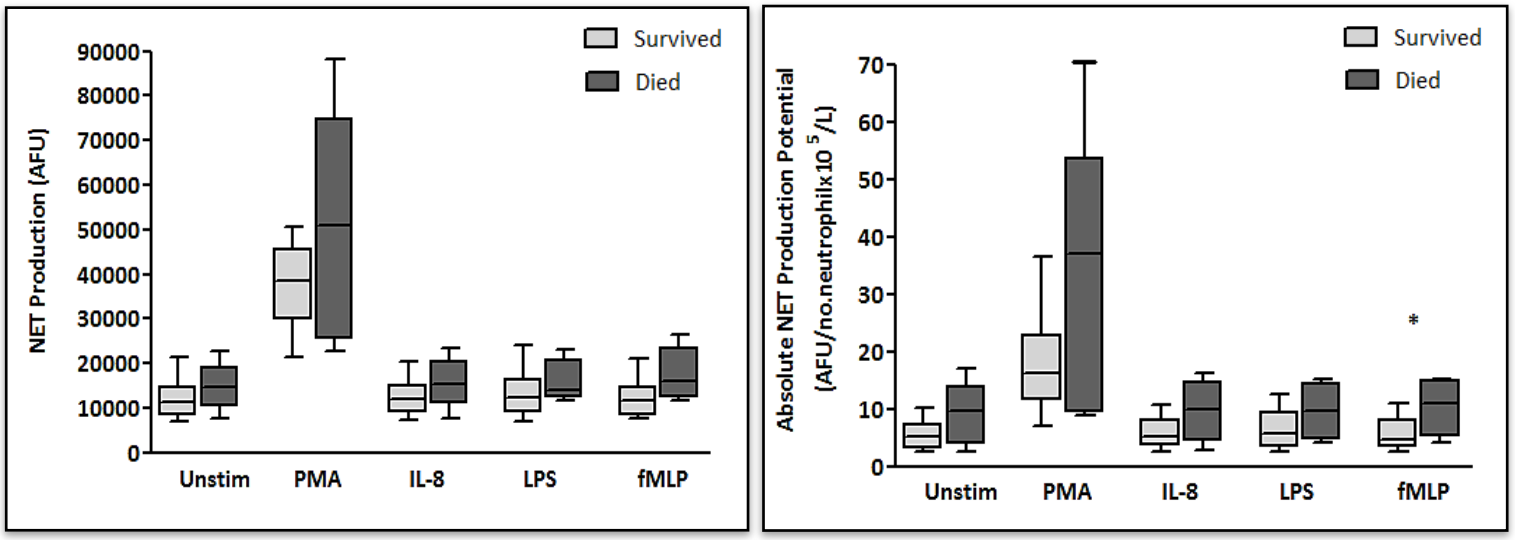


Figure 4.10 NET Production and Absolute NET Production Potential in Patients who in Survived vs. Patients who Died

Box and Whisker Plots (10-90 percentile) of NET production and Absolute NET Production Potential in who Survived vs. Patients who Died following Colorectal Cancer Resection to a median follow-up of 21.3 months (IQR 16.7-23.5 months in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

An increase in both NET production and ANPP was evident in patients who went on to develop significant complications (Clavien-Dindo ≥3) compared to patients who experienced minor or no complications (Clavien-Dindo <3). This reached statistical significance in response to fMLP for both NET production and ANPP.

Similarly, an increase in NET production and ANPP was evident in patients with a LOS > 5 days compared to those patients with a LOS ≤ 5 days. Again, this reached statistical significance is response to fMLP for NET production and ANPP.

Likewise, an increase in NET production and ANPP was evident in patients who died compared to those patients who survived, but this only reached statistical significance in response to fMLP for ANPP.

It appears that adverse outcomes (mortality, Clavien-Dindo ≥3, LOS > 5 days) are all associated with increased pre-operative NET production.

No statistical significant differences were identified when comparing absolute neutrophil counts between the groups.

To assess the potential diagnostic ability of the quantification of NET formation assay in predicting post-operative complications, a prolonged hospital stay and mortality, ROC curve analyses were performed in patients with colorectal cancer who underwent surgical resection. When stimulated with fMLP, the area under the ROC curve (AUC) values for the development of a significant complication (Clavien-Dindo ≥ 3), LOS > 5 days and mortality were 0.7906 (95%CI=0.6259-0.9553, p=0.0232), 0.6851 (95%CI=0.5049-0.8652, p=0.0463) and 0.7700 (95%CI=0.6018-0.9382, p= 0.0512) respectively. The ROC curve analysis of NET

production in response to stimulation with fMLP for the development of significant post-operative complications (Clavien-Dindo ≥ 3) is displayed in Figure 4.11.

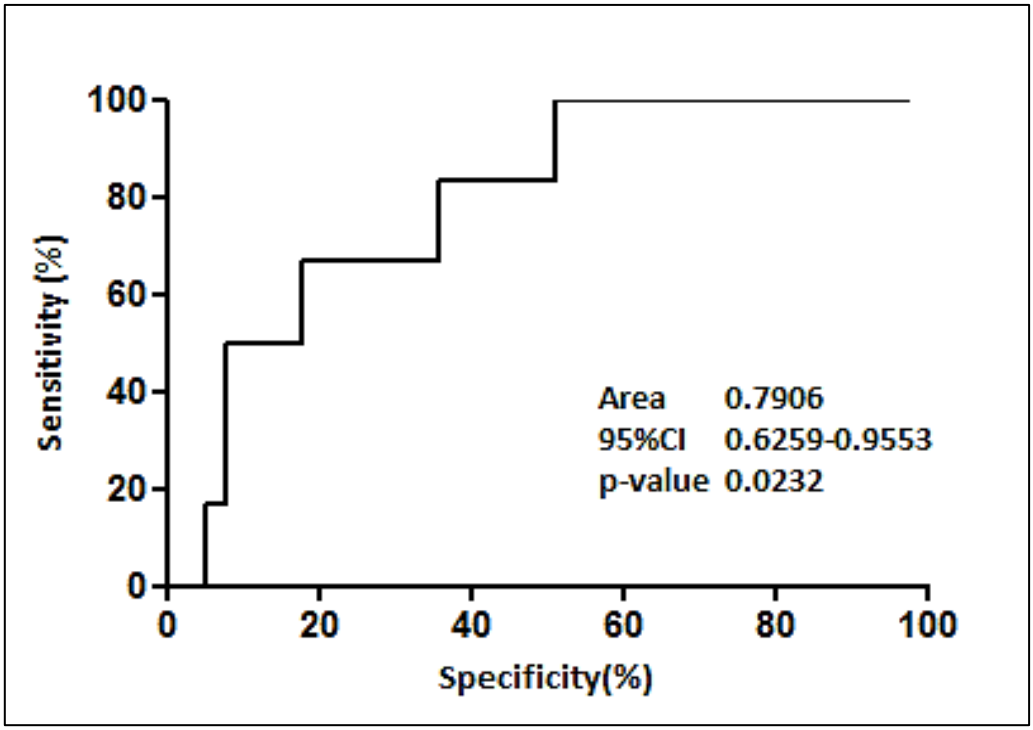


Figure 4.11 ROC Curve Analysis of the Quantification of NET Formation Assay for the development of Significant Post-operative Complications (Clavien-Dindo ≥3)

To assess the diagnostic ability of the quantification of NET formation assay in predicting post-operative complications ROC curve analysis was performed in patients with colorectal cancer who underwent surgical resection. When stimulated with fMLP, the area under the ROC curve (AUC) values for the development of a significant complication (Clavien-Dindo ≥ 3) was 0.7906 (95%CI=0.6259-0.9553, p=0.0232).

4.4.7 The Association Between NET Production and Validated Prognostic Scores

The association between NET production and NLR and mGPS was investigated.

Firstly, it was explored whether differences in NET production existed when categorised by NLR (NLR < 5 vs. NLR ≥ 5) as summarised in Table 4.15 and Figure 4.12. It was then investigated whether NET production and NLR correlated and this is displayed in Table 4.16 and Figure 4.13. The association and correlation between NLR and mGPS was also interrogated.

Secondly, it was explored whether differences in NET production and ANPP existed when categorised by mGPS (mGPS < 2 vs. mGPS ≥ 2) as outlined in Table 4.17 and Figure 4.14. It was then determined whether correlation existed between NET production and mGPS and ANPP and mGPS, as summarised in Table 4.18. The association and correlation between mGPS and NLR and mGPS and absolute neutrophil count was also calculated.

Table 4.15 NET Production and Modified Glasgow Prognostic Score in Patients with NLR < 5 vs. Patients with NLR ≥ 5

	NLR < 5 (n=36)	*NLR ≥ 5 (n=9)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	11220 (8067-14910)	12840 (8879-15330)	0.5049
PMA	37790 (29430-45400)	43820 (30990-55570)	0.2746
IL-8	11880 (9004-15740)	13340 (9250-15280)	0.8315
LPS	12290 (9368-16580)	10510 (13900-16520)	0.7875
fMLP	11990 (8345-16600)	12440 (10380-14960)	0.7875
Modified Glasgow Prognostic Score, median (IQR)	0.00 (0.00-0.00)	1.00 (0.00-2.00)	**0.0147**

A comparison of NET Production and Modified Glasgow Prognostic Score in Patients with a NLR < 5 (n=36) versus Patients with a NLR ≥ 5 (n=9). Statistical significance was measured by the Mann Whitney U test.

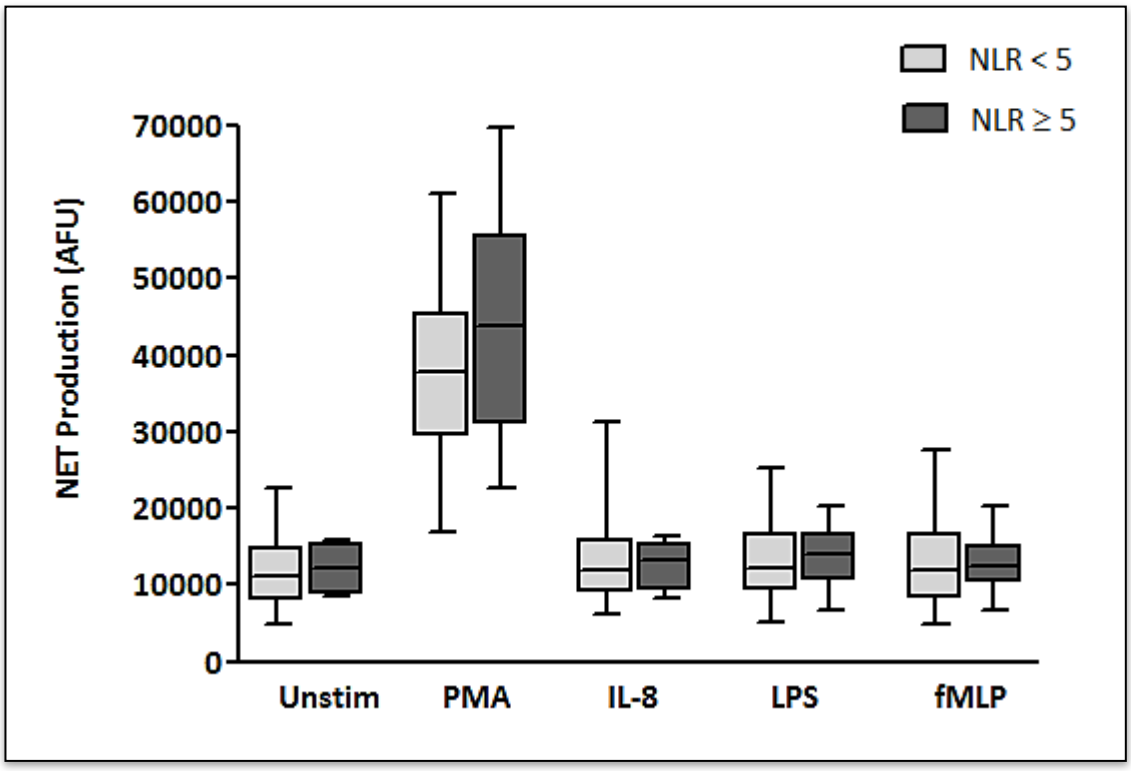
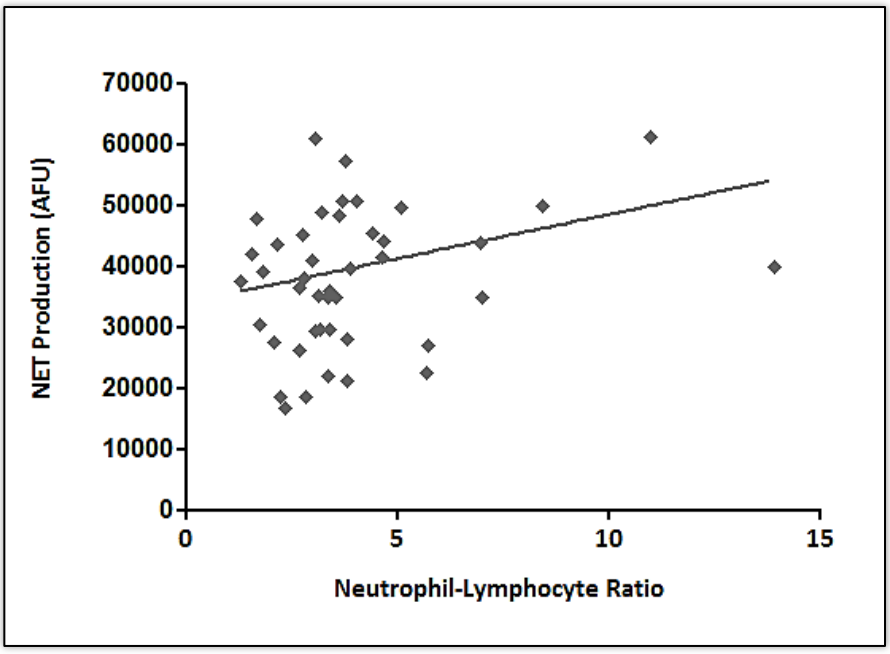


Figure 4.12 NET Production and Modified Glasgow Prognostic Score in Patients with NLR < 5 vs. Patients with NLR ≥ 5

Box and Whisker Plot (10-90 percentile) of NET production in Patients with NLR < 5 versus Patients with NLR ≥ 5 in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 4.16 Spearman's Rank Correlation Analysis of NET Production and Modified Glasgow Prognostic Score with Neutrophil-Lymphocyte Ratio in Patients with Colorectal Cancer

	Spearman r	*95% Confidence Interval*	*P-value*
NET Production			
Unstimulated	0.0311	-0.2732 – 0.3298	0.8393
PMA	0.3015	-0.0003 – 0.5530	**0.0441**
IL-8	-0.0573	-0.3530 - 0.2487	0.7084
LPS	0.0569	-0.2491 – 0.3526	0.7104
fMLP	0.0484	-0.2572 - 0.3451	0.7524
Modified Glasgow Prognostic Score	0.3531	0.5749 - 0.5918	**0.0174**



Spearman Rank Correlation	
Spearman r	0.3015
95% Confidence Interval	-0.0003 – 0.5530
P-value	**0.0441**

Figure 4.13 NET Production vs. Neutrophil-Lymphocyte Ratio

A scatter plot of NET production in response to PMA and Neutrophil Lymphocyte Ratio demonstrating a line of best fit. Spearman's Rank Correlation Analysis indicates a weakly positive, but statistically significant correlation (Spearman r = 0.3015, 95%CI=-0.0003-0.5530, p=0.0441).

No significant differences in NET production were identified when categorised by NLR. As expected, the documented association between NLR and mGPS was apparent in the studied population.

Spearman's Rank Correlation test was performed to determine if a correlation existed between NET production and NLR. It was identified that a weak, but significant correlation existed between NET production and NLR in response to PMA (Spearman r = 0.3015, p = 0.0441). As anticipated, the mGPS and NLR were also positively and significantly correlated (Spearman r = 0.3531, p = 0.0174), although the positive correlation was weak.

Table 4.17 NET Production, Absolute NET Production Potential, Absolute Neutrophil Count and Neutrophil-Lymphocyte Ratio in Patients with mGPS < 2 vs. Patients with mGPS ≥ 2

	mGPS < 2 (n=37)	*mGPS ≥ 2 (n=8)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	12250 (9139–14710)	11350 (8125-15260)	0.6034
PMA	38670 (25710-43260)	39240 (29500-48530)	0.7106
IL-8	12630 (10950-15100)	11840 (8422-15610)	0.4143
LPS	13540 (9858-14680)	12290 (9106-17270)	0.9882
fMLP	12700 (8742-16050)	11760 (8602-15690)	0.6034
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	5.15 (2.98-7.28)	8.16 (5.243-10.08)	**0.0364**
PMA	15.76 (9.93-24.11)	20.56 (12.81-38.86)	0.1675
IL-8	4.95 (3.51-8.03)	8.34 (6.07-11.77)	**0.0082**
LPS	5.98 (3.05-9.19)	8.12 (5.38-13.19)	0.0935
fMLP	4.63 (3.37-7.92)	8.25 (4.83-13.50)	**0.0391**
Absolute Neutrophil Count (x10^9/L), median (IQR)	4.23 (2.96 -5.43)	6.05 (4.14-8.99)	**0.0314**
Neutrophil-Lymphocyte Ratio, median (IQR)	3.54 (2.57-4.55)	3.07 (2.76-5.14)	0.7106

A comparison of NET Production, Absolute NET Production Potential, Absolute Neutrophil Count and Neutrophil-Lymphocyte Ratio in Patients with mGPS < 2 (n=37) versus Patients with mGPS ≥ 2 (n=8). Statistical significance was measured by the Mann Whitney U test.

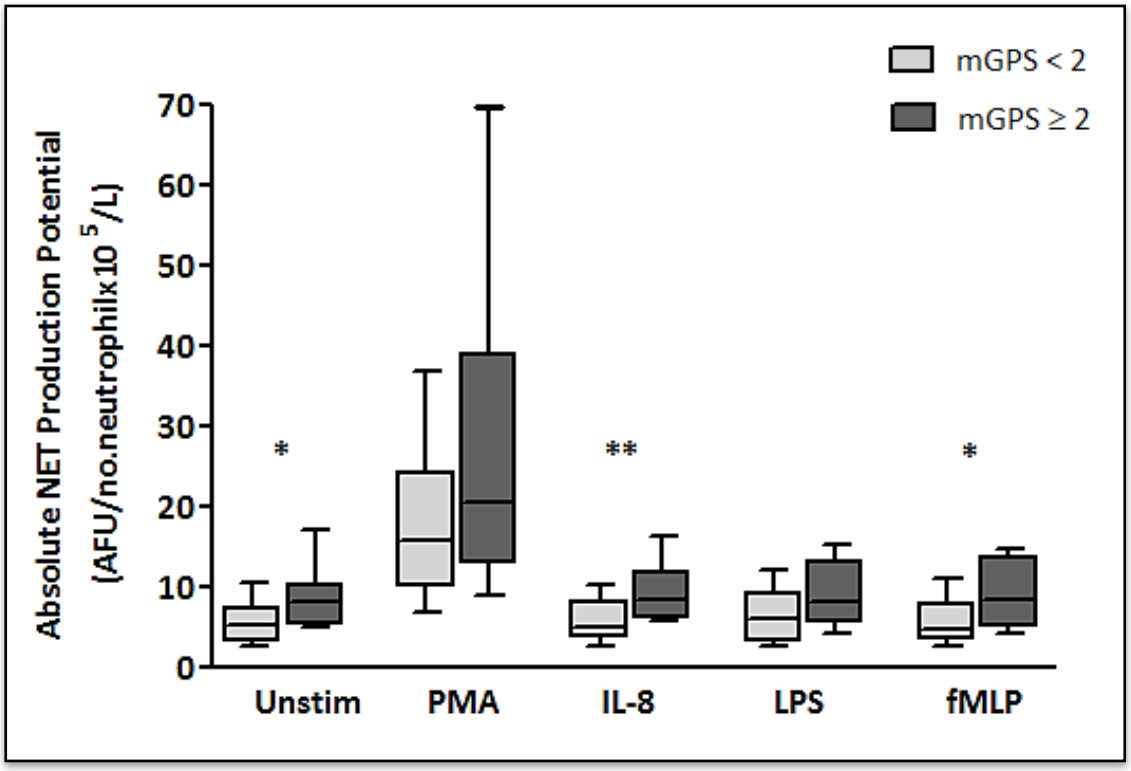


Figure 4.14 Absolute NET Production Potential in Patients with mGPS < 2 vs. Patients with mGPS ≥ 2

Box and Whisker Plot (10-90 percentile) of NET production in Patients with mGPS <2 (n=37) versus Patients with mGPS ≥ 2 (n=8) in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 4.18 Spearman's Rank Correlation Analysis of NET Production, Absolute NET Production Potential, Absolute Neutrophil Count and Neutrophil-Lymphocyte Ratio with Modified Glasgow Prognostic Score in Patients with Colorectal Cancer

	Spearman r	*95% Confidence Interval*	*P-value*
NET Production			
Unstimulated	0.1863	-0.1223 – 0.4621	0.2205
PMA	0.0979	0.2101 – 0.3882	0.5224
IL-8	0.1667	-0.1422 – 0.4460	0.2737
LPS	0.0617	-0.2446 – 0.3568	0.6871
fMLP	0.1538	-0.1551 – 0.4354	0.3131
Absolute NET Production			
Unstimulated	0.4226	0.1386 – 0.6425	**0.0038**
PMA	0.3475	0.0511 – 0.5876	**0.0193**
IL-8	0.4633	0.1878 – 0.6712	**0.0014**
LPS	0.3377	0.0400 – 0.5803	**0.0233**
fMLP	0.4032	0.1143 – 0.6277	**0.0062**
Absolute Neutrophil Count	0.4197	0.1351 - 0.6404	**0.0041**
Neutrophil-Lymphocyte Ratio	0.3531	0.0575 - 0.5918	**0.0173**

No significant differences in NET production were identified when categorised by mGPS. However, when taking into account the variation in circulating neutrophils, significant increases in ANPP were identified in patients with an mGPS ≥ 2 in response to No stimulant, IL-8 and fMLP. This may be accounted for by the significant increase in absolute neutrophil count in patients with an mGPS ≥ 2. Interestingly, no demonstrable differences were identified in NLR when categorised by mGPS.

Spearman's Rank Correlation test was performed to determine whether a correlation existed between NET production and mGPS. No significant correlation was identified. Significant weak to moderate positive correlation did exist between ANPP and mGPS in response to No stimulant, PMA, IL-8, LPS and fMLP. Significant moderate positive correlation was also identified between absolute neutrophil count and mGPS. As

anticipated, NLR and mGPS were significantly and positively correlated, although only weak positive correlation was evident.

4.5 Discussion

The experiements performed in this chapter defined NET production, Neutrophil Apoptosis and Neutrophil Phagocytosis in a population of patients with a confirmed diagnsis of Colorectal Cancer (pre-operatively) which will enable serial analysis of neutrophil function over the peri-operative period as oulined later in this thesis.

It has been proposed that primary tumours can facilitate NET production in circulating neutrophils. This effect has been attributed, in part, to granulocyte-colony stimulating factor (G-CSF) production by tumours [178]. In addition, TNF-α and IL-8 have been shown to facilitate NET formation and these cytokines are highly expressed by a number of tumour types, including colorectal cancer [173-175]. The findings of this chapter provide additional support to this theory, particulary as neutrophils primed with TNF-α had significantly increased NET production under controlled experimental conditions in response to all stimulants.

Despite the accumulating evidence that suggests an association between NETs and tumour progression, the experimental evidence outlined in this study revealed no significant differences in NET production in patients with more advanced disease when patients with early stage and late stage colorectal cancer were compared. In addition, no significant differences were identified in NET production in patients treated with neo-adjuvant therapy compared to patients who went straight to surgery or when comparing cancer location.

Cancer associated inflammation, both in the systemic circulation and in the tumour microenvironment, is now widely recognised to be a key determinant of disease progression and survival in cancer. A chronic dysregulation of the immune system may account for the persistently elevated systemic inflammatory response, either as a consequence of its

activation by micro-metastases or as a result of disease which induces tissue injury [35, 36]. It is now established that the systemic inflammatory response to a tumour is a negative prognostic factor in primary operable [40] and metastatic colorectal cancer [16, 41-44]. It is also known that infectious complications in patients with cancer are associated with adverse oncological outcomes and an increased mortality as a consequence of metastatic disease [187-191].

The analysis performed in this study suggests that adverse patient outcomes (post-operative complications, prolonged hospital recovery and mortality) are associated with increased pre-operative NET production, which indicates increased neutrophil activation, conceivably as a result of cancer associated inflammation. Significant increases in NET production in response to stimulation with fMLP were demonstrated in patients who developed a post-operative complication (Clavien-Dindo ≥ 3) and in patients who had a prolonged hospital stay (LOS > 5 days). ROC curve analyses were also able to distinguish between patients who developed post-operative complications and had a prolonged hospital stay with significance. NET production, particularly in response to stimulation with fMLP, therefore has potential prognostic significance and further investigation into their predictive value is justified. fMLP stimulation of neutrophils activates a wide variety of intra-cellular signalling pathways mediated by phospholipase C (PLC), phospholipase D (PLD), phospholipase A_2 (PLA_2), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs) to induce various cellular functions [291]. Both PLC and PLD have been reported to be involved in superoxide generation and degranulation of neutrophils [292, 293] and this may explain the experimental findings observed in this analysis.

The ANPP is a novel calculation that attempts to account for the variation in circulating neutrophils. It attempts to quantify the 'potential' NET formation in an individual which is inferred from the laboratory experiments. It is an interesting concept which attempts to provided a measure of the quanity of circulating NETs in-vivo and merits further review, particularly with regard to its prognostic value in predicting post-operative complications, a prolonged hospital length of stay and mortality.

In view of the potential prognostic value of pre-operative NET production on patient outcomes, as outlined above, a correlation analysis was undertaken with existing validated prognostic scores; NLR and mGPS. A significant, weak positive correlation was demonstrated between NET production and NLR in response to PMA, but no significant correlation was identified between NET production and mGPS, although a significant, moderate positive correlation did exist between ANPP and mGPS. This may suggest that the absolute number of circulating neutrophils are more important in prognostication, particularly as the NLR and mGPS are also positively correlated. The evidence presented in this chapter certainly indicates that further investigation into the prognostic value of NETs and ANPP is justified.

This experimental study has a number of limitations. Firstly, the population evaluated was small and heterogeneous with regard to patient demographics, patient co-morbidities, tumour characteristics and operative characteristics making interpretation of the results difficult and limiting the generalisability of the study findings. Secondly, comparative analyses stratifying patients according to cancer location, cancer stage and patient outcomes is subject to misinterpretation as potential confounding variables were not accounted for. Thirdly, the number of adverse patient outcomes in the study population

was low making interpretation of patient outcomes challenging. Fourthly, in-vitro experiments performed to assess neutrophil function were conducted outside of the biological context and consequently there are challenges in extrapolating the results and it must be acknowledged that they cannot be readily transposed to, and predict the reaction of, the entire organism in-vivo. Lastly, the patient group was not compared to a control population and therefore it was not possible to determine if differences in neutrophil function existed in patients with colorectal cancer when compared to a matched healthy control population.

4.6 Conclusion

It has been demonstrated that adverse patient outcomes; post-operative complications, prolonged hospital recovery and mortality were all associated with increased pre-operative NET production. It is therefore conceivable that NET production may play a pathophysiological role for the development of adverse outcomes. NETs represent potential therapeutic targets and merit further investigation in the context of colorectal cancer.

Chapter 5

SERIAL CHARACTERISATION OF NEUTROPHIL FUNCTION AFTER COLORECTAL CANCER RESECTION

5.1 Introduction

It is increasingly appreciated that outcomes in patients with cancer are not exclusively determined by the characteristics of the tumour. Cancer-associated inflammation, both in the systemic circulation and in the tumour microenvironment, is now widely recognised to be a key determinant of disease progression and survival in cancer [35, 36]. In the context of cancer surgery, an overwhelming systemic inflammatory response (SIR) may suppress anti-tumour immunity and promote tumour progression and dissemination [58]. The SIR can be assessed by examining changes in concentrations of circulating acute phase proteins, such as CRP, serum cytokines (TNF-α, IL-6, IL-8, IL-10) and low levels of circulating albumin [17, 18]. Pre-operatively, these factors have been demonstrated to be stage-independent prognostic factors in many cancer types [19-24]. The cellular components of the SIR, such a neutrophils, lymphocytes, monocytes and platelets have all been reported to have prognostic value in patients with cancer [26-30]. The SIR results in changes in circulating white blood cells and the neutrophil-lymphocyte ratio (NLR) has been used to predict overall and cancer specific survival in numerous solid organ malignancies, including colorectal cancer [31].

Loco-regional control in the form of a complete oncological resection remains the mainstay of treatment for the majority of solid tumours and provides improved disease-free and overall survival [182]. Cancer surgery, however, elicits a high-grade, non-specific SIR. This overwhelming, systemic inflammation suppresses systemic cell-mediated immunity and consequently compromises the tumour immunity in the host [58, 59]. This has been described as the 'immune-hit' [58]. The immune-hit can be exacerbated by pre-operative SIR, for example, an emergency presentation with colonic obstruction, bleeding or

perforation, confers a higher risk of disease recurrence independent of the stage of disease [40]. Additionally, the development of post-operative complications and the associated post-operative SIR increases the risk of disease recurrence [38] and is associated with poor oncological outcomes and increased mortality secondary to metastatic disease [187-191]. It is therefore appreciated that major surgical resection for colorectal cancer presenting as an emergency or in the presence of a post-operative infective complication results in further compromise of the immune response to residual disease [40, 58]. This is consistent with the negative prognosis associated with the presence of SIR in patients affected by most tumour types at any stage of disease [40].

It is believed that neutrophils play a vital role in circulating tumour cell metastases. This has been demonstrated in-vitro and in-vivo where neutrophils facilitate circulating tumour cell adhesion to both pulmonary and hepatic endothelial surfaces [174, 176, 179-181] and the direct contact of circulating tumour cells and neutrophils is thought to be an important precursor to the development of metastatic disease [179, 180]. Following dissemination, tumour cells must proliferate to form a stable metastatic foci and it is proposed that NETs may play a direct proliferative role and may inhibit tumour cell apoptosis [181].

This study was conducted to characterise neutrophil function in the peri-operative period of patients undergoing major colorectal cancer resection, focussing on NET formation, apoptosis and phagocytosis, and to determine whether differences in peri-operative NET formation, classified by cancer location, cancer stage, operative technique, operation type and patient outcome, existed.

5.2 Hypothesis

It was hypothesised that the impact of surgery would replicate the neutrophil functional changes observed in patients with sepsis:

1. Reduced NET formation
2. Reduced apoptosis
3. Increased phagocytosis

5.3 Objectives

The objectives of this chapter were:

1. To characterise neutrophil function in the peri-operative period with specific reference to:
    i. NET formation
    ii. Neutrophil apoptosis
    iii. Neutrophil phagocytosis
2. To evaluate the differences in peri-operative NET formation classified by operative technique and operation type.
3. To evaluate the differences in peri-operative NET formation classified by:
    i. Post-operative Complications
    ii. Total Hospital Length of Hospital Stay
    iii. Mortality

5.4 Methods

5.4.1 Acknowledgements

Dr. Hongxia Mei (International Research Fellow from the Hospital of Wenzouh Medical University, China) assisted with the Neutrophil Phagocytosis experiments. The short life-span of neutrophils meant that neutrophil functional experiments were conducted simultaneously and it was impossible for all experiments to be performed by one individual in entirety.

5.4.2 Recruitment of Patients

Patients undergoing an elective colorectal resection for cancer, within an established enhanced recovery programme, who satisfied the specific inclusion and exclusion criteria, were recruited to the study as outlined in Section 3.1.1.

Patient Demographics, Patient Co-morbidities, Pre-operative Risk Prediction, Tumour Characteristics and Operative Characteristics were recorded from study participants as outlined in Table 3.1.

Patient outcomes were collected prospectively and included:

1. Post-operative complications up to 30 days after surgery using pre-defined criteria and graded by the Clavien-Dindo classification system (Appendix 3).
2. Return to theatre within 28 days of the index procedure.
3. Admission to and length of stay on the Intensive Care Unit.
4. Total hospital length of stay.

5. Readmissions to hospital within 30 days of the index procedure requiring a hospital stay ≥ 24 hours.
6. 30 day and 90 day mortality.

Functional and quality of life was assessed using the Surgical Recovery Scale (Appendix 4).

Haematological and biochemical parameters were collected prospectively from the hospital pathology system. These parameters, in conjunction with physiological indices, were used to determine a CR-POSSUM (Appendix 5), a NLR, and an mGPS (Table 1.6) for each patient.

5.4.3 Sample Collection

Patients underwent peripheral blood tests pre-operatively (Day-0) and post-operatively (Day-1 and Day-3) to assess neutrophil function at these specific time points. Blood tests were performed by an experienced medical practitioner by peripheral venepuncture or from a peripheral arterial cannula. A total of 24mls of blood was taken from each patient at each time point and deposited into 4x6ml lithium-heparin vacutainers (Becton-Dickinson, Oxford, UK). The samples were then placed on ice and transported to the laboratory for processing.

5.4.4 Neutrophil Isolation

Processing began with 45 minutes of obtaining the blood sample. Neutrophils were isolated by following the method outlined in Section 3.2. A purity of ≥95% and a viability of ≥97% were routinely achieved.

5.4.5 Neutrophil Functional Assays

Quantification of Neutrophil Extracellular Trap Formation was conducted following the method outlined in Section 3.3.1. NETs were recorded in arbitrary fluorescent units (AFU).

To account for the variation in number of circulating neutrophils between individuals the Absolute NET Production Potential (ANPP) was determined. It was calculated as follows:

ANPP (AFU / no. of neutrophils x10^5 / L) = NETs per 100,000 neutrophils (AFU) x Absolute Neutrophil Count (x10^9 / L) ÷ 10,000

Neutrophil Apoptosis and Neutrophil Phagocytosis were assessed using commercially available assays which were carried out as per the manufacturer's instructions as outlined in Sections 3.3.2 and 3.3.3 respectively. Neutrophil Apoptosis was represented as the percentage of neutrophils in the different stages of apoptosis (alive, early apoptosis, late apoptosis and necrosis). Neutrophil phagocytotic function was represented by the Phagocytosis Index which provided a quantitative measure of neutrophil phagocytotic function. It was calculated as follows:

Phagocytosis Index = (Percentage of pHrodo 'bright' cells ÷ 100) x Median Fluorescent Intensity

5.4.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, California, USA).

Categorical data was analysed using Fisher's Exact tests for two variables or χ^2 tests for greater than two variables. Continuous data was analysed using non-parametric statistical models. Mann-Whitney U tests were used for independent samples and Wilcoxon Signed Rank tests for related samples for two groups. Freidmann's tests were used for related samples for greater than two groups. All statistical tests performed were two-tailed and results were considered significant when $p<0.05$.

5.5 Results

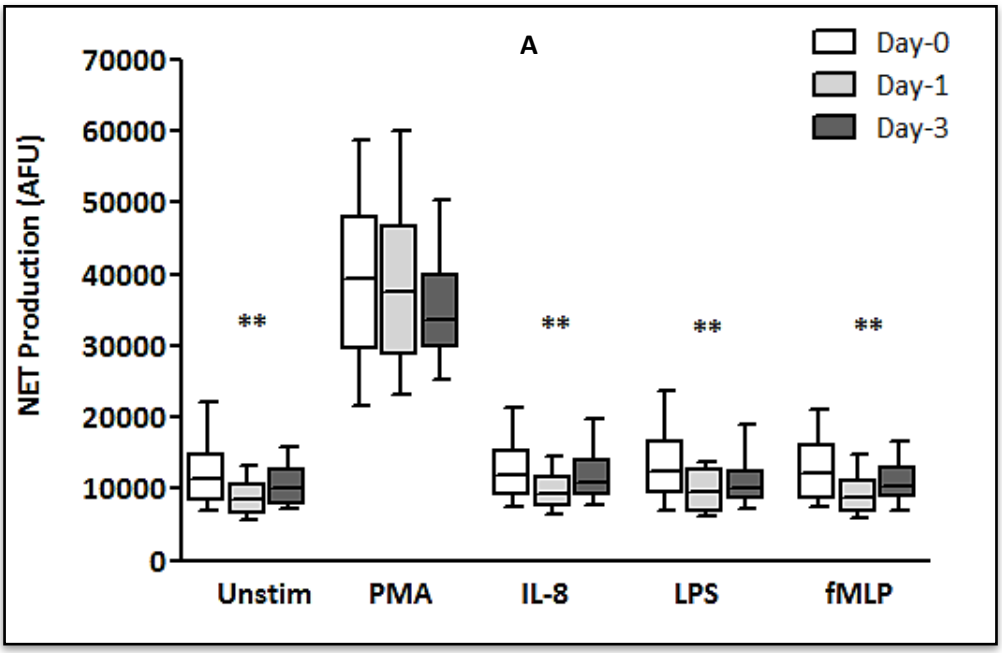
Neutrophil function assay results on sequential peri-operative days are displayed in full in the Appendix (Appendix 7).

5.5.1 Population Characteristics

55 patients were identified at the MDT meeting and approached for inclusion into the study. 45/55 patients (81.8%) were successfully recruited into the study and were followed up for a median of 21.3 months (IQR 16.7-23.5 months). Consent was refused from the remaining 10/55 patients (18.2%). 44/45 patients (97.8%) underwent surgical resection. 1/45 patient (2.2%) was deemed unfit for surgery on the day of surgery and did not progress to surgical resection. The baseline population characteristics, operative characteristics and patient outcomes are displayed in Table 4.1, Table 4.2 and Table 4.3 respectively.

5.5.2 Neutrophil Extracellular Trap Formation

Quantification of NET formation was conducted on Day-0 (n=45), Day-1 (n=44) and Day-3 (n=22) in an attempt to characterise NET production over the peri-operative period. In an attempt to account for the variation in number of circulating neutrophils between individuals the ANPP was also determined. NET production and ANPP on sequential peri-operative days is represented graphically in Figure 5.1 and Figure 5.2 respectively. The fold change, for both NET production and ANPP, from Day-0 to Day-1 and from Day-1 to Day-3 was calculated and is displayed in Figure 5.3 and Figure 5.4 respectively.



	P-value
Unstimulated	**0.0016**
PMA	0.3351
IL-8	**0.0045**
LPS	**0.0025**
fMLP	**0.0014**

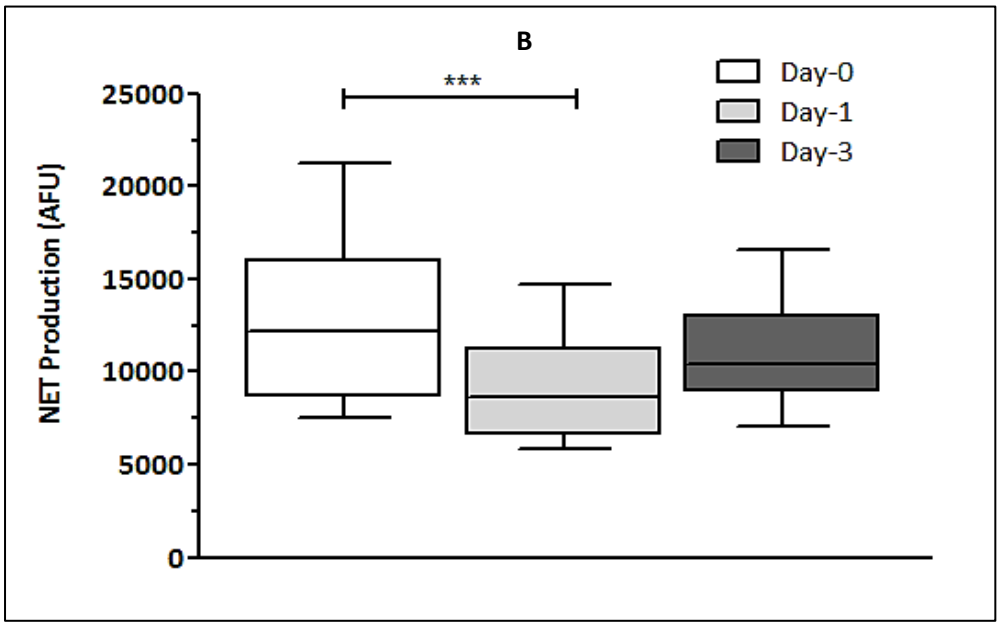
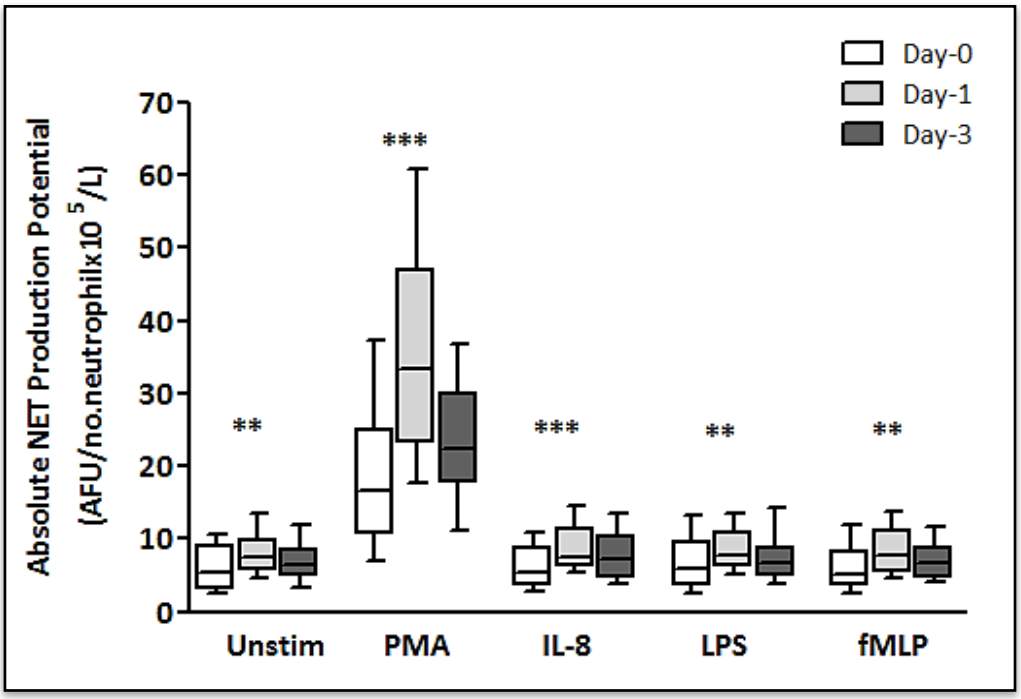


Figure 5.1 NET Production in patients with Colorectal Cancer on Sequential Peri-operative Days

Box and Whisker Plot (10-90 percentile) of NET Production in patients with Colorectal Cancer on sequential peri-operative days in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP (A). Statistical significance, measured by Friedman test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). NET Production in patients with Colorectal Cancer on sequential peri-operative days in response to fMLP (B). Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Statistically significant differences were identified in NET formation over the peri-operative period in response to No stimulant, IL-8, LPS and fMLP as determined by the Friedman test.

Statistically significant reductions in NET formation from Day-0 to Day-1 were identified in response to No stimulant (11,347AFU vs. 8,654AFU, p=0.0006), IL-8 (11,925AFU vs. 9,388AFU, p=0.0003), LPS (12,473AFU vs. 9,582AFU, p=0.0001) and fMLP (12,194AFU vs. 8,680AFU, p<0.0001) as calculated by the Wilcoxon Signed Rank test. Although demonstrable increases in NET production were seen from Day-1 to Day-3, these were not statistically significant.



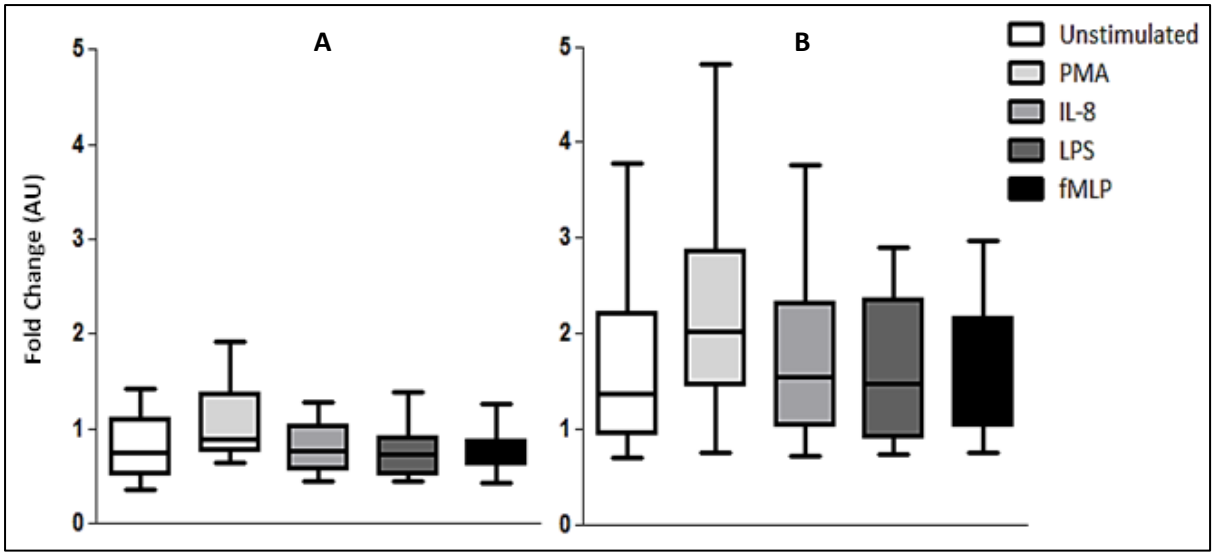
	P-value
Unstimulated	**0.0065**
PMA	**<0.0001**
IL-8	**0.0009**
LPS	**0.0049**
fMLP	**0.0050**

Figure 5.2 Absolute NET Production Potential in patients with Colorectal Cancer on Sequential Peri-operative Days.

Box and Whisker Plot (10-90 percentile) of Absolute NET Production Potential in patients with Colorectal Cancer on sequential peri-operative days in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Friedman test, is denoted by * (p<0.05), ** (p<0.01) and *** (p<0.001).

Statistically significant differences were identified in ANPP over the peri-operative period in response to No stimulant, PMA, IL-8, LPS and fMLP as determined by the Friedman test. A significant difference in absolute neutrophil count was also observed over the peri-operative period ($p < 0.0001$).

Statistically significant increases in ANPP from Day-0 to Day-1 were identified in response to No stimulant (5.29 vs. 7.47, $p=0.0028$), PMA (16.53 vs. 33.32, $p<0.0001$), IL-8 (5.33 vs. 7.45, $p=0.0002$), LPS (5.98 vs. 7.87, $p=0.0012$) and fMLP (5.19 vs. 7.75, $p=0.0005$) and significant reductions in ANPP from Day-1 to Day-3 were detected to No stimulant (7.47 vs. 6.56, $p=0.0113$), PMA (33.32 vs. 22.35, $p=0.0002$), LPS (7.87 vs. 6.67, $p=0.0178$) and fMLP (7.75 vs. 6.68, $p=0.0064$) as calculated by the Wilcoxon Signed Rank test.



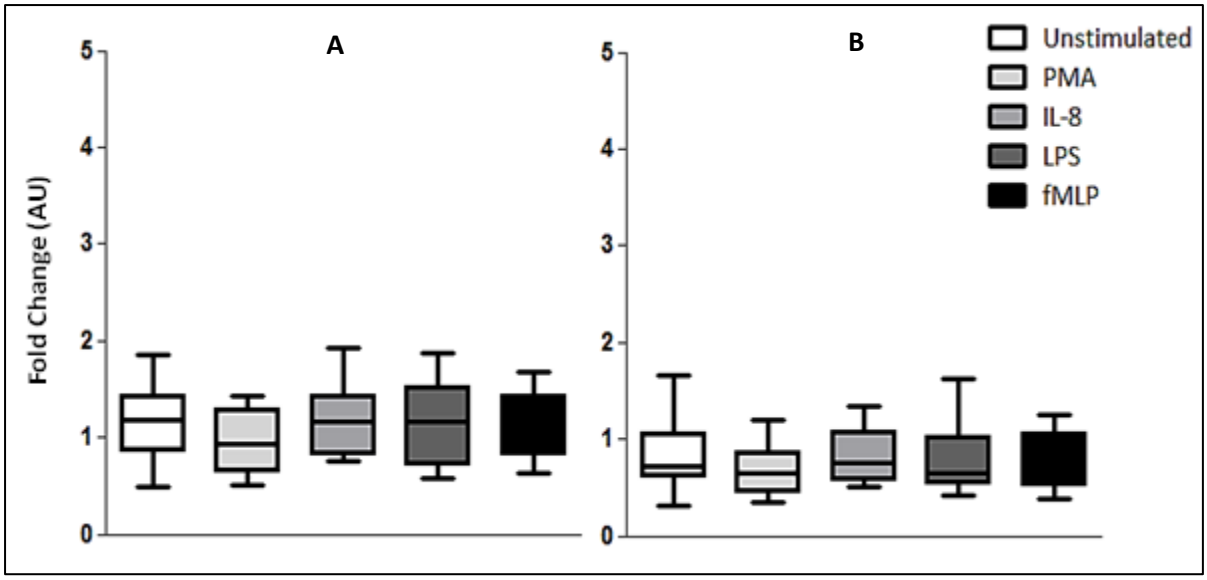
	Fold Change (AU) NET Production, median (IQR)	*Fold Change (AU) Absolute NET Production Potential, median (IQR)*
Unstimulated	0.756 (0.515 – 1.180)	1.368 (0.948 – 2.209)
PMA	0.898 (0.763 – 1.371)	2.021 (1.452 – 2.872)
IL-8	0.769 (0.575 – 1.030)	1.543 (1.029 – 2.316)
LPS	0.730 (0.514 – 0.907)	1.476 (0.904 – 2.355)
fMLP	0.740 (0.624 – 0.879)	1.488 (1.037 – 2.152)

Figure 5.3 Fold Change in NET Production (A) and Absolute NET Production Potential (B) from Day-0 to Day-1 following Colorectal Cancer Resection

Box and Whisker Plot (10-90 percentile) and tabulated results of Fold Change (AU) from Day-0 to Day-1 following Colorectal Cancer Resection in NET Production (A) and Absolute NET Production Potential (B).

From Day-0 to Day-1 there was a reduction in NET production as denoted by a fold change <1. As previously stated, the reductions in NET production were statistically significant in response to No stimulant ($p=0.0006$), IL-8 ($p=0.0003$), LPS ($p=0.0001$) and fMLP ($p<0.0001$) as calculated by the Wilcoxon Signed Rank test.

From Day-0 to Day-1 there was an increase in ANPP as indicated by a fold change > 1. The increases in ANPP were statistically significant in response to No stimulant ($p=0.0028$), PMA ($p<0.0001$), IL-8 ($p=0.0002$), LPS ($p=0.0012$) and fMLP ($p=0.0005$) as determined by the Wilcoxon Signed Rank test.



	Fold Change (AU) NET Production, median (IQR)	*Fold Change (AU) Absolute NET Production Potential, median (IQR)*
Unstimulated	1.175 (0.855 – 1.429)	0.719 (0.621 – 1.053)
PMA	0.927 (0.656 – 1.280)	0.654 (0.453 – 0.863)
IL-8	1.170 (0.820 – 1.430)	0.753 (0.573 – 1.083)
LPS	1.171 (0.728 – 1.527)	0.655 (0.536 -1.025)
fMLP	1.148 (0.832 – 1.437)	0.755 (0.532 – 1.055)

Figure 5.4 Fold Change in NET Production (A) and Absolute NET Production Potential (B) from Day-1 to Day-3 following Colorectal Cancer Resection

Box and Whisker Plot (10-90 percentile) and tabulated results of Fold Change (AU) from Day-1 to Day-3 following Colorectal Cancer Resection in NET Production (A) and Absolute NET Production Potential (B).

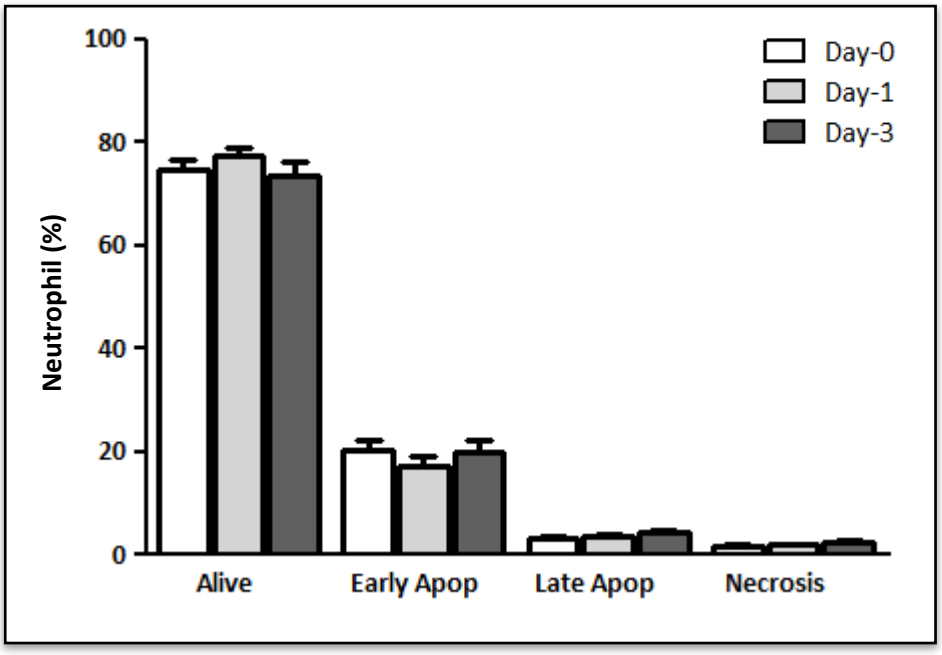
From Day-1 to Day-3 there was an increase in NET production as denoted by a fold change >1. Although demonstrable increases in NET production were seen from Day-1 to Day-3, these were not statistically significant.

From Day-1 to Day-3 there was a reduction in ANPP as indicated by a fold change < 1. Significant reductions in ANPP from Day-1 to Day-3 were detected to No stimulant

(p=0.0113), PMA (p=0.0002), LPS (p=0.0178) and fMLP (p=0.0064) as calculated by the Wilcoxon Signed Rank test.

5.5.3 Neutrophil Apoptosis

Neutrophil apoptosis was performed at 4-hours incubation on Day-0 (n=43), Day-1 (n=40) and Day-3 (n=22) and at 24-hours incubation on Day-0 (n=36), Day-1 (n=33) and Day-3 (n=12) in an attempt to characterise neutrophil apoptosis over the peri-operative period. The stage of apoptosis on sequential peri-operative days, at 4-hours and at 24-hours, is represented graphically in Figure 5.5 and Figure 5.6 respectively. The fold change, for apoptosis at 4-hours and 24-hours, from Day-0 to Day-1 and from Day-1 to Day-3 was calculated and is displayed in Figure 5.7 and Figure 5.8 respectively.

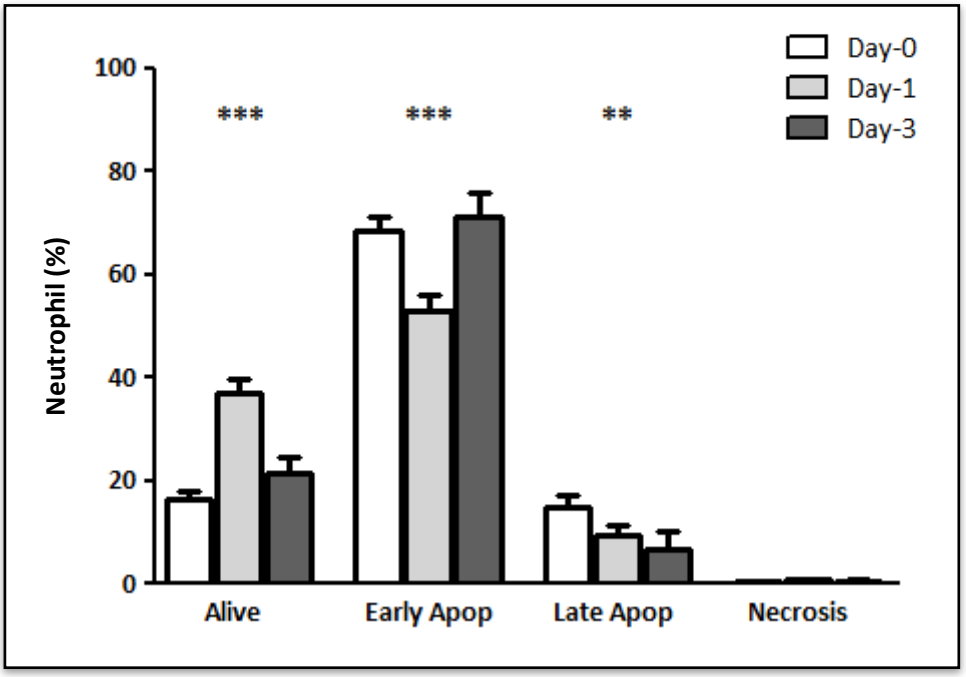


	P-value
Alive	0.3169
Early Apoptosis	0.3959
Late Apoptosis	0.1917
Necrosis	0.5632

Figure 5.5 Stage of Apoptosis at 4-hours in patients with Colorectal Cancer on sequential peri-operative days

Bar graph depicting the Stage of Apoptosis (Alive, Early Apoptosis [Early Apop], Late Apoptosis [Late Apop], Necrosis) of Neutrophils (Mean [%] + SEM) at 4-hours incubation in patients with Colorectal Cancer on sequential peri-operative days. Statistical significance, measured by Friedman test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

No statistically significant differences were identified in the stages of apoptosis at 4-hours on sequential peri-operative days as determined by the Friedman test. No statistically significant differences were detected in the stages of apoptosis at 4-hours from Day-0 to Day-1. From Day-1 to Day-3, however, there was a significant reduction in alive neutrophils (79.9% vs. 74.75%, p=0.0203), as calculated by the Wilcoxon Signed Rank test.



	P-value
Alive	**<0.0001**
Early Apoptosis	**0.0008**
Late Apoptosis	**0.0018**
Necrosis	0.0585

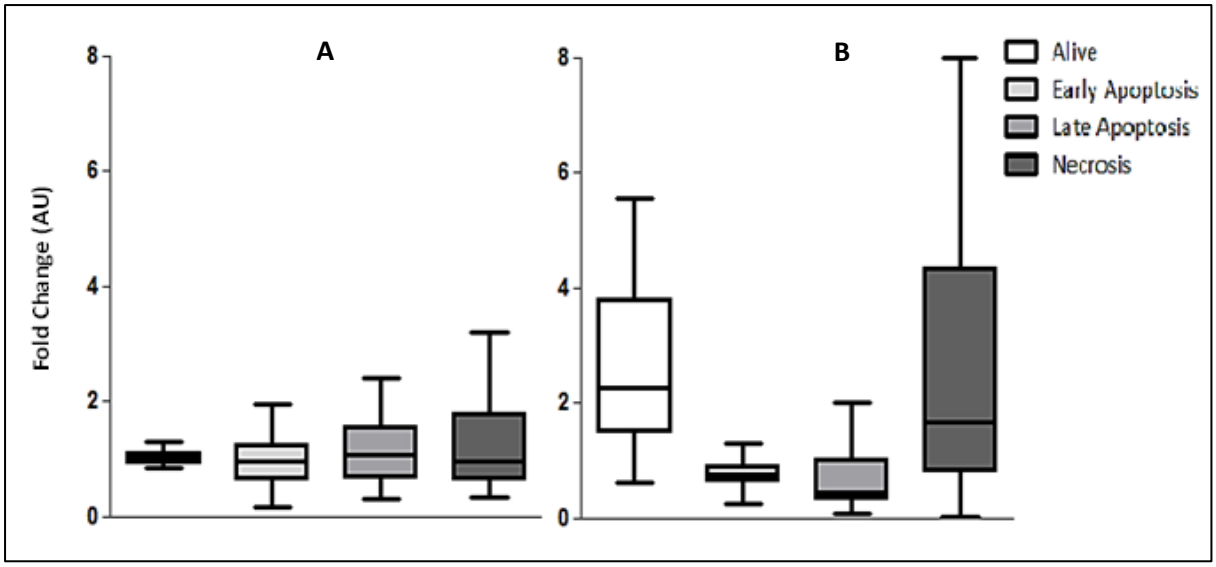
Figure 5.6 Stage of Apoptosis at 24-hours in patients with Colorectal Cancer on sequential peri-operative days

Bar graph depicting the Stage of Apoptosis (Alive, Early Apoptosis [Early Apop], Late Apoptosis [Late Apop], Necrosis) of Neutrophils (Mean [%] + SEM) at 24-hours incubation in patients with Colorectal Cancer on sequential peri-operative days. Statistical significance, measured by Friedman test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Statistically significant differences were identified in alive, early apoptotic and late apoptotic stages over the peri-operative period at 24-hours, as determined by the Friedman test.

Statistically significant differences were identified from Day-0 to Day-1 in alive (14.05% vs. 36.90%, $p<0.0001$), early apoptotic (71.85% vs. 51.80%, $p<0.0001$), late apoptotic (9.30% vs. 4.92%, $p=0.0130$) and necrotic (0.30% vs. 0.60%, $p=0.0147$) stages indicating impaired apoptosis as calculated by the Wilcoxon Signed Rank test.

From Day-1 to Day-3 significant differences in alive (36.9% vs. 18.10%, $p=0.0093$) and early apoptotic (51.80% vs. 76.79%, $p=0.0210$) stages indicating favoured (or restored) apoptosis as calculated by the Wilcoxon Signed Rank test.

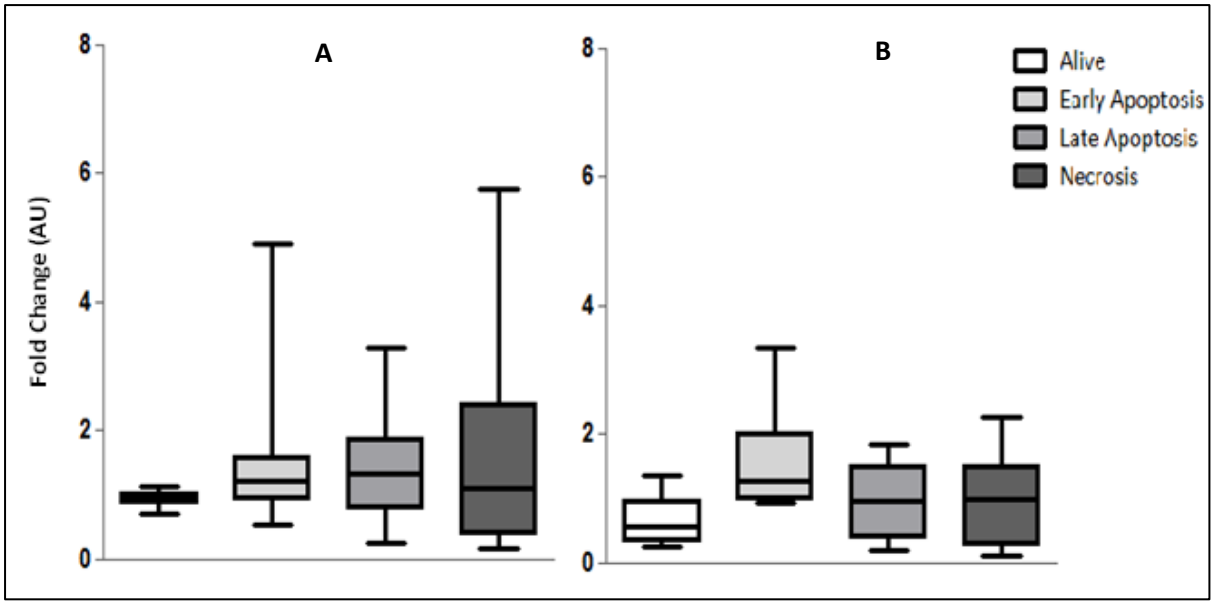


	Fold Change (AU) 4-hour Apoptosis, median (IQR)	*Fold Change (AU) 24-hour Apoptosis, median (IQR)*
Alive	1.005 (0.935 – 1.090)	2.265 (1.500 – 3.798)
Early Apoptosis	0.944 (0.643 – 1.252)	0.761 (0.642 – 0.911)
Late Apoptosis	1.082 (0.657 – 1.556)	0.456 (0.336 – 1.010)
Necrosis	0.942 (0.647 – 1.780)	1.667 (0.808 – 4.333)

Figure 5.7 Fold Change in Stage of Apoptosis at 4-hours (A) and 24-hours (B) from Day-0 to Day-1 following Colorectal Cancer Resection

Box and Whisker Plot (10-90 percentile) and tabulated results of Fold Change (AU) from Day-0 to Day-1 following Colorectal Cancer Resection in Stage of Apoptosis at 4-hours (A) and 24-hours incubation (B).

From Day-0 to Day-1, at 24-hours, fold changes are consistent with cell survival and a disruption in neutrophil apoptosis.



	Fold Change (AU) 4-hour Apoptosis, median (IQR)	*Fold Change (AU) 24-hour Apoptosis, median (IQR)*
Alive	0.942 (0.875 – 1.002)	0.548 (0.322 – 0.945)
Early Apoptosis	1.199 (0.926 – 1.584)	1.261 (0.984 – 2.010)
Late Apoptosis	1.329 (0.795 – 1.856)	0.961 (0.388 – 1.496)
Necrosis	1.101 (0.402 – 2.408)	0.981 (0.265 – 1.500)

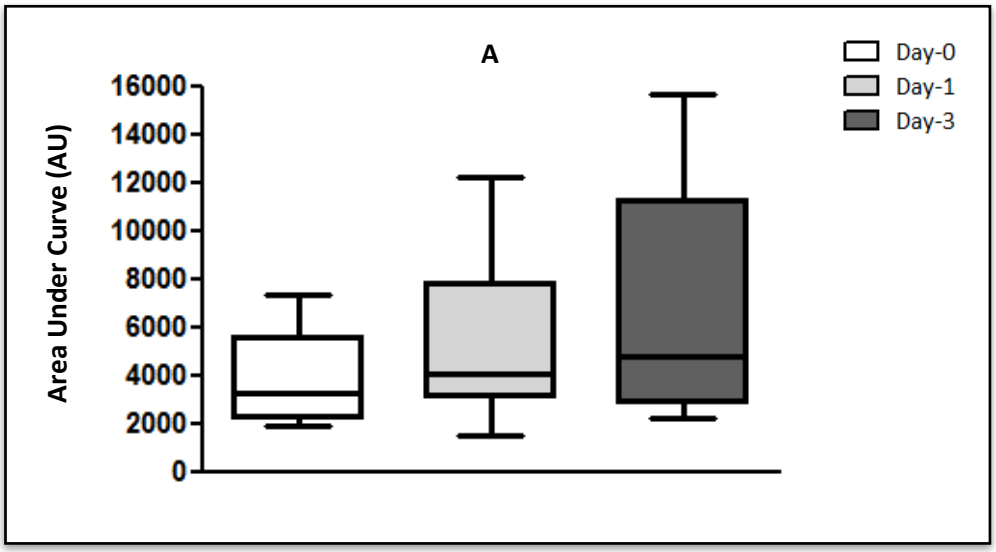
Figure 5.8 Fold Change in Stage of Apoptosis at 4-hours (A) and 24-hours (B) from Day-1 to Day-3 following Colorectal Cancer Resection

Box and Whisker Plot (10-90 percentile) and tabulated results of Fold Change (AU) from Day-1 to Day-3 following Colorectal Cancer Resection in Stage of Apoptosis at 4-hours (A) and 24-hours incubation (B).

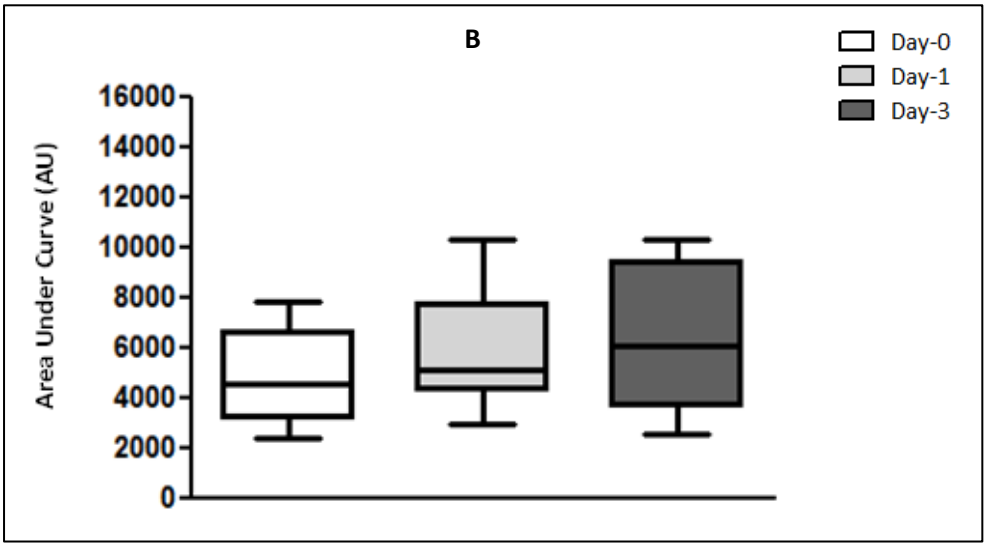
From Day-1 to Day-3, at 24-hours, fold changes are consistent with cell death and a resolution of the disrupted neutrophil apoptosis.

5.5.4 Neutrophil Phagocytosis

Neutrophil phagocytosis was performed on Day-0 (n=27), Day-1 (n=25) and Day-3 (n=9), in response to *E.Coli* and *S.Aureus* in an attempt to characterise neutrophil phagocytosis over the peri-operative period. The assay was performed at three time points (30 minutes, 45 minutes and 60 minutes) and the Phogocytosis Index was calculated for each time point. The Phagocytosis Index was represented as AUC which enabled comparisons to be made considering all time points. This is graphically displayed in Figure 5.9.



	P-value
30 minutes	0.1943
45 minutes	0.2227
60 minutes	0.1972
AUC (AU)	0.1828



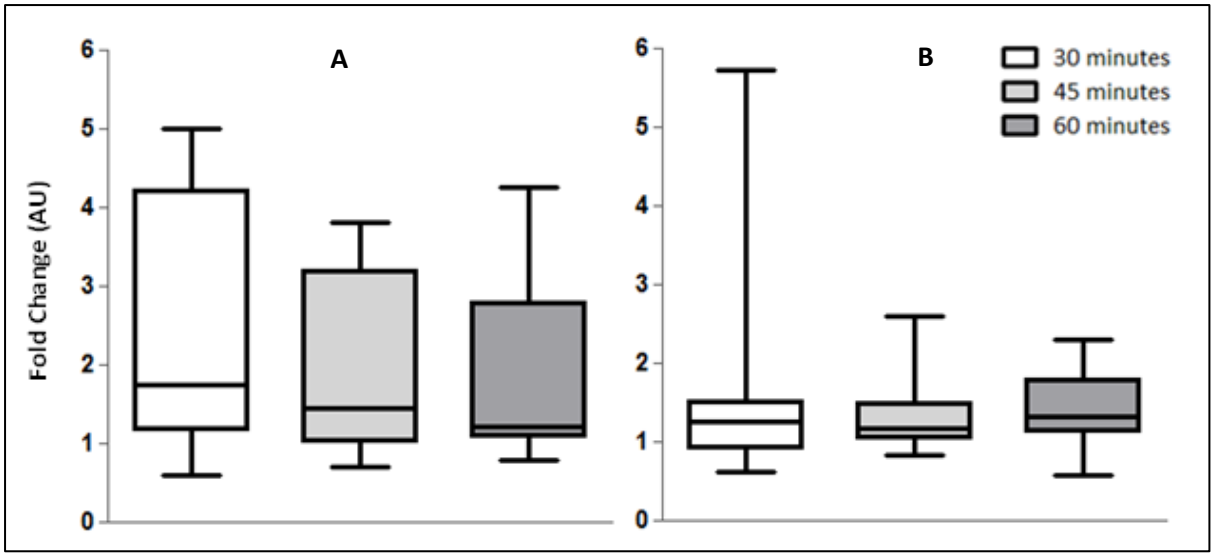
	P-value
30 minutes	0.2617
45 minutes	0.1455
60 minutes	0.3111
AUC (AU)	0.2313

Figure 5.9 Neutrophil Phagocytosis Index in response to *E.Coli* (A) and *S.Aureus* (B) in patients with Colorectal Cancer on Sequential Peri-operative days depicted by Area Under the Curve (AUC)

Box and Whisker Plot (10-90 percentile) of Phagocytosis Index represented by Area Under Curve (AU) in response to *E.Coli* (A) and *S.Aureus* (B) on sequential peri-operative days. Statistical significance, measured by Friedman test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Demonstrable increases in neutrophil phagocytotic activity were revealed on sequential peri-operative days, over the peri-operative period, but statistical significance was not attained in response to *E.Coli* (p=0.1828) and *S.Aureus* (p=0.2313). From Day-1 to Day-3, significant increases in the phagocytotic index were identified in response to *E.Coli* (p=0.0078) but not in response to *S.Aureus* (p=0.2031).

A cumulative increase in phagocytotic activity was identified from Day-0 to Day-3 in response to both *E.Coli* and *S.Aureus*. The positive fold changes from Day-0 to Day-3 are displayed graphically in Figure 5.10 indicating an increasing Phagocytosis Index with time.



	Fold Change (AU) E.Coli, median (IQR)	*Fold Change (AU) S.Aureus, median (IQR)*
30 minutes	1.742 (1.163 – 4.214)	1.257 (0.911 – 1.496)
45 minutes	1.439 (1.012 – 3.195)	1.165 (1.042 – 1.474)
60 minutes	1.196 (1.086 – 2.771)	1.302 (1.130 – 1.772)

Figure 5.10 Fold Change in Neutrophil Phagocytosis Index in response to *E.Coli* (A) and *S.Aureus* (B) from Day-0 to Day-3 following Colorectal Cancer

Box and Whisker Plot (10-90 percentile) and tabulated results of Fold Change (AU) from Day-0 to Day-3 following Colorectal Cancer Resection in Phagocytosis Index in response to *E.Coli* (A) and *S.Aureus* (B).

5.5.5 Changes in Peri-operative NET Production Defined by Operative Characteristics and Patient Outcomes

An evaluation of post-operative NET formation, on Day-1 and Day-3, defined by operative technique (laparoscopic vs. open) and operation type (rectal resection vs. segmental resection), was performed. To account for the variation in number of circulating neutrophils between individuals the ANPP was also determined.

Post-operative NET production was also analysed with regard to patient outcomes which included; post-operative complications (Clavien-Dindo <3 vs. Clavien-Dindo ≥3), total hospital length of stay (LOS ≤ 5 days vs. LOS > 5 days) and mortality (Survived vs. Died) after a median follow-up of 21.3 months (IQR 16.7-23.5 months). ANPP was also calculated and analysed.

NET production and ANPP on Day-1 defined by operative characteristics and patient outcomes is summarised in Table 5.1 and Table 5.2.

Table 5.1 NET Production on Day-1 following Colorectal Cancer Resection Categorised by Operative Characteristics and Patient Outcomes

NET Production (AFU) Day-1			
Operation Technique	**Laparoscopic (n=27)**	**Open (n=17)**	**P-value**
Unstimulated	8784	8277	0.3716
PMA	36210	39040	0.8460
IL-8	9792	8274	0.1506
LPS	10520	7267	0.0804
fMLP	11770	10820	0.3856
Operation Type	**Rectal (n=19)**	**Segmental (n=25)**	**P-value**
Unstimulated	8883	8525	0.8201
PMA	37500	40310	0.9195
IL-8	8000	9642	0.6860
LPS	9319	10420	0.7235
fMLP	10830	11770	0.4950
Complication	**CD < 3 (n=38)**	**CD ≥ 3 (n=6)**	**P-value**
Unstimulated	8685	8525	0.7267
PMA	35700	47880	0.0679
IL-8	9642	9031	0.9072
LPS	9435	10780	0.8460
fMLP	8188	11770	0.1740
Total Length of Stay	**LOS < 5 days (n=15)**	**LOS ≥ 5 days (n=29)**	**P-value**
Unstimulated	8883	8622	0.7132
PMA	31880	41620	**0.0381**
IL-8	9642	9084	0.7929
LPS	10420	9319	0.9581
fMLP	7607	9406	0.2375
Mortality	**Survived (n=39)**	**Died (n=5)**	**P-value**
Unstimulated	8685	8622	0.6412
PMA	36720	46910	0.3316
IL-8	9642	8660	0.9381
LPS	9435	10780	0.7857
fMLP	8188	10830	0.5343

Median values presented. Statistical significance is measured by Mann Whitney U test.

Table 5.2 Absolute NET Production Potential on Day-1 following Colorectal Cancer Resection Categorised by Operative Characteristics and Patient Outcomes

Absolute NET Production Potential (AFU/no.neutrophils x10^5/L) Day-1			
Operation Technique	**Laparoscopic (n=27)**	**Open (n=17)**	**P-value**
Unstimulated	6.98	9.06	0.1822
PMA	29.06	40.67	0.0804
IL-8	7.30	8.48	0.3579
LPS	7.97	7.87	0.7461
fMLP	7.63	8.11	0.2654
Operation Type	**Rectal (n=19)**	**Segmental (n=25)**	**P-value**
Unstimulated	7.24	7.88	0.9597
PMA	33.16	33.47	0.8596
IL-8	7.34	9.51	0.4950
LPS	7.21	8.56	0.6044
fMLP	6.64	8.40	0.6583
Complication	**CD < 3 (n=38)**	**CD ≥ 3 (n=6)**	**P-value**
Unstimulated	7.24	9.26	1.0000
PMA	32.21	46.34	0.1400
IL-8	7.38	9.81	0.4373
LPS	7.63	8.62	0.6551
fMLP	7.57	11.39	0.2439
Total Length of Stay	**LOS < 5 days (n=15)**	**LOS ≥ 5 days (n=29)**	**P-value**
Unstimulated	6.80	8.13	0.1279
PMA	28.08	40.39	**0.0136**
IL-8	7.20	8.18	0.0930
LPS	7.42	8.55	0.2535
fMLP	5.81	8.65	**0.0406**
Mortality	**Survived (n=39)**	**Died (n=5)**	**P-value**
Unstimulated	7.24	9.80	0.7267
PMA	33.16	46.34	0.3316
IL-8	7.44	11.31	0.5866
LPS	7.63	8.10	0.7121
fMLP	7.57	11.39	0.3512

Median values presented. Statistical significance is measured by Mann Whitney U test.

The post-operative NET production and ANPP were evaluated on post-operative Day-1 and Day-3 categorised by operative technique and operation type and patient outcomes. On Day-1, significant differences were identified only in the analysis of total hospital length of stay (LOS ≤ 5 days vs. LOS > 5 days). It was found that NET production was significantly increased in response to PMA ($p=0.0381$) and that ANPP was significantly elevated in response to PMA ($p=0.0136$) and fMLP ($p=0.0406$) in patients with LOS > 5 days. No significant differences were identified in post-operative NET production or ANPP on Day-3.

When appraising the peri-operative changes in NET production and ANPP it was evident that the greatest differences in NET production and ANPP occurred from Day-0 to Day-1. Consequently, fold changes were calculated to determine if 'the change' in NET production or ANPP, when categorised according to operative characteristics and patient outcomes, was significant. The fold changes in NET production and ANPP, from Day-0 to Day-1 are presented in Table 5.3 and Table 5.4 respectively.

Table 5.3 Fold Change in NET Production from Day-0 to Day-1 in Patients with Colorectal Cancer Categorised by Operative Characteristics and Patient Outcomes

Fold Change in NET Production (AU) Day-0 to Day-1			
Operation Technique	**Laparoscopic (n=27)**	**Open (n=17)**	**P-value**
Unstimulated	0.698	0.772	0.3999
PMA	0.878	1.035	0.1581
IL-8	0.751	0.823	0.5776
LPS	0.705	0.760	0.9690
fMLP	0.730	0.816	0.6135
Operation Type	**Rectal (n=19)**	**Segmental (n=25)**	**P-value**
Unstimulated	0.771	0.708	0.4637
PMA	0.879	0.911	0.8795
IL-8	0.771	0.757	0.9195
LPS	0.779	0.675	0.6311
fMLP	0.735	0.771	0.8795
Complication	**CD < 3 (n=38)**	**CD ≥ 3 (n=6)**	**P-value**
Unstimulated	0.765	0.469	0.1621
PMA	0.879	0.944	0.4373
IL-8	0.771	0.560	0.1507
LPS	0.748	0.675	0.2000
fMLP	0.771	0.572	0.1203
Total Length of Stay	**LOS < 5 days (n=15)**	**LOS ≥ 5 days (n=29)**	**P-value**
Unstimulated	0.772	0.725	0.6965
PMA	0.802	0.944	0.0715
IL-8	0.726	0.772	0.3600
LPS	0.863	0.673	0.3908
fMLP	0.735	0.775	0.8637
Mortality	**Survived (n=39)**	**Died (n=5)**	**P-value**
Unstimulated	0.765	0.469	0.2768
PMA	0.885	0.942	0.9381
IL-8	0.772	0.607	0.1400
LPS	0.748	0.569	0.1113
fMLP	0.783	0.572	**0.0219**

Median values presented. Statistical significance is measured by Mann Whitney U test.

Table 5.4 Fold Change in Absolute NET Production Potential from Day-0 to Day-1 in Patients with Colorectal Cancer Categorised by Operative Characteristics and Patient Outcomes

Fold Change in Absolute NET Production Potential (AU) Day-0 to Day-1			
Operation Technique	**Laparoscopic (n=27)**	**Open (n=17)**	**P-value**
Unstimulated	1.173	1.980	0.0850
PMA	1.693	2.395	**0.0326**
IL-8	1.341	2.101	0.0948
LPS	1.402	1.807	0.2386
fMLP	1.812	2.868	0.1112
Operation Type	**Rectal (n=19)**	**Segmental (n=25)**	**P-value**
Unstimulated	1.955	1.072	**0.0115**
PMA	2.479	1.592	**0.0188**
IL-8	2.256	1.340	**0.0245**
LPS	2.474	1.926	**0.0201**
fMLP	1.789	1.248	**0.0107**
Complication	**CD < 3 (n=38)**	**CD ≥ 3 (n=6)**	**P-value**
Unstimulated	1.625	1.012	0.1298
PMA	2.020	2.479	0.6412
IL-8	1.593	1.162	0.2943
LPS	1.718	1.153	0.2440
fMLP	1.580	1.000	0.1113
Total Length of Stay	**LOS < 5 days (n=15)**	**LOS ≥ 5 days (n=29)**	**P-value**
Unstimulated	1.781	1.346	0.9372
PMA	2.020	2.106	0.4465
IL-8	1.588	1.409	0.7728
LPS	1.926	1.365	0.5636
fMLP	1.580	1.462	0.7929
Mortality	**Survived (n=39)**	**Died (n=5)**	**P-value**
Unstimulated	1.625	1.012	0.2000
PMA	2.022	1.793	0.6977
IL-8	1.593	1.001	0.1740
LPS	1.718	1.153	0.1400
fMLP	1.616	1.000	**0.0360**

Median values presented. Statistical significance is measured by Mann Whitney U test.

The fold change in NET production was noted to be significantly reduced in patients who died compared to patients who survived, but only in response to fMLP. No other significant differences were identified when investigating operative characteristics and patient outcomes.

The fold change in ANPP was significantly increased in patients undergoing rectal resection compared to segmental resection in response to No stimulant, PMA, IL-8, LPS and fMLP. Similarly, the fold change was increased in patients undergoing open compared to laparoscopic resection, but only achieved statistical significance in response to PMA. Regarding patient outcomes, the fold change in ANPP from Day-0 to Day-1 was significantly reduced in patients who died compared to patients who survived, but only in response to fMLP.

5.6 Discussion

The experiments undertaken in this chapter help to provide a greater understanding of the serial changes in neutrophil function over the peri-operative period in patients undergoing major resectional surgery for colorectal cancer. The findings suggest that a novel neutrophil phenotype may exist in patients undergoing colorectal resection for cancer.

Firstly, a significant reduction in NET formation from Day-0 to Day-1 was detected. This observed NET reduction, as a consequence of the surgical insult, may indicate neutrophil activation as the findings are supported by in-vitro experiments which studied the neutrophil functional changes in patients with sepsis and severe sepsis [294]. When taking into account the number of absolute circulating neutrophils, despite an observed reduction in NET formation from Day-0 to Day-1, a significant increase in the ANPP was identified from Day-0 to Day-1. This calculation attempts to quantify the 'potential' NET formation in an individual which is inferred from the laboratory experiments. It is an interesting concept which attempts to provided a measure of the quanity of circulating NETs in-vivo. This might imply that the number of circulating neutrophils are also important when determining patient outcomes and this may also explain why the NLR has been used successfully to predict overall and cancer specific survival in numerous solid organ malignancies [99].

Secondly, significant changes indicating delayed apoptosis (increase in alive and reduction in apoptotic neutrophils) were detected from Day-0 to Day-1 and significant changes favouring apoptosis (reduction in alive and increase in apoptotic neutrophils) were identified from Day-1 to Day-3 at 24-hours incubation. Evidence supports reduced apoptosis of neutrophils in inflammation, which is thought to be caused by the activation of nuclear factor-kappa B which is associated with reduced activity of caspase-3 and caspase-9, and that this is

associated with poor patient outcomes [295, 296]. Therefore, delayed apoptosis from Day-0 to Day-1 is indicative of pro-inflammation and favoured apoptosis from Day-1 to Day-3 is suggestive of anti-inflammation. In this study it was not possible to identify any statistically significant results when analysing the stages of neutrophil apoptosis with adverse patient outcomes, however, it is hypothesised that neutrophils that exhibit a phenotype characterising a prolonged delayed apoptosis may be associated with adverse outcomes as it is essential that neutrophils are 'switched off', undergo apoptosis and are successfully cleared to minimise host damage and a state of chronic inflammation [295].

Thirdly, demonstrable increases in neutrophil phagocytotic activity were revealed on sequential peri-operative days, over the peri-operative period, but statistical significance was not achieved. Significant increases in phagocytotic index from Day-1 to Day-3 were observed to *E.Coli* which suggests an increase in phagocytotic activity up to the third post-operative day in response to stimulation with *E.Coli*.

It is increasingly recognised that circulating neutrophils are heterogenous comprising of cell populations that might favour inflammation or the resolution of inflammation. The polarisation of neutrophils towards a pro-inflammatory or anti-inflammatory phenotype, as a consequence of the response to the surgical insult, may determine short-term patient outcomes and longer-term oncological outcomes. Neutrophil phenotypic plasticity might therefore help to explain the findings that a pre-operative SIR has been shown to be independently associated with the risk of developing infective post-operative complications in patients undergoing curative colorectal cancer resection [39] and it is well established as a negative prognostic factor in primary operable [40] and metastatic colorectal cancer [1,

51-54] and that following colorectal cancer resection post-operative septic complications increase the risk of disease recurrence [37, 38].

The experimental findings suggest that neutrophils adopt a distinctive phenotype, characterised by a reduction in NET formation, inhibition of the apoptosis and increase in phagocytosis in response to *E.Coli*, as a consequence of the surgical insult. The resultant accumulation of activated neutrophils in the circulation following surgery may cause more extensive collateral tissue damage and potential organ dysfunction. The increase in NET formation (and reduction in the ANPP) and the restoration of apoptosis in the late post-operative phase (Day-3) coincides with apparent surgical recovery and therefore an early phenotypic switch (from dysregulated to normal neutrophil function) may be desirable. The experimental findings outlined in this study are supported by a specific neutrophil phenotype described in the context of inflammation associated with sepsis which demonstrates a failure of migration, a propensity to generate ROS with preserved phagocytic capacity while suppressing NET formation and suspending apoptosis [294].

In this chapter it was also investigated whether differences in peri-operative NET production were apparent when classifed by operative technique and operation type and when classified by post-operative complication, total length of hosital stay and mortality. No significant differences in peri-operative NET production were identified in the analysis of operative technique or operation type. Significant increases in NET production were, however, associated with an increased hospital stay and prolonged hospital recovery in response to PMA. No differences were identified in patients who experienced significant post-operative complications or died during the study follow-up. It was also demonstrated that the ANPP was significantly elevated in patients with a prolonged hospital recovery in

response to PMA and fMLP which may allude to a potential prognostic role of this novel measure. The fold changes in NET production and ANPP from Day-0 to Day-1 were also evaluated. A significant reduction in the change in NET production and ANPP was observed in patients who died in response to fMLP. This might imply that a reduced fold change in NET production as a consequence of surgery may have a role in prognostication and mortality prediction. The evidence indicates that further investigation into the prognostic value of NET production is justified and the change in NET production over the peri-operative may be a promising area to study in relation to patient outcomes.

This experimental study has a number of limitations. Firstly, the population evaluated was small and heterogeneous with regard to patient demographics, patient co-morbidities, tumour characteristics and operative characteristics making interpretation of the results difficult and limiting the generalisability of the study findings. Secondly, in-vitro experiments performed to assess neutrophil function were conducted outside of the biological context and consequently there are challenges in extrapolating the results and it must be acknowledged that they cannot be readily transposed to, and predict the reaction of, the entire organism in-vivo. Thirdly, comparative analyses stratifying patients according to cancer location, cancer stage, operative technique, operation type and patient outcomes is subject to misinterpretation as potential confounding variables were not accounted for. Fourthly, evidence suggests that anaesthetic choice is important in peri-operative immunosuppression and has been linked to cancer recurrence and survival. Total intravenous anaesthetics appear to reduce surgical stress, peri-operative immunosuppression and angiogenesis compared with volatile inhalational anaesthetics [306, 307]. A causal link between anaesthetics, immune function and survival, however,

remains to be elucidated. The type of anaesthetic and the possible impact of different anaesthetic agents were not investigated in this study. Lastly, the number of adverse patient outcomes in the study population was low making interpretation of patient outcomes challenging.

The benefit of studying a surgical population, compared to patients with other inflammatory conditions, for example in patients with sepsis, is that the true impact of a provoked SIR can be identified as pre-operative and post-operative evaluation is undertaken. It is also appreciated that the surgical insult is relatively consistent between individuals, particularly with the universal implementation of a standardised enhanced recovery peri-operative care pathway.

5.7 Conclusion

A novel neutrophil phenotype has been described in patients undergoing resectional surgery for colorectal cancer which demonstrates reduced NET formation, delayed apoptosis and increased phagocytosis in response to *E.Coli*.

As a consequence of impaired cell death, either by disrupted apoptosis or impaired NETosis, an accumulation of activated neutrophils in the circulation could be potentially harmful to the host following surgery. The restoration of the apoptosis and NET production in the later post-operative period appears to coincide with surgical recovery and therefore an early phenotypic switch (from dysregulated to normal neutrophil function) may be desirable. Clinical and experimental evidence suggests that activated neutrophils may facilitate metastatic progression and therefore therapeutic strategies aimed at modifying tumour-neutrophil interactions may maximise the therapeutic benefit of surgery.

Chapter 6

THE IMPACT OF HMG-COA REDUCTASE INHIBITORS ON NEUTROPHIL FUNCTION

6.1 Introduction

Cancer surgery elicits a high-grade, non-specific systemic inflammatory response (SIR). This overwhelming, systemic inflammation suppresses systemic cell-mediated immunity and consequently compromises the tumour immunity in the host [58, 59]. This has been described as the 'immune-hit' [58]. The immune-hit can be exacerbated by pre-operative SIR, for example, an emergency presentation with colonic obstruction, bleeding or perforation, confers a higher risk of disease recurrence independent of the stage of disease [40]. Additionally, the development of post-operative complications and the associated post-operative SIR increases the risk of disease recurrence [38] and is associated with poor oncological outcomes and increased mortality secondary to metastatic disease [187-191].

Peri-operative immune-modulatory therapies aim to suppress non-specific systemic inflammation and maintain an effective anti-tumour, cell-mediated immunity of the host which may assist the clearance of circulating tumour cells and the development of occult metastases [58]. Accumulating evidence suggests that simple immune-modulatory strategies (anti-inflammatories [non-steroidal anti-inflammatory drugs] or immune-modulatory therapies [corticosteroids, HMG-CoA reductase inhibitors (statins)]) could be safely implemented in the peri-operative period, although concerns surrounding their side effect profile exist [60].

Statins have been extensively studied and have proven efficacy in the primary and secondary prevention of cardiovascular morbidity and mortality in a variety of populations [212-217]. Their main mechanism of action is competitive inhibition of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase leading to a reduction in endogenous cholesterol biosynthesis and a reduction in low-density lipoprotein which is a major risk factor for

cardiovascular disease [15, 16]. The cholesterol independent effects of statins are known as pleiotropic effects and they have been shown to modulate the innate and adaptive immune systems and have anti-inflammatory effects which counteract the detrimental effects of inflammation [223, 225].

In the context of colorectal surgery, statin use is associated with a significantly lower incidence of SIR and a lower incidence of surgical site infection (SSI) [248]. There appears to be no difference in overall mortality, total complications or median hospital length of stay between statin users and non-statin users undergoing major colorectal resection [246, 21], despite statin users having a greater baseline peri-operative risk compared to non-statin users, and it has been proposed that peri-operative statin therapy may reduce morbidity after colorectal resection [248, 250].

There is increasing evidence that statins are able to modulate neutrophil function. In sepsis it has been demonstrated that statins reduce the neutrophil infiltrate following lipopolysaccharide (LPS) challenge in bronchoalveolar lavage fluid taken from patients receiving simvastatin [297]. In addition, the reduction in neutrophil counts was not associated with a decrease in pro-inflammatory mediator expression, which suggests a reduced neutrophil response or increased neutrophil apoptosis [297]. Statins have also been shown to increase NET formation in healthy individuals with the consequent elimination of bacteria in-vitro [298]. Furthermore, during bacterial pneumonia associated sepsis, NET production is suppressed with improvements occurring during sepsis resolution [299]. It has been proposed that neutrophil functional defects may be restored by the administration of statins [299].

This is particularly relevant in the context of cancer surgery as neutrophils are appreciated to have an important role in cancer progression and dissemination [158]. Neutrophils play a vital role in circulating tumour cell metastases. This has been shown in-vitro and in-vivo where neutrophils facilitate circulating tumour cell adhesion to both pulmonary and hepatic endothelial surfaces [174, 176, 179-181]. The direct contact of circulating tumour cells and neutrophils is an important precursor to the development of metastatic disease [179, 180]. Following dissemination, tumour cells must be able to proliferate to form stable metastatic foci. NETs have been implicated in a direct proliferative role and have also been demonstrated to inhibit tumour cell apoptosis [158]. The ability of tumours to predispose neutrophils to produce NETs has been demonstrated and various tumour types have predisposed circulating neutrophils to produce NETs [178].

The evidence supports the theory that primary tumours can facilitate NET production in circulating neutrophils and we propose that neutrophil functional defects in cancer may be modulated by statin therapy. It is anticipated that in-vitro treatment with statin will attenuate the neutrophil functional changes observed in patients with a diagnosis of colorectal cancer and modulate the neutrophil functional changes exhibited as a consequence of the surgical insult. This may help to explain the observed benefits associated with statin therapy in the context of surgery for colorectal cancer.

6.2 Hypothesis

It was hypothesised that HMG-CoA reductase inhibitors would attenuate the neutrophil functional changes exhibited in response to the surgical insult, demonstrating:

1. Increased NET formation
2. Increased apoptosis
3. Reduced phagocytosis

6.3 Objectives

The objectives of this chapter were:

1. To evaluate the effect of HMG-CoA Reductase Inhibitors on neutrophil function, in-vivo, over the peri-operative period with specific reference to:
    i. NET formation
    ii. Neutrophil apoptosis
    iii. Neutrophil phagocytosis
2. To investigate the effect of in-vitro treatment with 1µM Simvastatin on NET formation and Neutrophil apoptosis pre-operatively (Day-0) and post-operatively (Day-1).

6.4 Methods

6.4.1 Acknowledgements

Dr. Hongxia Mei (International Research Fellow from the Hospital of Wenzouh Medical University, China) assisted with the Neutrophil Phagocytosis experiments. The short life-span of neutrophils meant that neutrophil functional experiments were conducted simultaneously and it was impossible for all experiments to be performed by one individual in entirety.

6.4.2 Recruitment of Patients

Patients undergoing an elective colorectal resection for cancer, within an established enhanced recovery programme, who satisfied the specific inclusion and exclusion criteria, were recruited to the study as outlined in Section 3.1.1.

Patient Demographics, Patient Co-morbidities, Pre-operative Risk Prediction, Tumour Characteristics and Operative Characteristics were recorded from study participants as outlined in Table 3.1.

Patient outcomes were collected prospectively and included:

1. Post-operative complications up to 30 days after surgery using pre-defined criteria and graded by the Clavien-Dindo classification system (Appendix 3).
2. Return to theatre within 28 days of the index procedure.
3. Admission to and length of stay on the Intensive Care Unit.
4. Total hospital length of stay.

5. Readmissions to hospital within 30 days of the index procedure requiring a hospital stay ≥ 24 hours.
6. 30 day and 90 day mortality.

Functional and quality of life was assessed using the Surgical Recovery Scale (Appendix 4).

Haematological and biochemical parameters were collected prospectively from the hospital pathology system. These parameters, in conjunction with physiological indices, were used to determine a CR-POSSUM (Appendix 5), a NLR, and an mGPS (Table 1.6) for each patient.

6.4.3 Sample Collection

Patients underwent peripheral blood tests pre-operatively (Day-0) and post-operatively (Day-1 and Day-3) to assess neutrophil function at these specific time points. Blood tests were performed by an experienced medical practitioner by peripheral venepuncture or from a peripheral arterial cannula. A total of 24mls of blood was taken from each patient at each time point and deposited into 4x6ml lithium-heparin vacutainers (Becton-Dickinson, Oxford, UK). The samples were then placed on ice and transported to the laboratory for processing.

6.4.4 Neutrophil Isolation

Processing began with 45 minutes of obtaining the blood sample. Neutrophils were isolated by following the method outlined in Section 3.2. A purity of ≥95% and a viability of ≥97% were routinely achieved.

6.4.5 Neutrophil Functional Assays

Quantification of Neutrophil Extracellular Trap Formation was conducted following the method outlined in Section 3.3.1. NETs were recorded in arbitrary fluorescent units (AFU).

To account for the variation in number of circulating neutrophils between individuals the Absolute NET Production Potential (ANPP) was determined. It was calculated as follows:

ANPP (AFU / no. of neutrophils x10^5 / L) = NETs per 100,000 neutrophils (AFU) x Absolute Neutrophil Count (x10^9 / L) ÷ 10,000

Neutrophil Apoptosis and Neutrophil Phagocytosis were assessed using commercially available assays which were carried out as per the manufacturer's instructions as outlined in Sections 3.3.2 and 3.3.3 respectively. Neutrophil Apoptosis was represented as the percentage of neutrophils in the different stages of apoptosis (alive, early apoptosis, late apoptosis and necrosis). Neutrophil phagocytotic function was represented by the Phagocytosis Index which provided a quantitative measure of neutrophil phagocytotic function. It was calculated as follows:

Phagocytosis Index = (Percentage of pHrodo 'bright' cells ÷ 100) x Median Fluorescent Intensity

6.4.6 In-vitro Investigation with HMG-CoA Reductase Inhibitors

The HMG-CoA reductase inhibitor used in the in-vitro experiments was simvastatin (Sigma-Aldrich). The dose used to investigate the in-vitro effects of simvastatin was 1µM. The rationale for the dose selection is outlined in Section 3.1.1. The solvent Dimethyl Sulfoxide (DMSO; Sigma-Aldrich) is the vehicle control for simvastatin. The rationale for its selection is outlined in Section 3.1.2.

Quantification of Neutrophil Extracellular Trap Formation (3.3.1) and Neutrophil Apoptosis (3.3.2) assays were repeated after isolated neutrophils, suspended in RPMI-1640 (Sigma-Aldrich) at a concentration of 1x10^6/ml, were incubated with 1µM simvastatin (Sigma-Aldrich) for 40 minutes at 37^{o}C with 5% CO_2.

6.4.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, California, USA).

Categorical data was analysed using Fisher's Exact tests for two variables or χ^2 tests for greater than two variables. Continuous data was analysed using non-parametric statistical models. Mann-Whitney U tests were used for independent samples and Wilcoxon Signed Rank tests for related samples for two groups. All statistical tests performed were two-tailed and results were considered significant when $p<0.05$.

6.5 Results

6.5.1 Population Characteristics According to Statin Use

55 patients were identified at the MDT meeting and approached for inclusion into the study. 45/55 patients (81.8%) were successfully recruited into the study and were followed up for a median of 21.3 months (IQR 16.7-23.5 months). Consent was refused from the remaining 10/55 patients (18.2%). 44/45 patients (97.8%) underwent surgical resection. 1/45 patient (2.2%) was deemed unfit for surgery on the day of surgery and never progressed to surgical resection. The baseline population characteristics, operative characteristics and patient outcomes are displayed in Table 4.1, Table 4.2 and Table 4.3 respectively.

The patients were analysed according to statin use; patients receiving peri-operative statin (statin users) and patients not receiving peri-operative statin (non-statin users). Statin users were defined as, 'patients who were prescribed and had taken a statin ≤ 5 days before the index procedure and who were prescribed and received a statin ≤ 5 days after the index procedure'. All statins currently available for use in the UK were included (atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin).

20/45 patients (44.4%) were statin users as determined by the criteria above. The majority of statin users were on simvastatin (70.0% [14/20]) and the remainder were on atorvastatin (25.0% [5/20]) and pravastatin (5.0% [1/20]). The majority of patients were on moderate-dose statin (80.0% [16/20]) and the remainder were on low-dose (20.0% [4/20]). No Statin Users were prescribed high-dose statins. The median number of statin doses omitted during the index admission was 1 (IQR = 0.0 – 1.0).

Statin Users and Non-statin Users were similar in their population characteristics, but as expected Statin Users were significantly more co-morbid.

The population characteristics according to statin use are summarised in Table 6.1.

Table 6.1 Population Characteristics According to Statin Use

Patient Demographics	*Statin User (N=20)*	*Non-statin User (N=25)*	*P-Value*
Age, median (IQR)	68.0 (62.5-73.0)	71.0 (63.5–76.0)	0.583
Gender, n (%), M/F	14 / 7 (70.0 / 30.0)	12 / 13 (48.0 / 52.0)	0.224
BMI, median (IQR), kg/m^2	24.1 (22.2-27.7)	27.7 (23.1-30.2)	0.107
Smoking Status, n (%), 1/2/3[a]	7 / 4 / 9 (35.0 / 20.0 / 45.0)	2 / 5 / 18 (8.0 / 20.0 / 72.0)	0.067
ASA, n (%), 1/2/3/4	1 / 12 / 6 / 1 (5.0 / 60.0 / 30.0 / 5.0)	3 / 13 / 8 / 1 (12.0 / 52.0 / 32.0 / 4.0)	0.854
Co-morbidity, n (%), 0/1/2/≥3	0 / 11 / 8 / 1 (0.0 / 55.0 / 40.0 / 5.0)	10 / 10 / 2 / 3 (40.0 / 40.0 / 8.0 / 12.0)	**0.003**
Medications, n, 1/2/3/4[b]	9 / 4 /8 / 4	12 / 5 / 3 / 3	0.578
CR-POSSUM, %, median (IQR)	2.14 (1.33-4.78)	2.58 (1.80-3.58)	0.731
mGPS, n (%), 0/1/2	16 / 1 / 3 (80.0 / 5.0 / 15.0)	18 / 2 / 5 (72.0 / 8.0 / 20.0)	0.819
NLR, median (IQR)	3.52 (2.78-5.72)	3.38 (2.68-3.96)	0.568
SRS (Pre-operative [%]), median (IQR)	72.6 (71.2 – 83.6)	77.4 (64.4 – 88.7)	1.000
Presentation	***Statin User (N=20)***	***Non-statin User (N=25)***	***P-value***
Presentation, n (%), 1/2[c]	18 / 2 (90.0 / 10.0)	21 / 4 (84.0 / 16.0)	0.678
Cancer Location, n (%), 1/2[d]	11 / 9 (55.0 / 45.0)	16 / 9 (64.0 / 36.0)	0.559

y = yes, 1/2/3[a] = active / former / never, 1/2/3/4[b] = antihypertensive / β-antagonist / anti-platelet / oral hypoglycaemic , 1/2[c] = symptomatic / screened , 1/2[d] = colon / recto-sigmoid junction and rectum

Statin Users and Non-statin Users were well matched in their tumour and operative characteristic and no significant differences were identified.

The tumour and operative characteristics according to statin use are displayed in Table 6.2.

Table 6.2 Tumour and Operative Characteristics According to Statin Use

Tumour Characteristics	*Statin User (N=20)*	*Non-statin User (N=24)*	*P-Value*
Neo-adjuvant Therapy, n (%), y	5 (25.0)	6 (25.0)	1.000
T-Stage, n (%), 0/1/2/3/4	1 / 0 / 4 / 12 / 3	1 / 2 / 5 / 10 / 6	0.5656
	(5.0 / 0.0 / 20.0 / 60.0 / 15.0)	(4.2 / 8.3 / 20.8 / 41.7 / 25.0)	
N-Stage, n (%), 0/1/2	14 / 3 / 3	18 / 5 / 1	0.4382
	(70.0 / 15.0 / 15.0)	(75.0 / 20.8 / 4.2)	
M-Stage, n (%), 0/1	19 / 1	23 / 1	1.000
	(95.0 / 5.0)	(95.8 / 4.2)	
Dukes' Stage, n(%), A/B/C/D	3 / 11 / 5 / 1	7 / 10 / 6 / 1	0.709
	(15.0 / 55.0 / 25.0 / 5.0)	(29.2 / 41.7 / 25.0 / 4.2)	
Differentiation, n (%), 1/2[a]	18 / 2	21 / 3	1.000
	(90.0 / 10.0)	(87.5 / 12.5)	
EMVI, n(%), y	9 (45.0)	5 (20.8)	0.112
Operative Characteristics	*Statin User (N=20)*	*Non-statin User (N=24)*	*P-value*
Operation Type, n (%), 1/2[b]	10 / 10	15 / 9	0.543
	(50.0 / 50.0)	(62.5 / 37.5)	
Operation technique, n (%), 1/2[c]	13 / 7	14 / 10	0.760
	(65.0 / 35.5)	(58.3 / 41.7)	
Stoma Formation, n (%), y	8 (40.0)	8 (33.3)	0.757
Length of Operation (minutes), median (IQR)	170.7 (139.5-201.8)	212.7 (180.8-244.6)	0.061
Post-operative Level of Care, n (%), 1/2[d]	13 / 7	14 / 10	0.760
	(65.0 / 35.0)	(58.3 / 41.7)	

y = yes, 1/2[a] = well and moderately differentiated / poorly differentiated, 1/2[b] = segmental / rectal, 1/2[c] = laparoscopic / open and laparoscopic converted to open, 1/2[d] = ward based care / Critical Care

Despite being a more co-morbid patient group, Statin Users were identified to have equivalent patient outcomes as outlined in Table 6.3.

Table 6.3 Patient Outcomes According to Statin Use

Patient Outcomes	*Statin User (N=20)*	*Non-statin User (N=24)*	*P-value*
Complication, n (%), y	13 (65.0)	9 (37.5)	0.129
Clavien-Dindo Classification, n (%),	9 / 1 / 1 / 1 / 1	3 / 3 / 1 / 1 / 1	0.496
I/II/III/IV/V	(45.0 / 5.0 / 5.0 / 5.0 / 5.0)	(12.5 / 12.5 / 4.2 / 4.2 / 4.2)	
Re-operation, n (%)	2 (10.0)	1 (4.2)	0.583
Critical Care Admission, n (%), y	8 (40.0)	11 (45.8)	0.766
Total LOS, median (range, IQR)	8.5 (5.3-10.8)	7.0 (4.0-8.5)	0.070
SRS (Post-operative [%]), median (IQR)	39.7 (35.8 – 45.9)	43.3 (32.9 – 56.2)	0.408
Readmission, n (%), y	1 (5.0)	2 (8.3)	1.000
Disease Recurrence, n (%)	1 (5.0)	3 (12.5)	0.614
Mortality, n (%), y			
30-day	0 (0.0)	1 (4.2)	1.000
90-day	1 (5.0)	1 (4.2)	1.000
12-month	2 (10.0)	1 (4.2)	0.583
24-month	3 (15.0)	2 (8.3)	0.646

Y = yes

6.5.2 Neutrophil Extracellular Trap Formation

6.5.2.1 Neutrophil Extracellular Traps In-vivo

Quantification of NET formation was conducted on Day-0 (n=45), Day-1 (n=44) and Day-3 (n=22). The population was stratified and analysed according to statin use in an attempt to identify differences in NET production between Satin Users and Non-statin Users in-vivo. To account for the variation in number of circulating neutrophils between individuals the ANPP was also determined.

NET production and ANPP, according to statin use, on Day-0, Day-1 and Day-3 are displayed in Table 6.4 and Figure 6.1, Table 6.5 and Figure 6.2, and Table 6.6 and Figure 6.3 respectively.

Table 6.4 NET Production, Absolute NET Production Potential and Absolute Neutrophil Count in Statin Users and Non-statin Users on Day-0

	Statin User (n=20)	*Non-statin User (n=25)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	11380 (10230 – 15340)	10400 (7759 – 14780)	0.3917
PMA	42790 (31040 – 48100)	35110 (28790 – 47170)	0.3204
IL-8	13770 (8632 – 15750)	11820 (9534 – 14190)	0.3792
LPS	13540 (11890 – 17590)	11280 (8881 – 16100)	0.2880
fMLP	13060 (10590 – 16660)	11120 (8281 – 14300)	0.1124
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	5.40 (4.16 – 9.01)	4.87 (2.62 – 8.13)	0.3432
PMA	19.33 (11.98 – 24.91)	15.62 (8.92 – 26.37)	0.3204
IL-8	5.68 (4.01 – 9.11)	5.07 (2.86 – 9.14)	0.2729
LPS	6.06 (4.13 – 9.65)	5.36 (2.94 – 9.33)	0.2582
fMLP	5.97 (4.39 – 10.00)	4.16 (2.90 – 8.19)	0.1024
Absolute Neutrophil Count (x10^9/L), median (IQR)	4.82 (4.05-5.92)	4.19 (2.94-6.05)	0.3094

Statistical significance, measured by Mann Whitney U test.

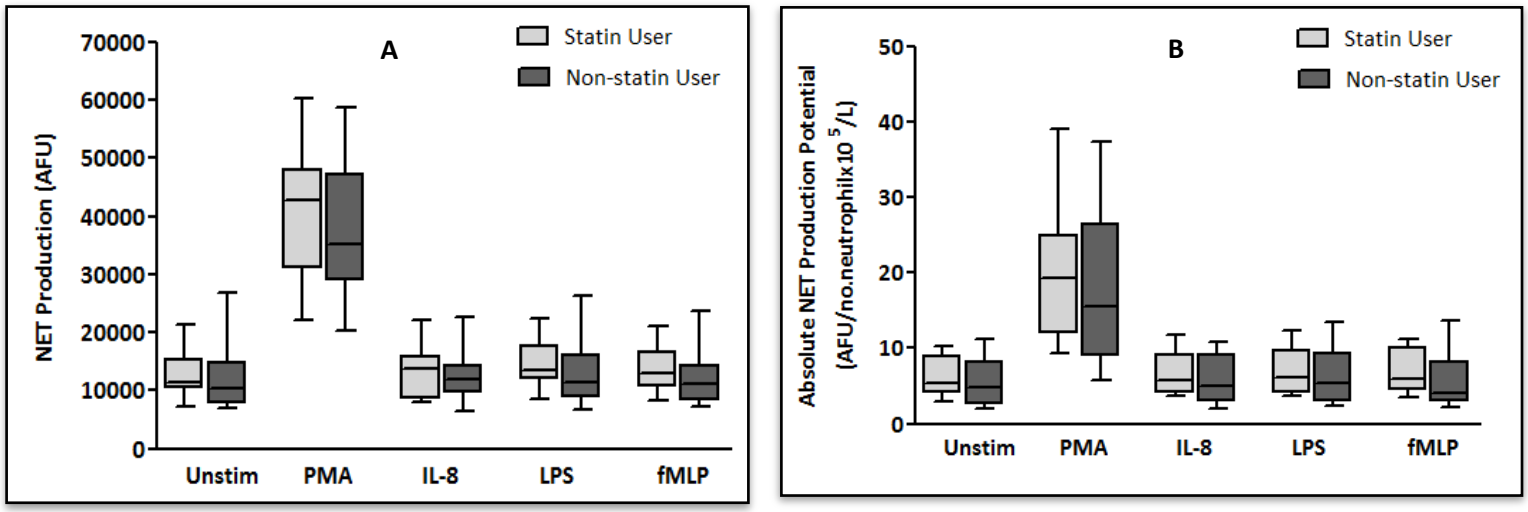


Figure 6.1 NET Production (A) and Absolute NET Production Potential (B) in Statin Users and Non-statin Users on Day-0

Box and Whisker Plots (10-90 percentile) of NET Production (A) and Absolute NET Production Potential (B) in Statin Users and Non-statin Users on Day-0 in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 6.5 NET Production and Absolute NET Production Potential in Statin Users and Non-statin Users on Day-1

	Statin User (n=20)	*Non-statin User (n=24)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	8883 (7167 – 10370)	8622 (6236 – 10840)	0.7425
PMA	42250 (33760 – 54510)	31880 (26300 – 41620)	**0.0164**
IL-8	10120 (8000 – 11720)	8236 (7391 – 10700)	0.2156
LPS	10420 (8154 – 13040)	7622 (6507 – 12790)	0.2772
fMLP	10530 (8509 – 11790)	7097 (6164 – 10630)	**0.0338**
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	7.88 (5.74 – 10.73)	6.83 (5.34 – 9.85)	0.4484
PMA	40.39 (28.09 – 56.69)	29.35 (19.32 – 40.95)	**0.0383**
IL-8	9.77 (6.31 – 13.23)	7.29 (6.01 – 9.63)	0.1498
LPS	10.48 (6.43 – 12.51)	7.41 (6.03 – 10.17)	0.0953
fMLP	10.05 (6.32 – 11.91)	6.65 (5.25 – 9.47)	**0.0419**
Absolute Neutrophil Count (x10^9/L), median (IQR)	9.54 (7.67-10.50)	8.52 (6.91-10.66)	0.5111

Statistical significance is measured by Mann Whitney U test.

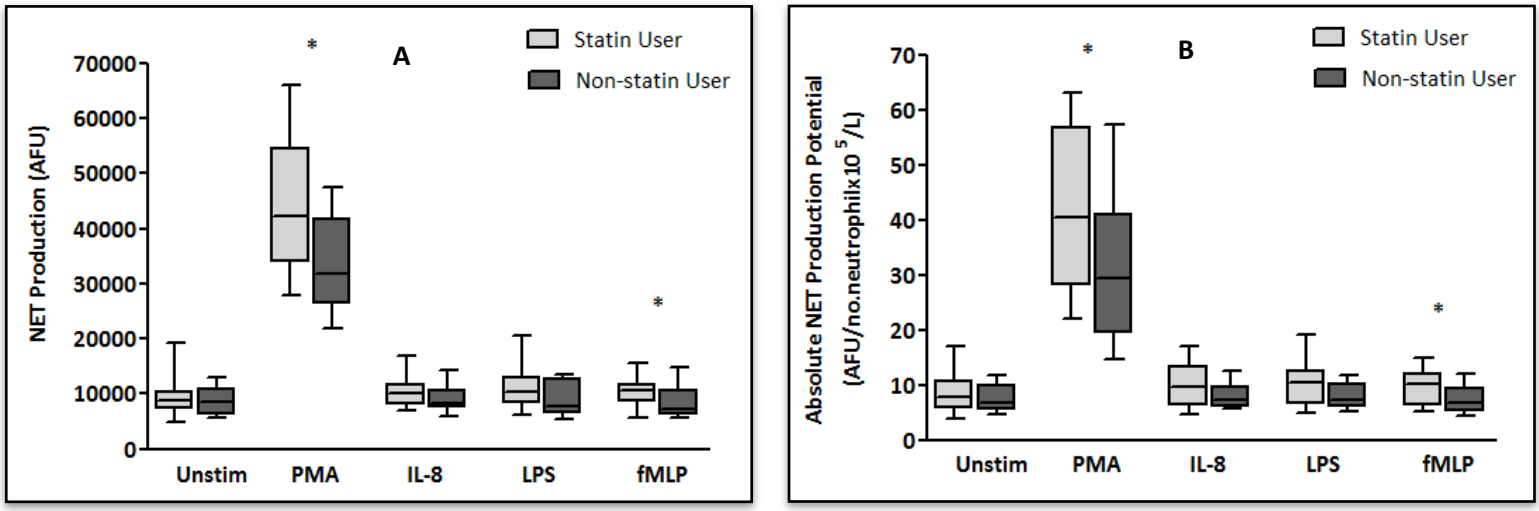


Figure 6.2 NET Production (A) and Absolute NET Production Potential (B) in Statin Users and Non-statin Users on Day-1

Box and Whisker Plots (10-90 percentile) of NET Production (A) and Absolute NET Production Potential (B) in Statin Users and Non-statin Users on Day-1 in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 6.6 NET Production and Absolute NET Production Potential in Statin Users and Non-statin Users on Day-3

	Statin User (n=10)	*Non-statin User (n=12)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	9882 (7816 – 10570)	10270 (7503 – 15850)	0.5752
PMA	33170 (27950 – 36750)	34550 (30860 – 42540)	0.3734
IL-8	10410 (9109 – 14140)	10860 (8780 – 15380)	0.8691
LPS	10410 (9436 – 12810)	9841 (8333 – 12550)	0.6682
fMLP	10830 (9466 – 13080)	9827 (8023-13100)	0.5310
Absolute NET Production (AFU / no. neutrophils x 10^5/L), median (IQR)			
Unstimulated	6.16 (5.21 – 8.33)	7.16 (4.02 – 9.47)	0.9212
PMA	24.52 (18.13 – 28.20)	21.24 (15.86 – 33.38)	0.7667
IL-8	7.13 (5.43 – 1.75)	7.37 (4.48 – 10.12)	0.6209
LPS	8.11 (4.84 – 8.79)	6.18 (4.54 – 8.79)	0.5310
fMLP	7.71 (4.75 – 9.32)	6.35 (4.42 – 8.20)	0.2766
Absolute Neutrophil Count (x10^9/L), median (IQR)	7.33 (5.75-7.97)	5.63 (4.31-8.27)	0.6418

Statistical significance is measured by Mann Whitney U test.

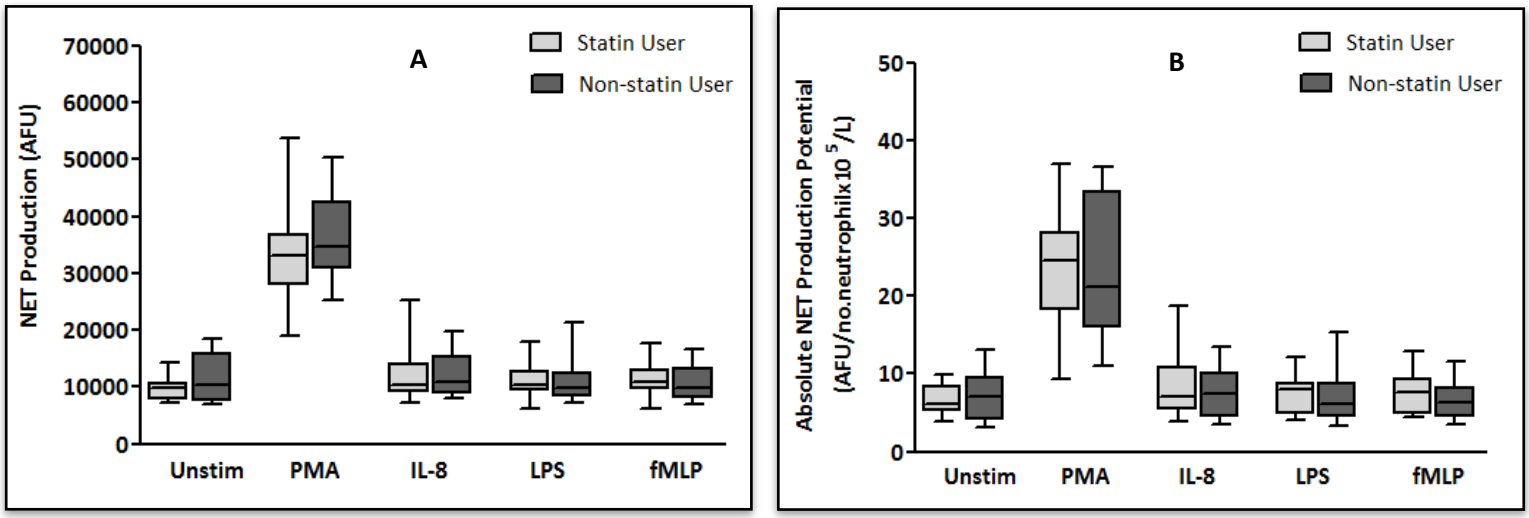


Figure 6.3 NET Production (A) and Absolute NET Production Potential (B) in Statin Users and Non-statin Users on Day-3

Box and Whisker Plots (10-90 percentile) of NET Production (A) and Absolute NET Production Potential (B) in Statin Users and Non-statin Users on Day-3 in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

No significant differences in NET production or ANPP were demonstrated between Statin Users and Non-statin Users on Day-0 or Day-3.

On Day-1 significant increases in NET production and ANPP were identified in response to stimulation with PMA and fMLP in Statin Users when compared to Non-statin Users.

No statistically significant differences were identified in absolute neutrophil counts on Day-0, Day-1 or Day-3 between the two groups.

6.5.2.2 Neutrophil Extracellular Traps In-vitro

Quantification of NET formation was performed with in-vitro statin treatment (1µM Simvastatin) or vehicle control (DMSO) on Day-0 (n=15) and Day-1 (n=14). The population characteristics of the patients who underwent in-vitro experiments are summarised in Table 6.7.

Table 6.7 Population Characteristics of Patients who Underwent In-vitro Experiments with Statin Treatment

Patient Demographics		*N=15*
Age, median (IQR)		72.0 (59.0-80.0)
Gender, n (%), M/F		11 / 4 (73.3 / 26.7)
BMI, median (IQR), kg/m^2		24.5 (21.6-27.8)
Smoking Status, n (%), 1/2/3[a]		3 / 3 / 9 (20.0 / 20.0 /60.0)
ASA, n (%), 1/2/3/4		1 / 6 / 6 / 2 (6.7 / 40.0 / 40.0 / 13.3)
Co-morbidity, n (%), 0/1/2/≥3		4 / 5 / 3 / 3 (26.7 / 33.3 / 20.0 / 20.0)
Medications, n, 1/2/3/4[b]		7 / 7 / 6 / 1
Statin User, n (%), y		5 (33.3)
CR-POSSUM, (%), median (IQR)		3.1 (1.3-4.95)
Dukes' Stage, n (%), A/B/C/D		4 / 7 / 7 / 0 (26.7 / 46.7 / 46.7 / 0.0
Operation Type, n (%), 1/2[c]		9 / 6 (60.0 / 40.0)
Operation technique, n (%), 1/2[d]		10 / 5 (66.6 / 33.3)
Clavien-Dindo Classification, n (%), I/II/III/IV/V		5 / 1 / 2 / 0 / 1 (33.3 / 6.7 / 13.3 / 0.0 / 6.7)
Total LOS, median (range, IQR)		9.0 (7.0-16.0)
Disease Recurrence, n (%)		1 (6.7)
Mortality, n (%), y		
	30-day	1 (6.7)
	90-day	2 (13.3)

y = yes, 1/2/3[a] = active / former / never, 1/2/3/4[b] = antihypertensive / β-antagonist / anti-platelet / oral hypoglycaemic, 1/2[c] = segmental / rectal, 1/2[d] = laparoscopic / open and laparoscopic converted to open

NET production in patents who underwent in-vitro treatment with statin treatment on Day-0 and Day-1 are displayed in Table 6.8 and Figure 6.4 and Table 6.9 and Figure 6.5 respectively.

Table 6.8 NET Production with in-vitro treatment with Statin Treatment (1µM Simvastatin) or Vehicle Control (DMSO) on Day-0

	Statin Treatment (1µM Simvastatin)	*Vehicle Control (DMSO)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	12730 (8986 – 14680)	14560 (10040 – 15910)	**0.0256**
PMA	35730 (29930 – 44080)	41930 (36030 – 50640)	**0.0015**
IL-8	14350 (11740 – 17210)	15190 (9438 – 17620)	0.3303
LPS	12190 (10390 – 14110)	15280 (11210 – 18640)	**0.0302**
fMLP	12580 (9887 – 14540)	14540 (11760 – 19030)	**0.0029**

Statistical significance, measured by Wilcoxon Signed Rank test.

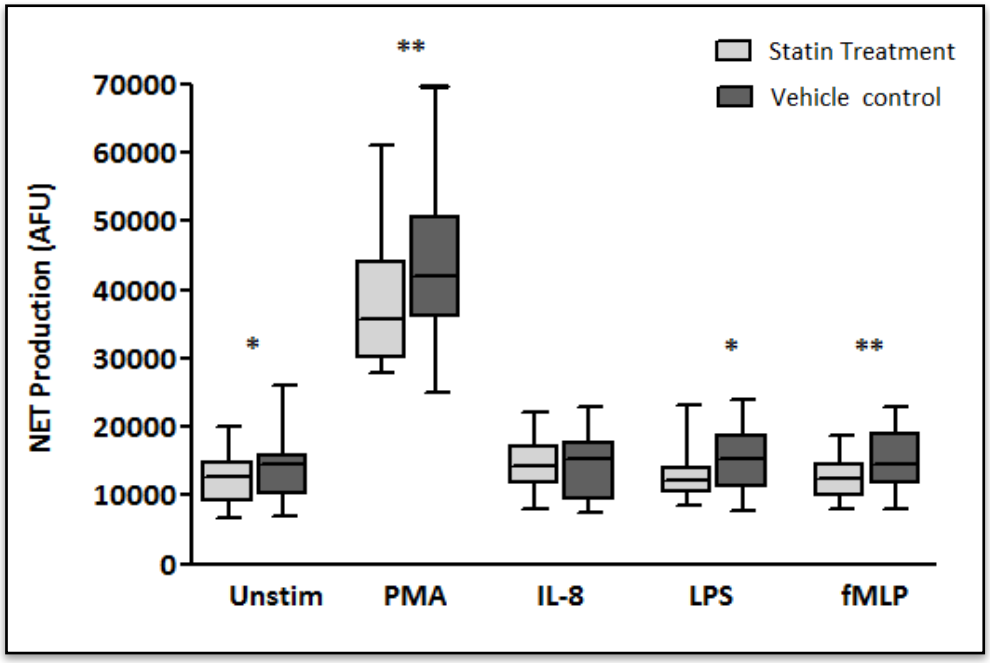


Figure 6.4 NET Production with in-vitro Statin Treatment (1µM Simvastatin) vs. Vehicle Control (DMSO) on Day-0

Box and Whisker Plot (10-90 percentile) of NET Production with in-vitro Statin Treatment (1µM Simvastatin) vs. Vehicle Control (DMSO) on Day-0 in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 6.9 NET Production with in-vitro treatment with Statin Treatment (1µM Simvastatin) or Vehicle Control (DMSO) on Day-1

	Statin Treatment (1µM Simvastatin)	*Vehicle Control (DMSO)*	*P-value*
NET Production (AFU), median (IQR)			
Unstimulated	8147 (6720 – 10300)	10240 (8793 – 12010)	**0.0107**
PMA	36280 (30780 – 51210)	48280 (39680 – 58840)	**0.0494**
IL-8	8891 (6943 – 12330)	11690 (10220 – 14580)	**0.0134**
LPS	9479 (7185 – 11360)	12850 (9626 – 13660)	**0.0245**
fMLP	8986 (7247 – 12540)	11640 (10210 – 13240)	**0.0419**

Statistical significance, measured by Wilcoxon Signed Rank test.

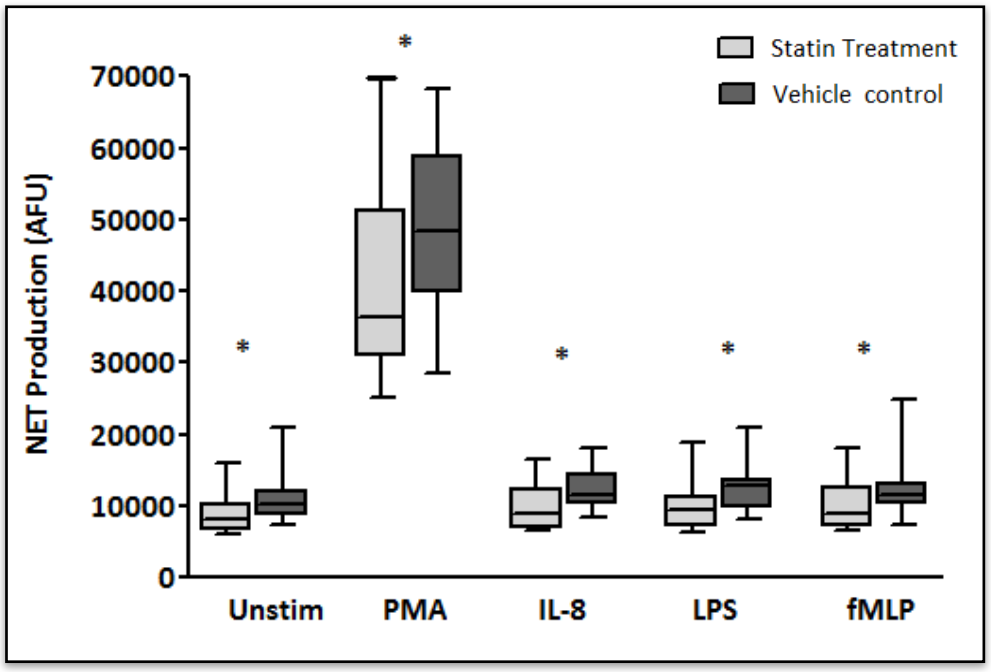


Figure 6.5 NET Production with in-vitro Statin Treatment (1µM Simvastatin) vs. Vehicle Control (DMSO) on Day-1

Box and Whisker Plot (10-90 percentile) of NET Production with in-vitro Statin Treatment (1µM Simvastatin) vs. Vehicle Control (DMSO) on Day-1 in response to No stimulant (Unstim), PMA, IL-8, LPS and fMLP. Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

The experiments revealed a reduction in NET production in patients treated with 1µM Simvastatin in-vitro at all time points. This NET reduction was significant to No stimulant, PMA, LPS and fMLP on Day-0 and to all stimulants on Day-1.

As outlined in Chapter 4, when neutrophils from a colorectal cancer population were primed with TNF-α, NET production was significantly increased in response to No stimulant ($p=0.0021$), IL-8 ($p=0.0007$), LPS ($p=0.0233$) and fMLP ($p=0.0002$) (Table 4.1).

When neutrophils from a colorectal cancer population were treated with 1µM Simvastatin the converse is true with a significant reduction in NET production in response to No stimulant ($p=0.0256$), PMA ($p=0.0015$), LPS ($p=0.0302$) and fMLP ($p=0.0029$) (Table 6.8). This finding is also maintained in the post-operative period where a significant reduction in NET production was identified in response to all stimulants on Day-1 (Table 6.9).

Figure 6.6 demonstrates a comparison of unprimed versus primed NET production and vehicle control versus statin treatment NET production in a colorectal cancer population on Day-0 in response to stimulation with fMLP.

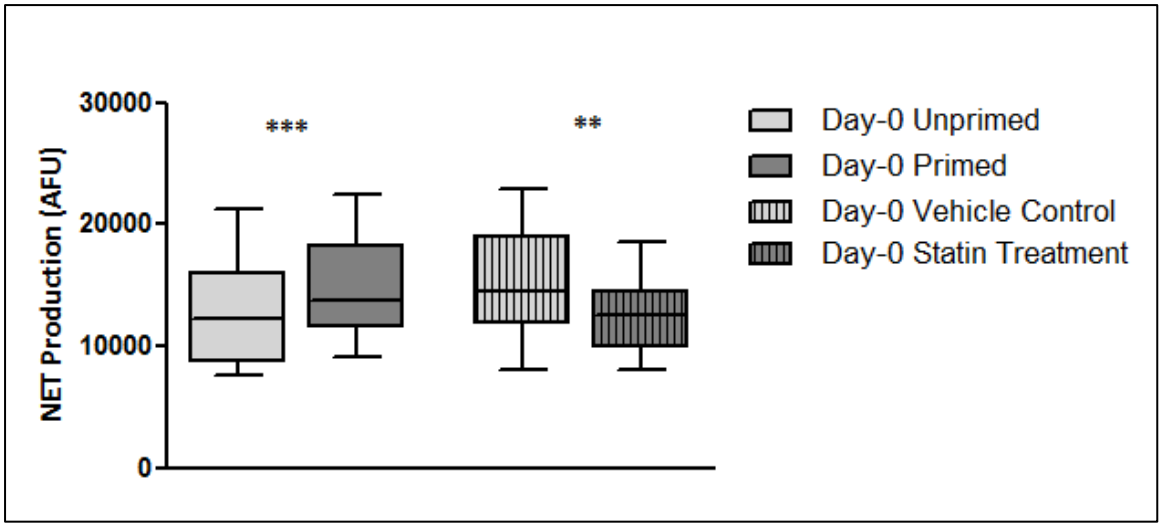


Figure 6.6 Unprimed vs. Primed NET Production and Vehicle Control vs. Statin Treatment NET Production on Day-0 in response to stimulation with fMLP

Box and Whisker Plot (10-90 percentile) of Unprimed versus Primed (TNF-α) NET Production and in-vitro Statin Treatment (1μM Simvastatin) vs. Vehicle Control (DMSO) NET Production in a colorectal cancer population on Day-0 in response to stimulation with fMLP. Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

6.5.3 Neutrophil Apoptosis

6.5.3.1 Neutrophil Apoptosis In-vivo

Neutrophil apoptosis was performed at 4-hours incubation on Day-0 (n=43), Day-1 (n=40) and Day-3 (n=22) and at 24-hours incubation on Day-0 (n=36), Day-1 (n=33) and Day-3 (n=12). The population was stratified and analysed according to statin use in an attempt to identify differences in neutrophil apoptosis between Satin Users and Non-statin Users.

Neutrophil apoptosis, according to statin use, on Day-0, Day-1 and Day-3 is displayed in Table 6.10 and Figure 6.7, Table 6.11 and Figure 6.8, and Table 6.12 and Figure 6.9 respectively.

Table 6.10 Stage of Apoptosis at 4-hours and 24-hours in Statin Users and Non-statin Users on Day-0

	Statin User (n=19)	*Non-statin User (n=24)*	*P-value*
Stage of Apoptosis (%) 4-hours			
Alive	76.90 (72.10 – 84.80)	77.0 (69.43 – 83.18)	0.7413
Early Apoptosis	15.90 (8.10 – 25.10)	17.17 (12.30 – 27.68)	0.4706
Late Apoptosis	3.40 (1.20 – 4.50)	3.10 (1.85 – 4.68)	0.9026
Necrosis	2.20 (0.90 – 2.70)	1.30 (0.65 – 2.18)	0.1744
	Statin User (n=18)	*Non-statin User (n=18)*	*P-value*
Stage of Apoptosis (%) 24-hours			
Alive	13.50 (9.55 – 19.78)	15.30 (11.05 – 23.98)	0.5798
Early Apoptosis	73.05 (54.93 – 83.45)	71.85 (57.00 – 78.40)	0.9874
Late Apoptosis	9.90 (5.68 – 26.68)	8.80 (3.95 – 16.58)	0.3425
Necrosis	0.35 (0.18 – 0.55)	0.30 (0.10 – 0.65)	0.8234

Statistical significance, measured by Mann Whitney U test.

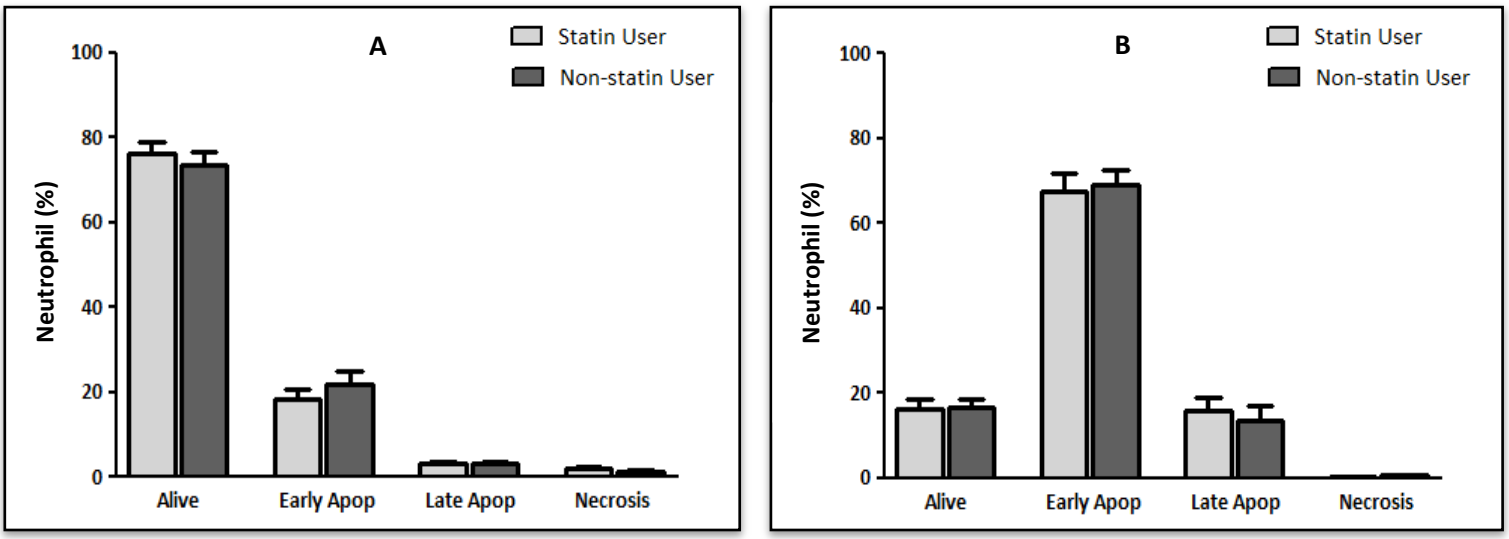


Figure 6.7 Stage of Apoptosis at 4-hours (A) and 24-hours (B) in Statin Users and Non-statin Users on Day-0

Bar graph depicting the Stage of Apoptosis (Alive, Early Apoptosis [Early Apop], Late Apoptosis [Late Apop], Necrosis) of Neutrophils (Mean [%] + SEM) at 4-hours (A) and 24-hours (B) incubation in Statin Users and Non-statin Users on Day-0. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 6.11 Stage of Apoptosis at 4-hours and 24-hours in Statin Users and Non-statin Users on Day-1

	Statin User (n=18)	*Non-statin User (n=22)*	*P-value*
Stage of Apoptosis (%) 4-hours			
Alive	80.20 (73.02 – 85.45)	78.70 (68.79 – 82.98)	0.3210
Early Apoptosis	14.30 (5.80 – 19.83)	16.25 (11.80 – 23.02)	0.2370
Late Apoptosis	3.25 (1.98 – 6.15)	3.00 (2.08 – 5.05)	0.7752
Necrosis	1.35 (1.03 – 2.80)	1.45 (0.75 – 2.23)	0.5492
	Statin User (n=17)	*Non-statin User (n=16)*	*P-value*
Stage of Apoptosis (%) 24-hours			
Alive	35.40 (23.90 – 55.35)	38.0 (21.65 – 42.19)	0.9282
Early Apoptosis	47.58 (37.31 – 69.32)	54.60 (37.68 – 69.88)	1.0000
Late Apoptosis	4.20 (1.86 – 13.43)	5.61 (2.73 – 17.69)	0.2564
Necrosis	0.58 (0.30 – 1.30)	0.74 (0.15 – 1.00)	0.8145

Statistical significance, measured by Mann Whitney U test.

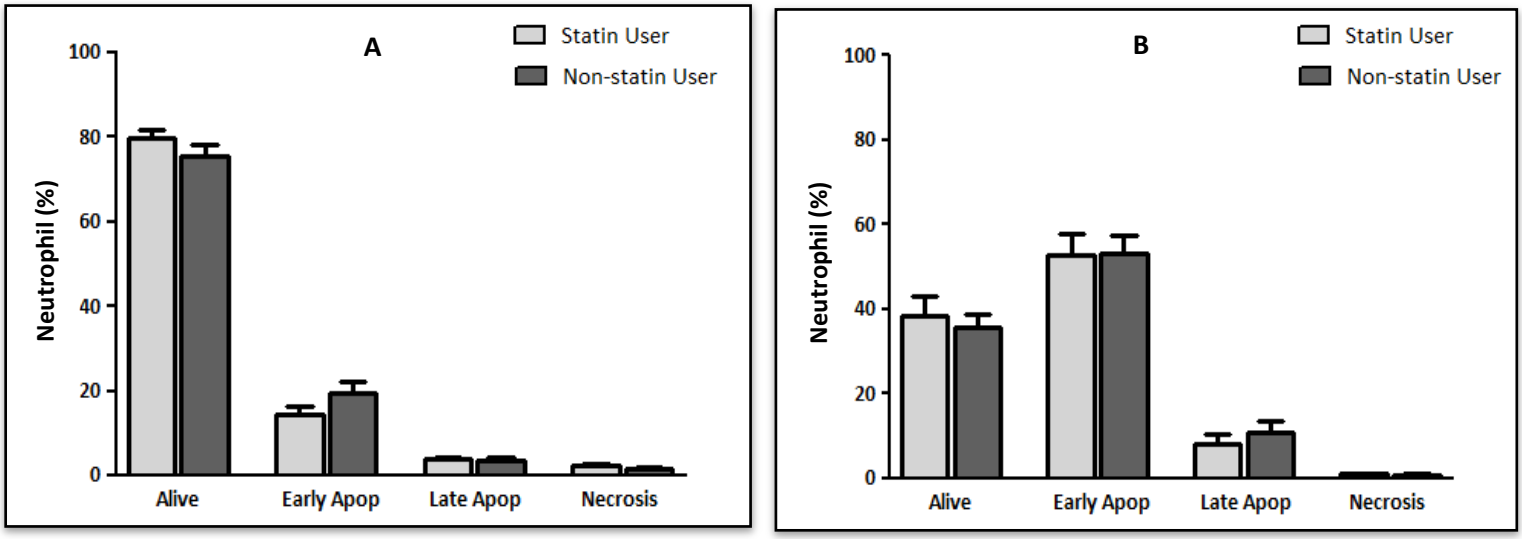


Figure 6.8 Stage of Apoptosis at 4-hours (A) and 24-hours (B) in Statin Users and Non-statin Users on Day-1

Bar graph depicting the Stage of Apoptosis (Alive, Early Apoptosis [Early Apop], Late Apoptosis [Late Apop], Necrosis) of Neutrophils (Mean [%] + SEM) at 4-hours (A) and 24-hours (B) incubation in Statin Users and Non-statin Users on Day-1. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 6.12 Stage of Apoptosis at 4-hours and 24-hours in Statin Users and Non-statin Users on Day-3

	Statin User (n=10)	*Non-statin User (n=12)*	*P-value*
Stage of Apoptosis (%) 4-hours			
Alive	74.70 (65.53 – 82.58)	74.80 (62.33 – 81.83)	0.9212
Early Apoptosis	18.80 (13.40 – 25.73)	16.50 (10.60 – 29.95)	0.8691
Late Apoptosis	2.60 (1.60 – 4.53)	5.40 (4.63 – 6.85)	**0.0056**
Necrosis	2.70 (0.60 – 4.70)	1.60 (1.23 – 2.98)	0.3907
	Statin User (n=9)	*Non-statin User (n=3)*	*P-value*
Stage of Apoptosis (%) 24-hours			
Alive	17.60 (15.80 – 22.25)	21.64 (14.10 – 48.10)	0.6000
Early Apoptosis	79.78 (68.30 – 82.10)	71.86 (46.80 – 82.10)	0.6000
Late Apoptosis	2.60 (1.35 – 6.21)	3.70 (3.50 – 6.32)	0.2818
Necrosis	0.50 (0.15 – 0.90)	0.30 (0.18 – 1.50)	0.9262

Statistical significance, measured by Mann Whitney U test.

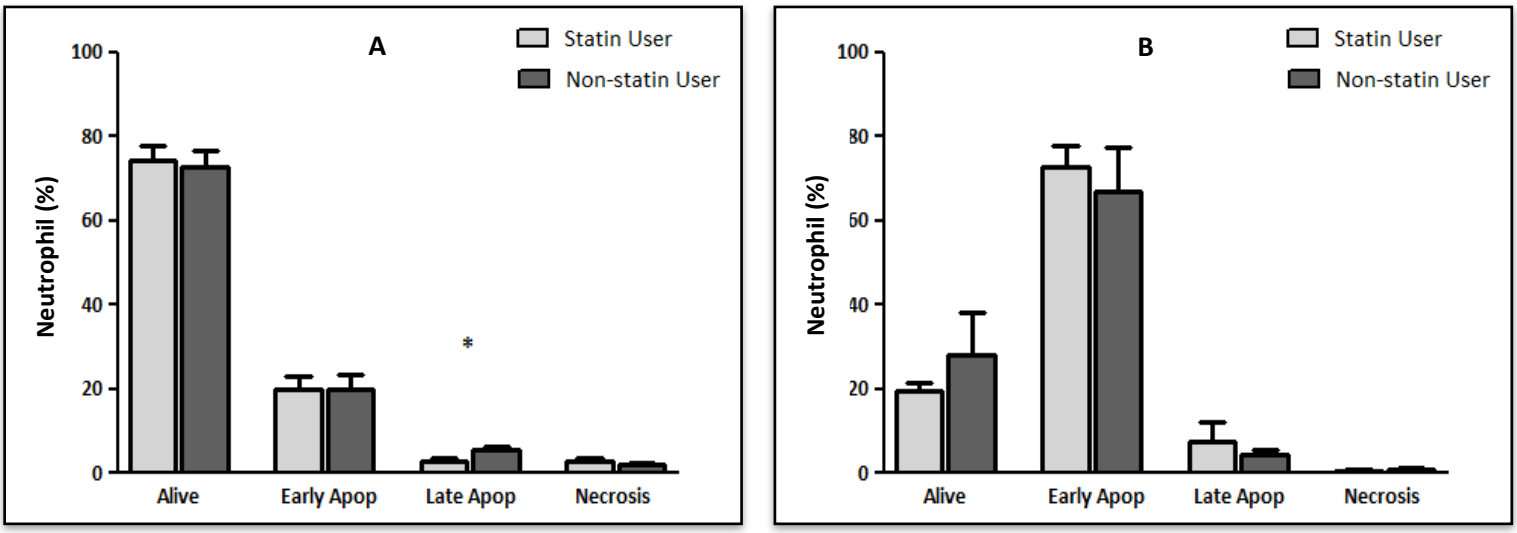


Figure 6.9 Stage of Apoptosis at 4-hours (A) and 24-hours (B) in Statin Users and Non-statin Users on Day-3

Bar graph depicting the Stage of Apoptosis (Alive, Early Apoptosis [Early Apop], Late Apoptosis [Late Apop], Necrosis) of Neutrophils (Mean [%] + SEM) at 4-hours (A) and 24-hours (B) incubation in Statin Users and Non-statin Users on Day-3. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

It was not possible to demonstrate differences in the stages of apoptosis between Statin Users and Non-statin Users at 4-hours and 24-hours incubation on Day-0 and Day-1.

A significant result was attained on Day-3 with a demonstrable reduction in late apoptotic neutrophils at 4-hours incubation in Statin Users.

6.5.3.2 Neutrophil Apoptosis In-vitro

Neutrophil Apoptosis was performed with in-vitro treatment with 1μM Simvastatin (Statin Treatment) or DMSO (Vehicle Control) on Day-0 (n=13) and Day-1 (n=12) at 4-hour and 24-hour time points.

Neutrophil apoptosis in patents who underwent in-vitro treatment with 1μM Simvastatin on Day-0 and Day-1 are displayed in Table 6.13, Figure 6.10, and Table 6.14.

Table 6.13 Stages of Apoptosis with in-vitro treatment with Statin Treatment (1µM Simvastatin) or Vehicle Control (DMSO) on Day-0

	Statin Treatment (1µM Simvastatin)	*Vehicle Control (DMSO)*	*P-value*
Stage of Apoptosis (%) 4-hours			
Alive	76.60 (71.40 – 83.55)	76.50 (72.45 – 82.30)	0.7354
Early Apoptosis	18.50 (13.35 – 27.00)	18.50 (14.15 – 23.40)	0.7344
Late Apoptosis	1.40 (1.15 – 3.10)	1.70 (1.15 – 3.80)	0.7834
Necrosis	0.80 (0.45 – 1.15)	0.90 (0.25 – 1.20)	0.8933
Stage of Apoptosis (%) 24-hours			
Alive	14.70 (8.20 – 24.00)	12.20 (9.40 – 17.50)	0.2163
Early Apoptosis	71.00 (65.30 – 85.20)	79.60 (72.10 – 84.85)	**0.0266**
Late Apoptosis	6.50 (5.60 – 15.20)	6.10 (4.70 – 9.40)	**0.0426**
Necrosis	0.20 (0.05 – 0.30)	0.20 (0.1 – 0.35)	0.3233

Statistical significance, measured by Wilcoxon Signed Rank test.

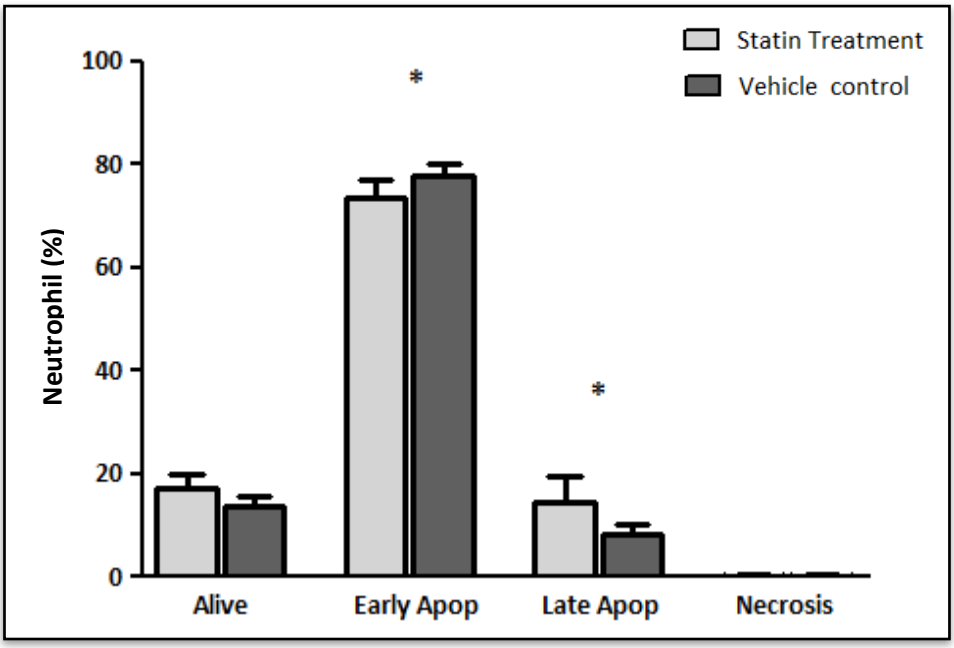


Figure 6.10 Stages of Apoptosis at 24-hours incubation with in-vitro treatment with Statin Treatment (1µM Simvastatin) or *Vehicle* Control (DMSO) on Day-0

Bar graph depicting the Stage of Apoptosis (Alive, Early Apoptosis [Early Apop], Late Apoptosis [Late Apop], Necrosis) of Neutrophils (Mean [%] + SEM) at 24-hours incubation with in-vitro Statin Treatment (1µM Simvastatin) vs. Vehicle Control (DMSO) on Day-0. Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 6.14 Stages of Apoptosis with in-vitro treatment with Statin Treatment (1µM Simvastatin) or Vehicle Control (DMSO) on Day-1

	Statin Treatment (1µM Simvastatin)	*Vehicle Control (DMSO)*	*P-value*
Stage of Apoptosis (%) 4-hours			
Alive	82.10 (77.28 – 85.65)	80.15 (77.88 – 86.58)	0.6660
Early Apoptosis	15.50 (12.15 – 17.68)	15.10 (11.28 – 19.35)	0.7555
Late Apoptosis	1.75 (1.40 – 3.25)	1.85 (1.18 – 3.45)	0.7002
Necrosis	0.90 (0.60 – 1.08)	0.90 (0.63 – 1.28)	0.9593
Stage of Apoptosis (%) 24-hours			
Alive	30.28 (23.85 – 51.63)	27.45 (21.15 – 49.75)	0.1099
Early Apoptosis	66.75 (45.08 – 71.00)	67.95 (46.83 – 74.58)	0.0522
Late Apoptosis	2.29 (1.53 – 4.62)	2.80 (1.76 – 4.82)	0.9097
Necrosis	0.43 (0.30 – 0.61)	0.39 (0.30 – 0.66)	0.6460

Statistical significance, measured by Wilcoxon Signed Rank test.

The experiments revealed a significant reduction in Early Apoptotic neutrophils and a significant increase in Late Apoptotic neutrophils in response to in-vitro treatment with 1µM Simvastatin at 24-hours incubation on Day-0. This finding is consistent with changes of favoured apoptosis and a restoration of impaired apoptosis in patients with colorectal cancer. No significant differences were demonstrated in response to in-vitro treatment with 1µM Simvastatin on Day-1 at 4-hour or 24-hour time points.

6.5.4 Neutrophil Phagocytosis

Neutrophil phagocytosis was performed on Day-0 (n=27), Day-1 (n=25) and Day-3 (n=9), in response to *E.Coli* and *S.Aureus.*

The assay was performed at three time points (30 minutes, 45 minutes and 60 minutes) and the Phogocytosis Index was calculated for each time point. The Phagocytosis Index was also represented as AUC which enabled comparisons to be made considering all time points.

The population was stratified and analysed according to statin use in an attempt to identify differences in neutrophil apoptosis between Satin Users and Non-statin Users.

Neutrophil phagocytosis, according to statin use, on Day-0, Day-1 and Day-3 is displayed in Table 6.15 and Figure 6.11, Table 6.16 and Table 6.17 respectively.

Table 6.15 Neutrophil Phagocytosis Index in response to *E.Coli* and *S.Aureus* in Statin Users and Non-statin Users on Day-0

	Statin User (n=15)	*Non-statin User (n=12)*	*P-value*
E.Coli (Phagocytosis Index)			
30 minutes	3446 (2349 – 7174)	3378 (2244 – 8050)	0.8262
45 minutes	6615 (4396 – 11670)	6773 (4703 – 9960)	0.8644
60 minutes	9915 (6463 – 14870)	9581 (6338 – 13830)	0.7884
E.Coli (Area Under Curve)	5861 (3588 – 10240)	3159 (2362 – 5293)	**0.0120**
S.Aureus (P)			
30 minutes	5463 (2122 – 8605)	4607 (2701 – 9730)	0.7511
45 minutes	8240 (6253 – 13760)	8198 (6048 – 12650)	0.8262
60 minutes	14760 (10330 – 18540)	12990 (8461 – 16940)	0.2319
S.Aureus (Area Under Curve)	4795 (3185 – 6744)	4031 (3030-6435)	0.6783

Statistical significance, measured by Mann Whitney U test.

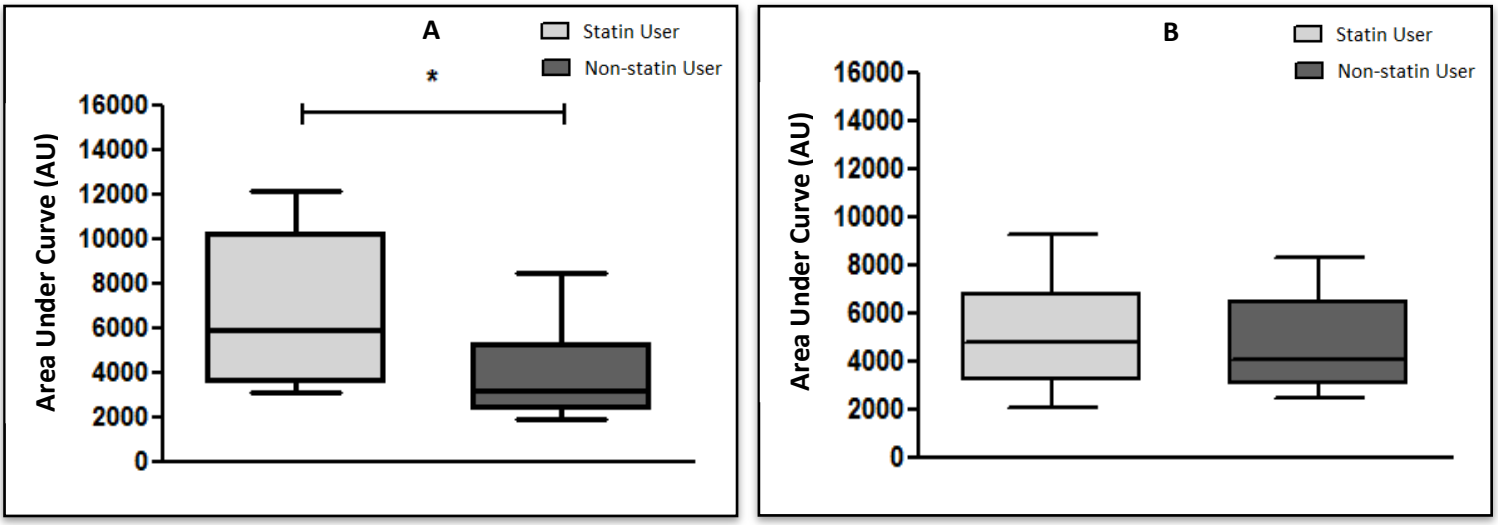


Figure 6.11 Neutrophil Phagocytosis Index in response to *E.Coli* (A) and *S.Aureus* (B) in Statin Users and Non-statin Users on Day-0 depicted by Area Under the Curve (AUC)

Box and Whisker Plot (10-90 percentile) of Phagocytosis Index represented by Area Under Curve (AU) in response to *E.Coli* (A) and *S.Aureus* (B) in Statin Users and Non-statin Users on Day-0. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Table 6.16 Neutrophil Phagocytosis in response to *E.Coli* and *S.Aureus* in Statin Users and Non-statin Users on Day-1

	Statin User (n=15)	*Non-statin User (n=10)*	*P-value*
E.Coli (Phagocytosis Index)			
30 minutes	3812 (1796 – 9949)	7417 (3589 – 13750)	0.1572
45 minutes	7166 (4292 – 16200)	8094 (6979 – 17430)	0.3317
60 minutes	9276 (4866-15460)	13740 (8870 – 20720)	0.1924
E.Coli (Area Under Curve)	3483 (2252 – 7685)	4152 (3664 – 8713)	0.3048
S.Aureus (Phagocytosis Index)			
30 minutes	5904 (3322-12080)	6300 (3729 – 9054)	0.7184
45 minutes	10260 (8366 – 18780)	10420 (7171 – 14430)	0.6774
60 minutes	15080 (12970 – 22410)	13410 (9692 – 20520)	0.4212
S.Aureus (Area Under Curve)	5119 (4282 – 7825)	5069 (3470 – 7220)	0.5982

Statistical significance, measured by Mann Whitney U test.

Table 6.17 Neutrophil Phagocytosis Index in response to *E.Coli* and *S.Aureus* in Statin Users and Non-statin Users on Day-3

	Statin User (n=4)	*Non-statin User (n=5)*	*P-value*
E.Coli (Phagocytosis Index)			
30 minutes	3581 (3402 – 9630)	13550 (9980 – 14420)	0.1667
45 minutes	6683 (4845 – 14410)	23270 (17760– 23330)	0.1667
60 minutes	9811 (7182 – 21390)	26610 (26190 – 28020)	0.1667
E.Coli (Area Under Curve)	3344 (2536 – 7479)	9191 (9191-10900)	0.1667
S.Aureus (Phagocytosis Index)			
30 minutes	10070 (6155 – 12830)	5116 (1593 – 9468)	0.2619
45 minutes	17140 (10020 – 19300)	6860 (4027 – 11410)	0.0952
60 minutes	22150 (14910 – 25750)	11840 (10370 – 16200)	0.1667
S.Aureus (Area Under Curve)	8369 (5138 – 10120)	3835 (2501– 6061)	0.1667

Statistical significance, measured by Mann Whitney U test.

A significant increase in the Phagocytosis Index, when represented as AUC, was identified on Day-0 in Statin Users in response to *E.Coli* when compared to Non-statin Users. No differences in the Phagocytosis Index were identified in response to *S.Aureus* on Day-0.

It was not possible to demonstrate differences in the Phagocytosis Index between Statin Users and Non-statin Users in response to *E.Coli* or *S.Aureus* on Day-1 and Day-3.

6.6 Discussion

The experiments undertaken in this chapter reveal the potential for neutrophil immune-modulation with statin therapy.

Firstly, Statin Users and Non-statin Users were similar in their population, tumour and operative characteristics. As anticipated, Statin Users had significantly more co-morbidities than Non-Statin Users. Despite being a more co-morbid patient group, Statin Users were identified to have equivalent patient outcomes. This replicates the findings of the larger retrospective cohort study which was undertaken and presented in Chapter 2.

Secondly, the evaluation of neutrophil function in Statin Users compared to Non-statin Users in-vivo revealed; a significant increase in NET production in response to stimulation with PMA and fMLP on Day-1, a significant reduction in late apoptosis at 4-hours incubation on Day-3, and a significant increases in the Phagocytosis Index, when represented as AUC, in response to *E.Coli* on Day-0. This was a small, heterogeneous study population with many potential confounding variables and as a result firm conclusions cannot be drawn from the in-vivo analysis. It must also be acknowledged that 5/20 (25%) patients taking statins in the study population were also taking aspirin, which has been associated with positive survival outcome in patients undergoing surgery for colorectal cancer, thought to be as a consequence of its anti-inflammatory effects.

Thirdly, neutrophil functional changes were observed when neutrophils were stimulated with simvastatin in-vitro under controlled experimental conditions. On Day-0 it was demonstrated that NET production was reduced and apoptosis was promoted when neutrophils were treated with simvastatin. Following surgery, however, a further reduction

in NET production was revealed with statin treatment on Day-1. No differences were observed in stages of apoptosis on Day-1 with statin treatment. This is contrary to the experimental hypothesis which theorised that the neutrophil functional changes observed following surgery would be attenuated by in-vitro treatment with simvastatin. In fact this finding contradicts evidence which suggests that neutrophil immune paralysis seen in the development and progression of sepsis is amenable to correction with statin therapy [297-299]. The observed reduction in NET production on Day-1 with in-vitro treatment with simvastatin may, however, explain why statins are advantageous in the setting of cancer surgery. The reduction in NETs following surgery theoretically reduces the tumour-neutrophil interaction and the potential for disease dissemination and progression.

Neutrophils are appreciated to have an important role in cancer progression and dissemination. The high-grade, non-specific SIR associated with cancer surgery may be amenable to modulation with statin therapy. This might explain why patients taking peri-operative statins appear to have a greater baseline peri-operative risk compared to non-statin users but achieve equivalent outcomes [248, 250] and why there is a reduced risk of a variety of cancer types [233-235] and a reduced all cause and cancer specific mortality [237] associated with statin use.

As outlined in Chapter 4, when neutrophils from a colorectal cancer population were primed with TNF-α, NET production was significantly increased in response to No stimulant ($p=0.0021$), IL-8 ($p=0.0007$), LPS ($p=0.0233$) and fMLP ($p=0.0002$). When neutrophils from a colorectal cancer population were treated with 1μM Simvastatin the converse is true with a significant reduction in NET production in response to No stimulant ($p=0.0256$), PMA ($p=0.0015$), LPS ($p=0.0302$) and fMLP ($p=0.0029$). A reduction in NET production with statin

treatment opposes the effect of priming with TNF-α. This adds to the evidence supporting the anti-inflammatory effect of statins. A reduction in NET production was also identified on Day-1, which supports an anti-inflammatory action in the post-operative period.

This experimental study has a number of limitations. Firstly, the population evaluated was small and heterogeneous with regard to patient demographics, patient co-morbidities, tumour characteristics and operative characteristics making interpretation of the results difficult and limiting the generalisability of the study findings. Secondly, in-vitro experiments performed to assess neutrophil function were conducted outside of the biological context and consequently there are challenges in extrapolating the results and it must be acknowledged that they cannot be readily transposed to, and predict the reaction of, the entire organism in-vivo. Thirdly, neutrophils were treated with simvastatin in experimental conditions in-vitro and this may not replicate the effect on systemic neutrophils in-vivo, also the 1μM concentration of simvastatin used was a predicted concentration corresponding to a plasma concentration following ingestion of 40mg simvastatin. The benefit of in-vitro experimental methods in this context is that each patient acted as its own control and the true impact of simvastatin treatment was detected. This was supported further by the use of the vehicle control for simvastatin in the non-treatment group which assisted in determining the true impact of simvastatin on neutrophil function.

6.7 Conclusion

As surgical resection remains the mainstay of treatment for the majority of solid tumours it is appreciated that an uncontrolled inflammatory response should be avoided in an attempt to minimise damage to the host. The evidence suggests that it is conceivable to implement a therapeutic strategy in the peri-operative period to reduce the systemic inflammation associated with surgery and improve oncological outcomes. The interaction between tumour immunity and systemic immunity is likely to be complex, but the administration of immune-modulatory therapies in an attempt to modify the peri-operative inflammatory response could be utilised to enhance the anti-tumour immune competency of the host and merits further investigation in the context of cancer surgery. A therapeutic strategy aimed at modifying tumour-neutrophil interactions may maximise the therapeutic benefit of surgery. Statins are promising immune-modulatory agents in the context of cancer surgery. Further basic science research should be performed to ascertain which cancer types and patient populations are more likely to benefit from immune-modulatory therapies, such as statins, either as a primary chemopreventative intervention or as an adjunctive therapy.

Chapter 7

HISTONE DEACETYLASE ACTIVITY IN NEUTROPHILS

A Hypothesis Generation Exercise

7.1 Introduction

Histone acetylation regulates inflammatory gene expression and is responsible for a number of diverse functions including DNA repair, cell proliferation and apoptosis [251, 252]. In the resting cell, DNA is tightly compacted around a core of histones. Specific residues in the N-terminal tails of histones can be post-translationally modified by acetylation leading to the release of the tightly wound DNA. Conversely, histone deacetylation is thought to re-establish the tight nucleosomal structure [251, 252]. Histone acetylation is regulated by a dynamic balance between HAT, which is associated with an open chromatin state and transcriptional activation, and HDAC, which is associated with a closed chromatin state and transcriptional repression [253].

Changes in histone acetylation patterns have been described in the context of cancer. It has been demonstrated that HDACs regulate the expression of a large number of genes by directly interacting with transcription factors [254]. HDACs have also been implicated in the deacetylation of chromatin proteins, leading to altered gene transcription regulation and the deacetylation of non-histone proteins that regulate cellular homeostasis (cell-cycle progression and differentiation) [255]. HDAC inhibitors have subsequently been investigated for a potential anti-tumour effect.

Recent evidence from animal models has suggested that HDAC inhibitors may have the potential to act as anti-inflammatory agents. Although it was initially believed that HDAC inhibitors did not induce apoptosis in non-malignant cells [257, 258] this has been subsequently disproven where HDAC inhibitors have been found to enhance apoptosis in human neutrophils and eosinophils both in the presence and absence of survival-prolonging

cytokines [253]. This suggests that HDAC inhibitors could potentially resolve neutrophilic and eosinophilic inflammation by inducing apoptosis.

Eighteen HDAC enzymes have been identified and they can be subdivided into four different classes (Class I, Class II, Class III and Class IV) as outlined in Table 1.9.

HDACs can induce aberrant transcription of key genes in the context of cancer and inflammation causing dysregulation of cellular functions. Consequently, HDACs are promising therapeutic targets for cancer treatment and perhaps, for the resolution of inflammation.

It has been demonstrated that statins target epigenetic mechanisms including histone deacetylation and epigenetic studies have shown simvastatin to be a direct inhibitor of HDAC-1 and HDAC-2 and it has therefore been proposed that statins may act by this mechanism and inhibit epigenetically influenced diseases such as cancer [264].

From the existing published evidence it is speculated that changes in HDAC activity may be apparent; in those patients with cancer compared to healthy individuals, in patients undergoing surgery and in those patients taking statins. This investigation may help to explain the neutrophil phenotypic change that has been outlined in this thesis and could possibly assist in the generation of a mechanistic hypothesis for the observed pleiotropic effects of statins.

7.2 Hypothesis

In a hypothesis generation exercise, it was theorised that there would be a reduction in HDAC activity in neutrophils following the surgical insult, which would allow a disruption in the closed chromatin state within the nucleus of the neutrophil and a subsequent reduction in transcriptional repression and ensuing transcriptional activation.

It is proposed that HMG-CoA Reductase Inhibitors may inhibit HDAC activity as outlined in the medical literature.

7.3 Objectives

The objectives of this chapter were:

1. To investigate Histone Deacetylase activity and expression in the neutrophil of patients with Colorectal Cancer pre-operatively (Day-0_ and post-operatively (Day-1).
3. To evaluate whether HMG-CoA Reductase Inhibitors influence Histone Deacetylase activity and expression in neutrophils over the peri-operative period in-vivo.

7.4 Methods

7.4.1 Acknowledgements

Dr. Vijay D'Souza (Post-Doctoral Research Scientist of the Institute of Inflammation and Ageing at the University of Birmingham, UK) provided expert advice and assistance when conducting the Histone Deacetylase Activity Assay and Quantitative Real-time PCR. The experimental techniques utilised in this Chapter are challenging even to the experts in laboratory science. Utilising the skills of an expert Post-Doctoral Research Scientist with a vast experience in the experimental techniques was vital in achieving reliable results, particularly when using neutrophils which are notoriously experimentally intractable.

7.4.2 Recruitment of Patients

Patients undergoing an elective colorectal resection for cancer, within an established enhanced recovery programme, who satisfied the specific inclusion and exclusion criteria, were recruited to the study as outlined in Section 3.1.1.

Patient Demographics, Patient Co-morbidities, Pre-operative Risk Prediction, Tumour Characteristics and Operative Characteristics were recorded from study participants as outlined in Table 3.1.

Patient outcomes were collected prospectively and included:

1. Post-operative complications up to 30 days after surgery using pre-defined criteria and graded by the Clavien-Dindo classification system (Appendix 3).
2. Return to theatre within 28 days of the index procedure.
3. Admission to and length of stay on the Intensive Care Unit.

4. Total hospital length of stay.
5. Readmissions to hospital within 30 days of the index procedure requiring a hospital stay ≥ 24 hours.
6. 30 day and 90 day mortality.

7.4.3 Sample Collection

Patients underwent peripheral blood tests. Blood tests were performed by an experienced medical practitioner by peripheral venepuncture or from a peripheral arterial cannula. A total of 24mls of blood was taken from each patient and deposited into 4x6ml lithium-heparin vacutainers (Becton-Dickinson, Oxford, UK). The samples were then placed on ice and transported to the laboratory for processing.

7.4.4 Neutrophil Isolation

Processing began with 45 minutes of obtaining the blood sample. Neutrophils were isolated by following the method outlined in Section 3.2. A purity of ≥95% and a viability of ≥97% were routinely achieved.

7.4.5 Histone Deacetylase Activity Assay

HDAC activity of neutrophils was evaluated using an HDAC Fluorometric Activity Assay Kit (Cayman Chemical) as per manufacturer's instructions, as outlined in Section 3.5.1. The assay provides a fluorescent-based method of measuring HDAC activity. Firstly a lysine substrate is incubated with samples containing HDAC activity. Deacetylation sensitises the substrate such that treatment with the HDAC developer releases a fluorescent product that can be measured using a fluorescence plate reader.

The average fluorescence of each standard, positive control, positive control + Trichostatin A, sample, and sample + Trichostatin A was calculated.

The average fluorescence of Standard A was subtracted from itself and all other standards and plotted as a function of the deactylated standard (Table 3.2) as shown in Figure 3.10.

The average fluorescence of the Trichostatin A treated samples was subtracted from the fuorescence of its corresponding samples to give the corrected sample fluorescence (CSF).

The deacetylated concentration was then calculated as follows:

Deacetylated Compound (µM) = [(CSF – y-intercept)/slope]

The HDAC activity was calculated as follows:

HDAC Activity (nmol/min/ml) = [µM/30 minutes] x sample dilution

7.4.6 Quantitative Real-time PCR

Firstly, total RNA was extracted from neutrophils using NucleoSpin® RNA isolation kit (Macherney-Nagal) according to the manufacturer's protocol. Complementary DNAs (cDNAs) were then generated by reverse transcription using High Capacity RNA-to-cDNA Kit (Life Technologies). Finally, quantitative real-time PCR was performed under the expert guidance of Dr. Vijay D'Souza (Post-doctoral Research Scientist, School of Clinical and Experimental Medicine, University of Birmingham, UK) using a Roche Light Cycler 480 (Roche; Life Science), as outlined in Section 3.5.2.

Quantitative real-time PCR was performed on the cDNA samples using the TaqMan® Gene Expression Assay and SYBR-green reagent (Applied Biosystems) as per manufacturer's

instructions. SYBR-green dye is a double-stranded DNA binding dye that detects double-stranded DNA generated during PCR which negates the need to target specific probes.

GAPDH was used as the reference gene and the Class I (HDAC-1, HDAC-2, HADC-3) and Class II (HDAC-4, HDAC-7, HDAC-9) HDAC genes were the genes of interest.

Relative transcript abundance values of the genes of interest are expressed as ΔCt values (ΔCt = Ctreference – Cttarget) and they were used to calculate the statistical significance between groups.

The reciprocal values of the relative transcript abundance are expressed as 1/ΔCt and they were used to depict the data in graphical form.

7.4.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6 (La Jolla, California, USA).

Categorical data was analysed using Fisher's Exact tests for two variables or χ^2 tests for greater than two variables. Continuous data was analysed using non-parametric statistical models. Mann-Whitney U tests were used for independent samples and Wilcoxon Signed Rank tests for related samples for two groups. All statistical tests performed were two-tailed and results were considered significant when $p<0.05$.

7.5 Results

7.5.1 Population Characteristics

A total of 10 patients undergoing elective colorectal cancer resection, within an established enhanced recovery programme, underwent HDAC activity analysis and quantitative real-time PCR. The baseline population characteristics are presented in Table 7.1 and they are representative of all patients recruited to the study.

Patient outcomes were collected prospectively and included:

1. Post-operative complications up to 30 days after surgery using pre-defined criteria and graded by the Clavien-Dindo classification system (Appendix 3).
2. Return to theatre within 28 days of the index procedure.
3. Admission to and length of stay on the Intensive Care Unit.
4. Total hospital length of stay.
5. Readmissions to hospital within 30 days of the index procedure requiring a hospital stay ≥ 24 hours.
6. 30 day and 90 day mortality.

Table 7.1 Population Characteristics of Patients who Underwent HDAC Activity and Quantitative Real-time PCR Experiments

Patient Demographics		*N=10*
Age, median (IQR)		69.5 (59.8 – 75.3)
Gender, n (%), M/F		6 / 4 (60.0 / 40.0)
BMI, median (IQR), kg/m^2		26.8 (24.9 – 29.8)
Smoking Status, n (%), 1/2/3[a]		2 / 4 / 4 (20.0 / 40.0 / 40.0)
ASA, n (%), 1/2/3/4		0 / 9 / 1 / 0 (0.0 / 90.0 / 10.0 / 0.0)
Co-morbidity, n (%), 0/1/2/≥3		2 / 4 / 3 / 1 (20.0 / 40.0 / 30.0 / 10.0)
Medications, n, 1/2/3/4[b]		6 / 3 / 2 / 1
Statin User, n (%), y		5 / 5 (50.0 / 50.0)
CR-POSSUM, (%), median (IQR)		2.2 (1.7 – 3.7)
Dukes' Stage, n (%), A/B/C/D		3 / 2 / 4 / 1 (30.0 / 20.0 / 40.0 / 10.0)
Operation Type, n (%), 1/2[c]		8 / 2 (80.0 / 20.0)
Operation technique, n (%), 1/2[d]		7 / 3 (70.0 / 30.0)
Complication, n (%)		4 (40.0)
Clavien-Dindo Classification, n (%), I/II/III/IV/V		3 / 0 / 1 / 0 / 0 (30.0 /0.0 / 10.0 /0.0 / 0.0)
Total LOS, median (range, IQR)		7.5 (4.0 – 8.3)
Disease Recurrence, n (%)		1 (10.0)
Mortality, n (%), y		
	30-day	0 (0.0)
	90-day	0 (0.0)

y = yes, 1/2/3[a] = active / former / never, 1/2/3/4[b] = antihypertensive / β-antagonist / anti-platelet / oral hypoglycaemic , 1/2[c] = segmental / rectal, 1/2[d] = laparoscopic / open and laparoscopic converted to open

50% (5/10) of patients were receiving peri-operative statin, defined as, 'patients were prescribed and had taken a statin ≤ 5 days before the index procedure and who were prescribed and received a statin ≤ 5 days after the index procedure'. All statins currently available for use in the UK were included (atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin).

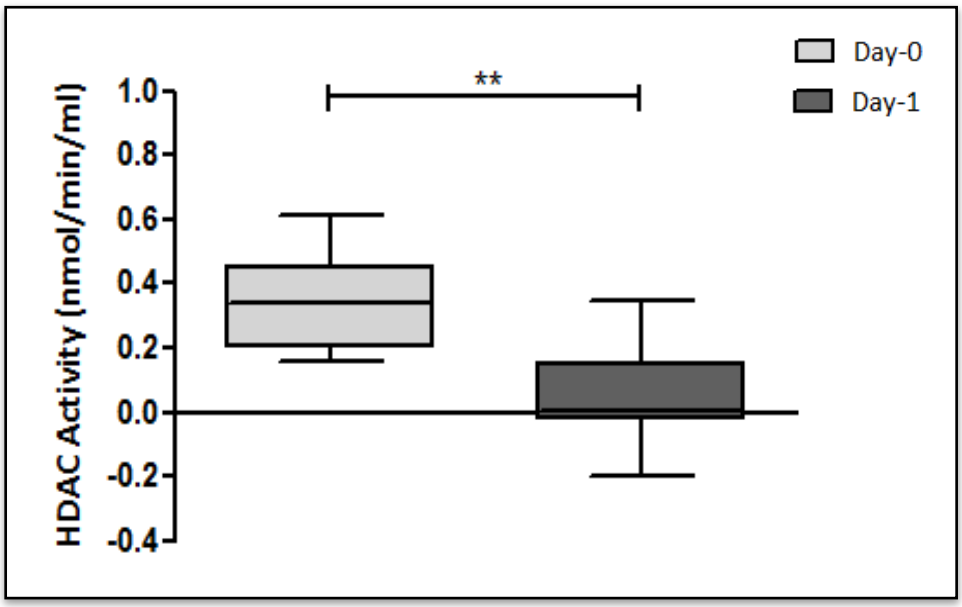
7.5.2 Histone Deacetylase Activity Assay

The HDAC activity assay was performed in patients with colorectal cancer (n=10). The baseline population characteristics and HDAC activity is summarised in Table 7.2.

Table 7.2 HDAC Activity in Patients with Colorectal Cancer

	Colorectal Cancer (n=10)
Age, median (IQR)	69.5 (59.8 – 75.3)
Gender, n (%), M/F	6 / 4 (60.0 /40.0)
HDAC activity (nmol/min/ml), median (IQR)	0.341 (0.200 – 0.449)

All patients with colorectal cancer underwent surgical resection. The HDAC activity was analysed pre-operatively (Day-0) and postoperatively (Day-1) and the results were compared. The results are displayed in Figure 7.1.



	P-value
HDAC Activity	**0.0039**

Figure 7.1 HDAC Activity in patients with Colorectal Cancer on Day-0 and Day-1

Box and Whisker Plot (10-90 percentile) of HDAC Activity in patients with Colorectal Cancer on Day-0 and Day-1. Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

A significant reduction in HDAC activity was observed, in neutrophils of patients with colorectal cancer, from the pre-operative to the immediate post-operative period.

The HDAC activity was then analysed, stratifying patients into Statin Users and Non-statin Users, on both pre-operative (Day-0) and post-operative (Day-1) days. The results are summarised in Table 7.3 and represented graphically in Figure 7.2. The fold change from Day-0 to Day-1 in Statin Users and Non-statin Users is displayed in Figure 7.3.

Table 7.3 HDAC Activity on Day-0 and Day-1 in Statin Users and Non-statin Users

	Statin User (n=5)	*Non-statin User (n=5)*	*P-value*
Day-0 HDAC activity (nmol/min/ml), median (IQR)	0.396 (0.239 – 0.545)	0.248 (0.186 – 0.402)	0.4206
Day-1 HDAC activity (nmol/min/ml), median (IQR)	-0.019 (-0.133 - -0.006)	0.125 (0.024 – 0.290)	**0.0079**

Statistical significance is measured by Mann Whitney U test.

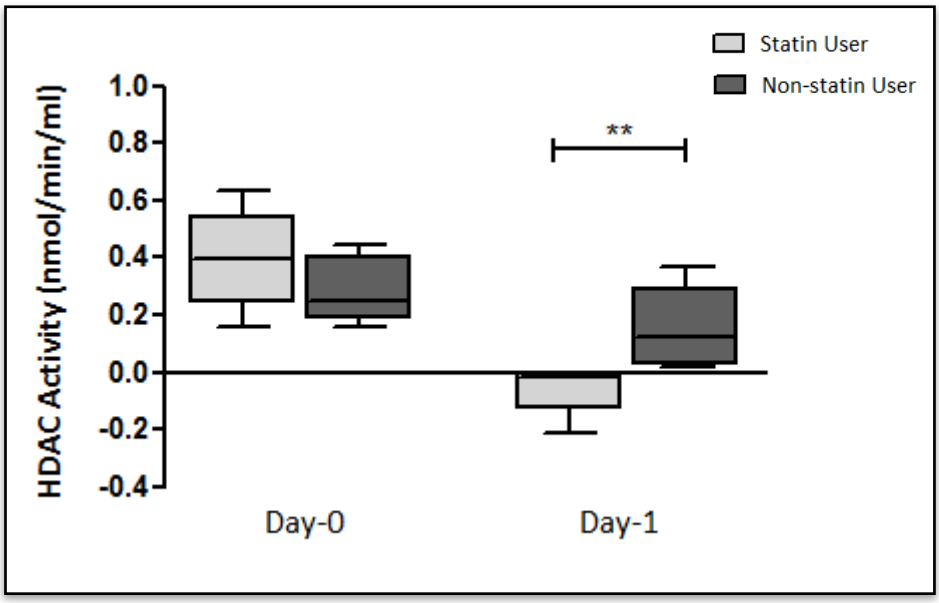
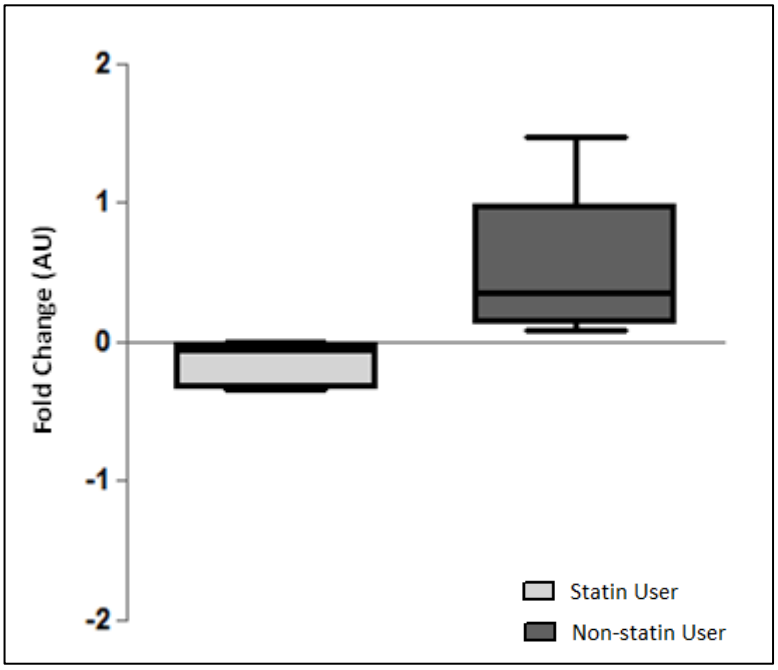


Figure 7.2 HDAC Activity on Day-0 and Day-1 in Statin Users and Non-statin Users

Box and Whisker Plot (10-90 percentile) of HDAC Activity in patients with Colorectal Cancer on Day-0 and Day-1 in Statin Users vs. Non-statin Users. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).



	Fold Change
HDAC activity	
Statin User	-0.060 (-0.332 - -0.014)
Non-statin User	0.347 (0.139 – 0.977)

Figure 7.3 Fold Change in HDAC Activity from Day-0 to Day-1 in Statin Users and Non-statin Users

Box and Whisker Plot (10-90 percentile) of Fold Change (AU) in HDAC Activity from Day-0 to Day-1 in Statin Users vs. Non-statin Users. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

On Day-0 no significant differences were identified between Statin and Non-statin Users, however, on Day-1 there was a significant reduction in HDAC activity in Statin Users in-vivo.

7.5.3 Quantitative Real-time PCR

Quantitative real-time PCR was performed in patients with colorectal cancer (n=10). The genes of interest analysed in Quantitative Real-time PCR analysis are outlined in Table 7.4. The relative transcript abundance of the HDAC genes of interest are summarised in Table 7.5.

Table 7.4 HDAC Genes of Interest for Quantitative Real-time PC

Class I	HDAC-1, HDAC-2, HDAC-3
Class IIa	HDAC-4, HDAC-7, HDAC-9

Table 7.5 Relative Transcript Abundance (ΔCt = Ctreference – Cttarget) of HDAC Genes of Interest in Patients with Colorectal Cancer

	Colorectal Cancer (n=10)
Age, median (IQR)	69.5 (59.8 – 75.3)
Gender, n (%), M/F	6 / 4 (60.0 / 40.0)
ΔCt, median (IQR)	
HDAC-1	3.733 (3.470 – 3.941)
HDAC-2	6.555 (6.096 – 7.008)
HDAC-3	5.255 (4.832 – 5.494)
HDAC-4	3.275 (3.231 – 3.539)
HDAC-7	2.365 (1.898 – 2.964)
HDAC-9	8.763 (8.261 – 9.878)

A comparison of the relative transcript abundance of the HDAC genes of interest are summarised on Day-0 and Day-1 are displayed in Table 7.6 and displayed graphically in Figure 7.4.

Table 7.6 Relative Transcript Abundance (ΔCt = Ctreference – Cttarget) of HDAC Genes of Interest on Day-0 and Day-1

	Day-0 (n=10)	*Day-1 (n=10)*	*P-value*
ΔCt, median (IQR)			
HDAC-1	3.733 (3.470 – 3.941)	4.210 (3.813 – 4.398)	0.1484
HDAC-2	6.555 (6.016 – 7.008)	6.595 (6.163 – 6.925)	0.8438
HDAC-3	5.255 (4.831 – 5.494)	5.645 (5.354 – 5.904)	0.1094
HDAC-4	3.275 (3.231 – 3.539)	3.498 (3.108 – 4.108)	0.4609
HDAC-7	2.365 (1.898 – 2.964)	2.475 (2.031 – 3.060)	0.4609
HDAC-9	8.763 (8.261 – 9.878)	10.45 (10.08 – 10.98)	**0.0207**

Statistical significance, measured by Wilcoxon Signed Rank test.

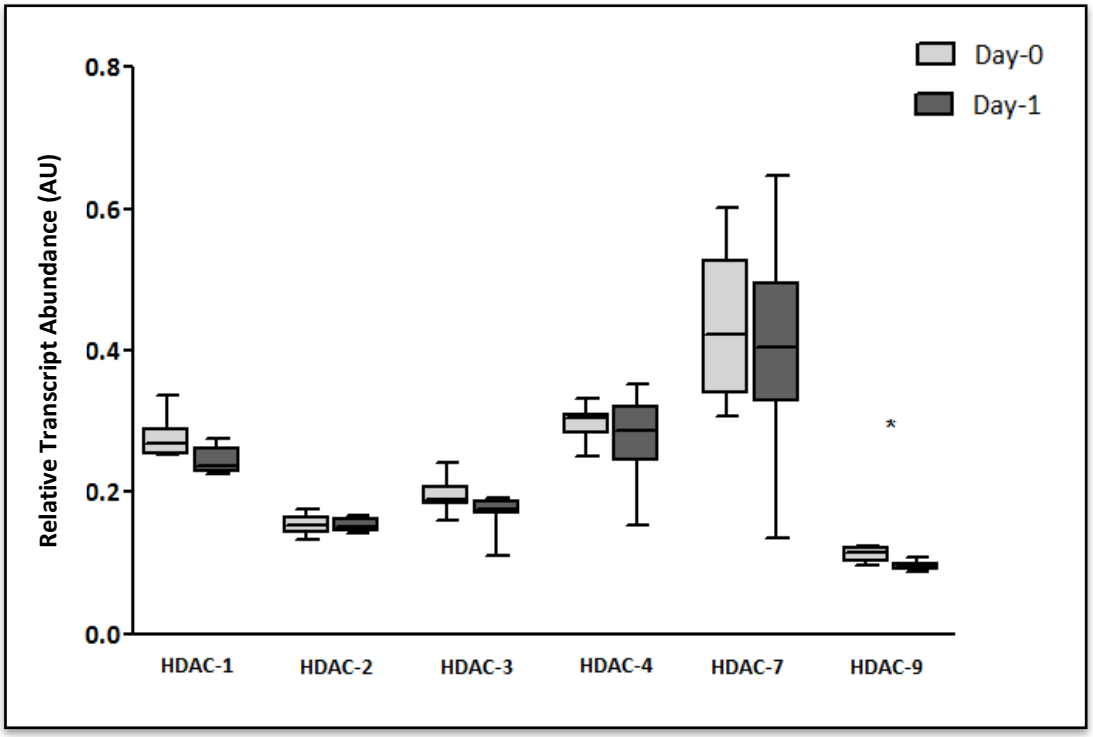


Figure 7.4 Relative Transcript Abundance (ΔCt = Ctreference – Cttarget) of HDAC Genes of Interest on Day-0 and Day-1

Box and Whisker Plot (10-90 percentile) of Relative Transcript Abundance (AU) (ΔCt = Ctreference – Cttarget) of HDAC Genes of Interest on Day-0 and Day-1. ΔCt values were used to calculate the statistical significance between groups. Statistical significance, measured by Wilcoxon Signed Rank test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). The reciprocal values of the relative transcript abundance (1/ΔCt) are used to depict the data in graphical form.

A significant difference in the relative transcript abundance of HDAC-9 was identified between in neutrophils of patients with colorectal cancer from Day-0 (pre-operative) and Day-1 (post-operative). A reduction in relative transcript abundance of all HDAC genes of interest was observed, but a statistically significant result was only identified for HDAC-9. Plotting reciprocal values of the transcript abundance demonstrates a visual reduction in HDAC expression in patients with colorectal cancer post-operatively (Day-1) compared to pre-operatively (Day-0) for HDAC-9.

The results obtained from the Quantitative real-time PCR were then analysed, stratifying patients into Statin Users and Non-statin Users, on both pre-operative (Day-0) and post-operative (Day-1) days. The results are summarised in Table 7.7 and represented graphically in Figure 7.5.

Table 7.7 Relative Transcript Abundance (ΔCt = Ctreference – Cttarget) of HDAC Genes of Interest on Day-0 and Day-1 in Statin Users and Non-statin Users

	Day-0			*Day-1*		
	Statin (n=5)	*Non-Statin (n=5)*	*P-value*	*Statin (n=5)*	*Non-Statin (n=5)*	*P-value*
ΔCt, median						
HDAC-1	3.923	4.960	0.7857	4.245	4.178	1.0000
HDAC-2	6.590	6.520	1.0000	6.550	6.730	0.3929
HDAC-3	5.650	5.640	1.0000	5.290	5.195	1.0000
HDAC-4	3.235	3.310	0.5714	3.485	3.510	1.0000
HDAC-7	2.385	1.995	0.7857	2.365	2.895	0.7857
HDAC-9	9.825	8.475	0.1429	10.96	10.08	**0.0357**

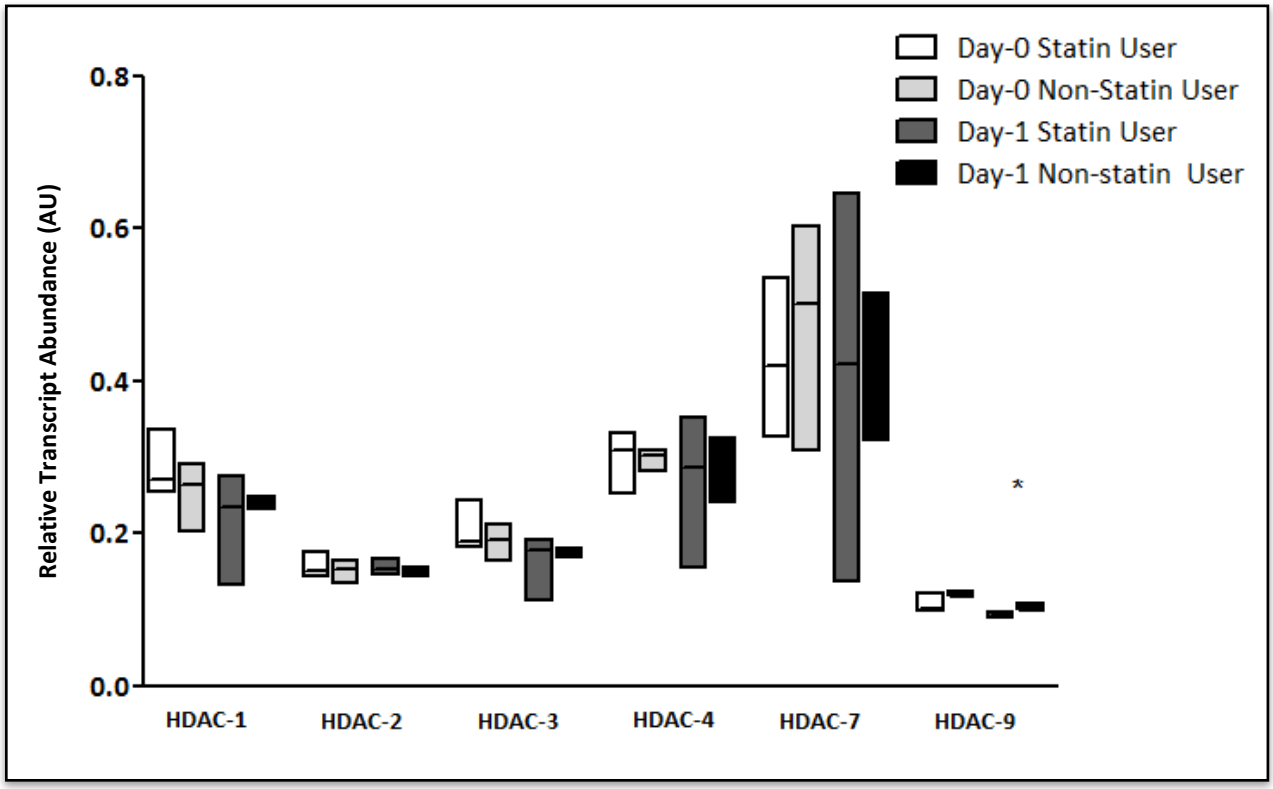


Figure 7.5 Relative Transcript Abundance (ΔCt = Ctreference – Cttarget) of HDAC Genes of Interest on Day-0 and Day-1 in Statin Users and Non-statin Users

Floating Bar Plot (Min / Max / Median) of Relative Transcript Abundance (AU) (ΔCt = Ctreference – Cttarget) of HDAC Genes of Interest on Day-0 and Day-1 in Statin Users and Non-statin Users. Statistical significance, measured by Mann Whitney U test, is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). The reciprocal values of the relative transcript abundance (1/ΔCt) are used to depict the data in graphical form.

When HDAC expression was analysed according to statin use over the peri-operative period, a significant reduction in HDAC-9 expression was observed in Statin Users compared to Non-statin Users ($p=0.0357$). This result corresponds to the reduction in HDAC activity witnessed in Statin Users compared to Non-statin Users when using the fluorometric HDAC activity assay ($p=0.0079$).

7.6 Discussion

The experiments in this chapter were undertaken to provide a greater understanding of the epigenetic changes in HDAC within the neutrophil of patients with colorectal cancer undergoing resectional surgery. The epigenetic changes in HDAC within the neutrophil were also explored to identify any differences in Statin Users compared to Non-statin Users in-vivo.

Firstly, it must be recognised that the experiments performed in this chapter were conducted in a small, heterogeneous study population with potentially confounding variables and therefore firm conclusions cannot be drawn from the analysis. The experiments, however, provide interesting results which may assist in the explanation of the distinct neutrophil phenotype described in this thesis and generate a possible hypothesis for the observed neutrophil phenotype changes as a consequence of the surgical insult.

A reduction in HDAC expression was observed in patients with colorectal cancer from the pre-operative to the immediate post-operative period for all genes of interest and this reached statistical significance when evaluating the HDAC-9 gene. In this experiment, the reduction in HDAC expression was supported by the demonstration of a significant reduction in HDAC activity in neutrophils of patients with colorectal cancer from Day-0 to Day-1. Epigenetic changes in HDAC expression in the neutrophil from pre-operative to the immediate post-operative period may help to explain the distinct pro-inflammatory neutrophil phenotype described in patients undergoing resectional surgery for colorectal cancer which demonstrates; reduced NET formation, reduced apoptosis and increased phagocytotic activity.

When HDAC expression was analysed according to statin use over the peri-operative period, a significant reduction in HDAC-9 expression was observed in Statin Users compared to Non-statin Users on Day-1. This result corresponds to the reduction in HDAC activity witnessed in Statin Users compared to Non-statin Users when using the fluorometric HDAC activity assay on Day-1. Reductions were appreciated on Day-0, but they did not achieve statistical significance. The reduction in HDAC expression and HDAC activity on Day-1 in Statin Users corresponds to the observed reduction in NET production and a restoration of apoptosis identified in the neutrophil functional assays performed in this thesis These findings support the published evidence which describe a promotion of neutrophil apoptosis with the inhibition of HDAC [253].

It was hypothesised that HDAC activity would decrease in patients with colorectal cancer following the surgical insult, which would allow a disruption in the closed chromatin state and ensuing transcriptional activation. The experiments performed in this chapter suggest that, in the nucleus of the neutrophil, reduced HDAC expression and subsequent transcriptional activation may contribute to the distinct neutrophil phenotype observed as a consequence of surgery.

The novel results reported in this chapter suggest that epigenetic changes in neutrophils may predispose neutrophils to specific phenotypes. These epigenetic changes may arise as a consequence of the chemokine landscape of the tumour micro-environment, the systemic inflammatory response associated with cancer or as a consequence of the surgical insult.

Hypothetically, statins may cause alterations in HDAC activity / expression within the nucleus of the neutrophil which may modulate neutrophil functions and predispose a

specific neutrophil phenotype, which consequently may impact upon patient outcomes and may be subject to modulataion to improve patient outcomes.

7.7 Conclusion

HDACs can induce aberrant transcription of key genes in the context of cancer and inflammation causing dysregulation of cellular functions. Consequently, HDACs are promising therapeutic target for cancer treatment and the resolution of inflammation.

As described in this thesis, the neutrophil has an important role in cancer progression and dissemination and it has been suggested that the chemokine landscape in the tumour microenvironment and the cancer-associated systemic inflammation may polarise neutrophils towards a pro-tumour neutrophil phenotype. It is also recognised that neutrophils may facilitate metastatic progression in the context of systemic inflammation and the inflammation associated with surgery.

The evidence presented in this chapter suggests that changes in HDAC activity / expression may contribute mechanistically to the the phenotypic changes seen in neutrophils in patients with colorectal cancer undergoing resectional surgery.

Chapter 8

GENERAL DISCUSSION AND THESIS CONCLUSIONS

8.1 General Discussion

Neutrophils are key mediators in the SIR and in the resolution of inflammation. It is appreciated that an uncontrolled inflammatory response should be avoided in an attempt to minimise damage to the host. In the context of cancer surgery, an overwhelming SIR may suppress the anti-tumour immunity and promote tumour progression and dissemination. It is also recognised that post-operative infective complications result in further compromise of the anti-tumour immune response to residual disease and are associated with a poor oncological outcome and increased mortality secondary to metastatic disease.

The interaction between tumour immunity and systemic immunity has been complex and the delivery of appropriate immune-modulatory therapies to modify the peri-operative inflammatory response could be utilised to preserve anti-tumour immune competency in the host. It has been proposed that the pleiotropic effects of statins could be utilised in the peri-operative period in an attempt to modulate the SIR and prevent infective complications. The use of statins in the context of cancer surgery may therefore influence oncological outcomes.

In the context of cancer, it is suggested that neutrophils have two distinct phenotypes, anti-tumour and pro-tumour, suggesting that neutrophils differ in their contribution to the progression and dissemination of cancer and are capable of phenotypic plasticity. This opens up the possibility of therapeutic intervention to enhance anti-tumour and suppress pro-tumour functional properties. Modifications in neutrophil function and phenotype have been demonstrated with simvastatin treatment in-vitro and in-vivo, but not in the context of cancer, or indeed cancer surgery, where the maximum therapeutic benefit may be achieved.

This study was conducted to:

1. Characterise neutrophil function in patients with colorectal cancer undergoing resectional surgery over the peri-operative period.
2. Investigate whether NET formation is associated with patient characteristics, operative characteristics, patient outcomes and existing validated prognostic scores.
3. Evaluate the effect of statins on neutrophil function in-vivo and in-vitro over the peri-operative period.
4. Explore the role of Histone Deacetylase as a potential epigenetic marker of neutrophil phenotype change.

Analysis performed in this thesis suggests that that adverse patient outcomes (post-operative complications, prolonged hospital recovery and mortality) are associated with increased pre-operative NET production when evaluating NET production in patients with colorectal cancer on Day-0 (pre-operatively). This indicates increased neutrophil activation which may, conceivably, occur as a consequence of cancer associated inflammation. Significant increases in NET production in response to stimulation with fMLP were demonstrated in patients who developed significant post-operative complications or who had a prolonged hospital stay. fMLP stimulation of neutrophils activates a wide variety of intra-cellular signalling pathways which causes superoxide generation and degranulation of neutrophils [292] and this may explain the experimental findings observed in this analysis. It is proposed that NET production, particularly in response to stimulation with fMLP, therefore has potential prognostic significance and further investigation into their predictive value is justified. Indeed, ROC curve analyses of NET production in response to stimulation with fMLP were also able to distinguish between patients who developed post-operative

complications and had a prolonged hospital stay with significance. Despite the accumulating evidence that suggests an association between NETs and tumour progression, the experimental evidence revealed no significant differences in NET production in patients with more advanced disease when patients with early stage and late stage colorectal cancer were compared.

An attempt to characterise serial changes in neutrophil function over the peri-operative period in patients undergoing major resectional surgery for colorectal cancer was then conducted. The findings suggest that a novel neutrophil phenotype may exist in patients undergoing colorectal resection for cancer as a result of the surgical insult which demonstrates reduced NET formation, impaired apoptosis and increased phagocytosis.

A significant reduction in NET formation from Day-0 to Day-1 was detected. This observed NET reduction, as a consequence of the surgical insult, appears to replicate and support the findings outlined in in-vitro experiments which studied the neutrophil functional changes in patients with sepsis and severe sepsis [294]. Significant changes indicating delayed apoptosis were detected from Day-0 to Day-1 and significant changes favouring apoptosis were identified from Day-1 to Day-3 at 24-hours incubation. Evidence supports reduced apoptosis of neutrophils in inflammation and this is associated with poor patient outcomes [295, 296]. Therefore, delayed apoptosis from Day-0 to Day-1 is indicative of pro-inflammation and favoured apoptosis from Day-1 to Day-3 is suggestive of anti-inflammation (resolution of inflammation). In this study it was not possible to identify any statistically significant results when analysing the stages of neutrophil apoptosis with adverse patient outcomes, however, it is hypothesised that neutrophils that exhibit a phenotype characterising a prolonged delayed apoptosis may be associated with adverse

outcomes as it is essential that neutrophils are 'switched off', undergo apoptosis and are successfully cleared to minimise host damage.

Demonstrable increases in neutrophil phagocytotic activity were revealed on sequential peri-operative days, over the peri-operative period. Significant increases in phagocytotic index from Day-1 to Day-3 were observed to *E.Coli* which suggests an increase in phagocytotic activity up to the third post-operative day in response to stimulation with *E.Coli*.

The experimental findings suggest that neutrophils adopt a distinctive phenotype, characterised by a reduction in NET formation, inhibition of the apoptosis and increase in phagocytosis in response to *E.Coli*, as a consequence of the surgical insult. The resultant accumulation of activated neutrophils in the circulation following surgery may cause more extensive collateral tissue damage and potential organ dysfunction. The increase in NET formation and the restoration of apoptosis in the late post-operative phase coincides with apparent surgical recovery and therefore an early phenotypic switch (from dysregulated to normal neutrophil function) may be desirable.

Peri-operative NET production was evaluated when classifed by operative technique and operation type and when classified by post-operative complication, total length of hosital stay and mortality. No significant differences in peri-operative NET production were identified in the analysis of operative technique or operation type. Significant increases in NET production were, however, associated with an increased hospital stay and prolonged hospital recovery in response to PMA. No differences were identified in patients who experienced significant post-operative complications or died during the study follow-up. The fold changes in NET production from Day-0 to Day-1 were also evaluated. A significant

reduction in the change in NET production was observed in patients who died in response to fMLP. This might imply that a reduced fold change in NET production as a consequence of surgery may have a role in prognostication and mortality prediction. The evidence indicates that further investigation into the prognostic value of NET production is justified and the change in NET production over the peri-operative may be a promising area to study in relation to patient outcomes.

The work undertaken in this thesis supports the pleiotropic effects of statin therapy in the context of colorectal cancer resection and strengthens the evidence to suggest that neutrophil immune-modulation with statin therapy is possible

It was investigated whether statin use is associated with reduced post-operative complications and improved clinical outcomes in patients undergoing elective colorectal cancer resection and whether a dose dependent effect on clinical outcomes could be demonstrated. A retrospective review of prospectively collected data was conducted for all elective colorectal cancer resections within an established enhanced recovery programme over a 5 year period. It was observed that statin users had equivalent outcomes in frequency of post-operative complications, post-operative antibiotic use, re-operations, re-admissions and there was no statistically significant difference in mortality after appropriate age and gender adjustments despite having a higher peri-operative risk. No statistically significant dose related differences were identified in patient outcomes although an overall risk reduction in mortality with increasing dose of statin was suggested. The results of a multi-variate analysis of a large retrospective data set provided evidence to further explore the potential benefit of the pleiotropic effects of statin therapy in the context of colorectal cancer surgery. This is particularly relevant as the peri-operative use of simvastatin has

recently been investigated in a prospective, double-blind, placebo-controlled RCT in the context of major colorectal surgery, including both malignant and benign resectional surgery. Although numbers were relatively small (65 simvastatin vs. 67 placebo) it was identified that peri-operative statin therapy attenuates the early pro-inflammatory response to surgery with significant reductions in plasma concentrations of IL-6, IL-8 and TNF-α and peritoneal concentrations in Il-6 and IL-8 on Day-1, but no differences in post-operative complications were identified (OR 0.71, 95% CI 0.33-1.52, p = 0.444) [300].

The evaluation of neutrophil function in Statin Users compared to Non-statin Users was performed in patients and experimental evidence revealed; a significant increase in NET production in response to stimulation with PMA and fMLP on Day-1, a significant reduction in late apoptosis at 4-hours incubation on Day-3, and a significant increase in the Phagocytosis Index, when represented as AUC, in response to *E.Coli* on Day-0. This was a small, heterogeneous study population with many potential confounding variables and as a result firm conclusions cannot be drawn from the in-vivo analysis.

Controlled, in-vitro experiments were then undertaken to assess neutrophil functional changes when neutrophils were directly stimulated with simvastatin. In patients with colorectal cancer a reduced NET production and favoured apoptosis (pro-apoptosis) was identified in response to treatment with simvastatin. Following surgery a further reduction in NET production was revealed with statin treatment on Day-1. This finding may help to explain why statins are advantageous in the setting of cancer surgery as a reduction in NETs following surgery theoretically reduces the tumour-neutrophil interaction and the potential for disease dissemination and progression.

Furthermore, when neutrophils from a colorectal cancer population were primed with TNF-α, a known pro-inflammatory cytokine, NET production was significantly increased when compared to a significant reduction in NET production when neutrophils from a colorectal cancer population were treated with simvastatin. A reduction in NET production with statin treatment opposes the effect of priming with TNF-α. This finding adds to the evidence to support the anti-inflammatory effect of statins (and improved patient outcomes in patients taking statins) in the context of colorectal cancer.

During analysis a novel calculation was undertaken (Absolute Neutrophil Production Potential) in an attempt to account for the variation in the number of circulating neutrophils and to predict the number of circulating NETs in-vivo. Although this is an unvalidated calculation it enables an assessment of the 'potential' NET production in an individual. ANPP is an interesting concept and merits further review. When taking into account the number of absolute circulating neutrophils, despite an observed reduction in NET formation from Day-0 to Day-1, a significant increase in the ANPP was identified from Day-0 to Day-1. This might imply that the number of circulating neutrophils are also important when determining patient outcomes and this may also explain why the NLR has been used successfully to predict overall and cancer specific survival in numerous solid organ malignancies [99].

Experiments were then conducted to provide a greater understanding of the epigenetic changes in HDAC within the neutrophil of patients with colorectal cancer undergoing resectional surgery. The epigenetic changes in HDAC within the neutrophil were also explored to identify any differences in Statin Users compared to Non-statin Users in-vivo.

A reduction in HDAC expression was observed in patients with colorectal cancer from the pre-operative to the immediate post-operative period for all genes of interest and this reached statistical significance when evaluating the HDAC-9 gene. In this experiment, the reduction in HDAC expression was supported by the demonstration of a significant reduction in HDAC activity in neutrophils of patients with colorectal cancer from Day-0 to Day-1. Again, epigenetic changes in HDAC expression in the neutrophil from pre-operative to the immediate post-operative period may help to explain the distinct pro-inflammatory neutrophil phenotype described in patients undergoing resectional surgery for colorectal cancer which demonstrates; reduced NET formation, reduced apoptosis and increased phagocytotic activity.

When HDAC expression was analysed according to statin use over the peri-operative period, a significant reduction in HDAC-9 expression was observed in Statin Users compared to Non-statin Users on Day-1. This result corresponds to the reduction in HDAC activity witnessed in Statin Users compared to Non-statin Users when using the fluorometric HDAC activity assay on Day-1. The reduction in HDAC expression and HDAC activity on Day-1 in Statin Users corresponds to a reduction in NET production and a restoration of apoptosis. These findings support the published evidence which indicates a promotion of neutrophil apoptosis with the inhibition of HDAC [253].

The novel results reported in this thesis suggest that epigenetic changes in neutrophils may predispose neutrophils to specific phenotypes. These epigenetic changes may arise as a consequence of the chemokine landscape of the tumour micro-environment, the systemic inflammatory response associated with cancer or as a consequence of the surgical insult. Hypothetically, statins may cause alterations in HDAC expression within the nucleus of the

neutrophil which may modulate neutrophil functions and predispose a specific neutrophil phenotype.

8.2 Study Limitations

Limitations to the study are recognised and include:

1. The population evaluated was small and heterogeneous with regard to patient demographics, patient co-morbidities, tumour characteristics and operative characteristics. This makes interpretation of the results difficult and limits the generalisability of the study findings.
2. Comparative analyses stratifying patients according to cancer location, cancer stage and patient outcomes is subject to misinterpretation as potential confounding variables were not accounted for. The number of adverse patient outcomes in the study population was low which makes interpretation of adverse patient outcomes challenging.
3. Comparative analyses which stratified patients according to statin use is also impeded by the influence of confounding variables. Although the groups appeared well matched, many factors could potentially impact on the results in a small cohort study, particularly the role of aspirin which has been implicated in the modulation of peri-operative inflammation and patient outcomes in colorectal cancer.
4. In-vitro experiments performed to assess neutrophil function were conducted outside of the biological context and consequently there are challenges in extrapolating the results and it must be acknowledged that they cannot be readily transposed to, and predict the reaction of, the entire organism in-vivo.
5. For all neutrophil functional assays blood was collected in lithium-heparin vacutainers. There is active debate about the anti-coagulant of choice for isolating neutrophils in preparation for neutrophil functional analysis. There are no firm

conclusions regarding the most appropriate anticoagulant. It is proposed that lithium-heparin may induce cell activation; however, this claim is not substantiated. All blood samples in this thesis were collected in lithium-heparin vacutainers and therefore the inter-sample variability is minimised. It is also acknowledged that neutrophils can be isolated in either lithium-heparin or ethylenediaminetetraacetic acid (EDTA) and subsequently be utilised effectively for neutrophil functional analysis [301].

6. Any protocol used to isolate neutrophils will result in a degree of neutrophil activation, although this can be minimised by isolating neutrophils with Percoll, which has been demonstrated to induce minimal neutrophil activation [285], as utilised when isolating neutrophils in the experiments conducted in this thesis.
7. The neutrophil functional assays were performed without blinding the experimenter to the use of statin therapy in-vivo or to the treatment with simvastatin in-vitro. This introduces the potential for experimenter and observer bias which could influence the results obtained. Inter-experimenter bias was minimised as all experiments were performed under the same experimental conditions and by the same individual (s) which maximised the validity and reliability of the experiments performed in this thesis. The results of some of the neutrophil functional experiments were validated by another trained individual who repeated the experiments and compared the results to ensure consistent results were obtained.
8. When neutrophils were treated with simvastatin in experimental conditions in-vitro a 1μM concentration of simvastatin used. This was only a predicted concentration corresponding to a plasma concentration following ingestion of 40mg simvastatin.

Importantly, all experiments were performed using the same experimental conditions which utilised a consistent 1µM concentration of simvastatin.

It should be noted that the benefit of studying a surgical population, compared to patients with other inflammatory conditions, for example in patients with sepsis, is that the true impact of a provoked SIR can be identified as pre-operative and post-operative evaluation is undertaken. It is also appreciated that the surgical insult is relatively consistent between individuals, particularly with the universal implementation of a standardised enhanced recovery peri-operative care pathway. The benefit of in-vitro experimental methods which utilised statin treatment is that each patient acted as its own control and the true impact of simvastatin treatment was detected. This was supported further by the use of the vehicle control for simvastatin in the non-treatment group which assisted in determining the true impact of simvastatin on neutrophil function.

8.3 Future Work

This thesis has characterised the serial changes in neutrophil function over the peri-operative period in patients undergoing resectional surgery for colorectal cancer and determined the effect of statins on neutrophil function in-vivo and in-vitro over the peri-operative period. The activity and expression of HDAC was also investigated which proposes a possible epigenetic change which may be, in part, responsible for distinct neutrophil phenotype described which may be a potential target for the immune-modulatory effects of statins.

This investigation has opened up many potential research opportunities and it is proposed that future work be conducted as follows:

1. An assessment of serum neutrophil elastase in patients with colorectal cancer and an evaluation of changes in serum neutrophil elastase over the peri-operative period is suggested to provide an assessment of the degree of neutrophil activation in both circumstances. Neutrophil elastase is abundantly secreted into the extracellular environment upon neutrophil activation at inflammatory sites and could be used to explain the neutrophil functional changes, in terms of neutrophil activation, identified in this thesis.
2. An evaluation of the ROS production in neutrophils in patients with colorectal cancer and over the peri-operative period by undertaking ROS production assays would clarify whether NETs are produced by an oxidant dependent (ROS dependent) pathway or oxidant independent pathway. It would be anticipated that ROS production would replicate the findings of NET production as the pathway of NET production in cancer and as a consequence of surgery is likely to be ROS dependent.

3. An evaluation of the migratory capacity of neutrophils in patients with colorectal cancer and following surgery could be conducted by undertaking neutrophil chemotaxis assays. The migratory capacity may be of paramount importance in determining whether neutrophils in cancer, or following cancer surgery, have migratory and therefore metastatic potential.
4. Following surgery, where possible, the function of neutrophils should be assessed from the peritoneal cavity (i.e. in those patients with an intra-peritoneal drain). This would characterise the neutrophil function of the transmigrated neutrophils at the site of tissue trauma and this could be compared to circulating neutrophils.
5. HDAC activity and expression assays should be performed in greater numbers to substantiate the findings in this thesis. Additionally, controlled experiments should be conducted to assess the direct impact of statin therapy on HDAC activity and expression by exposing neutrophils to statin treatment in-vitro prior to evaluation.
6. An evaluation of plasma cell-free DNA, a product of cell death and immune system activation, in patients with colorectal cancer and following surgery by undertaking a cell-free DNA quantification assay. This may assist our understanding of the 'quantity' of systemic inflammation associated with cancer and provide an insight into the changes following surgery. It is anticipated that the post-operative, pro-inflammatory response would reveal elevated plasma cell-free DNA. It may be possible to correlate this with the ANPP and attempt to validate its use in prognostication of outcomes following cancer surgery.
7. An assessment of local tumour inflammatory response, utilising the validated klintrup-Makinen scoring method, a 4 point grade which determines the inflammatory cell reaction in the central tumour and at the invasive margin or more

complex immunohistochemical techniques looking specifically for NETs could be used to determine whether the tumour inflammatory response is modified in patients taking statins. It is invisaged that large numbers of tumours would be required to be analysed to identify if patients on established statin therapy demonstrated differences in tumour inlammation when compared to non-statin users.

8. An evaluation of the effect of in-vivo statin therapy, in the peri-operative period of patients undergoing elective colorectal resection for cancer within an etablished enhanced recovery programme, on neutrophil function and on short-term and long-term outcomes, by conducting a double-blind, placebo controlled, randomised control trail. The trial could include a laboratory based analysis of neutrophil function (both circulating and trans-migrated neutrophils [peritoneal cavity]) and if feasible an evaluation of HDAC expression.

8.4 Thesis Conclusion

This study was conducted to investigate the neutrophil function in patients with colorectal cancer, to serially characterise the neutrophil function in patients undergoing colorectal cancer resection over the peri-operative period and to explore the impact of statin therapy on neutrophil function.

The experiments conducted in this thesis demonstrate a distinc neutrophil phenotype which exhibits reduced NET formation, reduced apoptosis and increased phagocytotic activity following resectional surgery in patients with colorectal cancer.

Epigenetic changes in HDAC activity and expression were identified from Day-0 to Day-1 which may contribute to the distinct neutrophil phenotype described.

The immune-modulatory capacity of statins was investigated over the peri-operative period. Following surgery, a reduction in NET production was identified with in-vitro treatment with simvastatin which is considered advantageous as NETs are strongly implicated in cancer progression and dissemination.

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Appendices

A1 Patient Information Sheet for STARS-CRC

STAtins in Reduction of Septic complications after ColoRectal Cancer resection

REC: 13/WM/0485

PATIENT INFORMATION SHEET

You are being invited to take part in a research study. Before you decide to take part it is extremely important you understand why the research is being conducted and exactly what it involves. You should understand the potential risks and benefits prior to making any decision. This process is known as informed consent. Please take time to read the following information sheet and if something is not clear or you do not fully understand, please do not hesitate to ask. Please take your time to decide if you would like to take part and thank you for taking the time to read this information sheet.

What is the purpose of the study?

This study aims to investigate how the function of the body's white blood cells are affected by surgery and why some patients develop infections and complications after surgery. White blood cells are the first line of defence against micro-organisms (like bacteria) which are responsible for infections in humans. It has been proposed that the function of these cells is altered following surgery and it has been suggested that this is why some patients develop infections and complications after surgery. Furthermore, it is recognised that surgery may compromise the body's defences against cancer and the defences may be weakened further in the presence of an infection. It is therefore important to minimise infections and complications following surgery.

Statins (cholesterol lowering medications) may be used for the treatment and prevention of infection and it has been suggested that they may reduce infections and complications after surgery. Statins may change the function of the body's white blood cells and this is why they may be beneficial.

This study will look at the white blood cells from samples of blood in patients undergoing bowel resection. This research aims to improve our understanding of what happens to these cells that leads to infections and complications after surgery.

This research area requires further investigation due to the lack evidence to support using statins routinely around the time of surgery. By increasing our understanding of these processes it may be possible to identify potential treatments that could improve outcomes in patients undergoing surgery.

What does this involve?

The study involves collecting a sample of your blood on up to three separate occasions, one before your operation and two afterwards. The blood will be taken from a vein, as for any blood test, and samples will be analysed and then stored in a freezer.

You will also be asked to fill in a questionnaire before and after your surgery to assess your recovery after your operation.

You will be given no additional medication within this research study other than the medications given to you by the doctors looking after you and no additional body tissue will be removed from you for the purposes of research.

Would my taking part in this study be kept confidential?

The information collected from this study will be stored in anonymous form on a password protected hospital computer and combined with the results from other study participants. In the future, the findings of this study may be discussed at a scientific meeting of doctors and published in a medical journal in order to share our research with the wider medical community. It will not be possible to identify you as an individual from any reports.

What is required from me?

We wish to seek your permission to allow us to take a blood sample on up to three separate occasions, one before your operation and two afterwards, and retain these samples to perform laboratory tests related to this study. Some of the samples will be stored for up to 20 years in a secure location at the University of Birmingham and may be used in future similar research studies.

We also request that you complete a questionnaire before and after your surgery to assess your recovery after your operation.

You are under no obligation to give your consent to this request and your future medical care will not be affected by your decision. In the unlikely event that you lose capacity (the ability to consent) during the study then you will be withdrawn from the study and no further research data or blood samples will be collected from you. The research team will retain data and blood samples already collected and continue to use it confidentially.

What happens if I have any questions about the study?

If you have any questions regarding the study, please ask the person presenting this form to you, or contact the Principal Investigator, Mr. Jonathan Richardson on (****) *** ****.

What if things go wrong?

If you have any comments, concerns or complaints about any aspect of the way you have been approached or treated during the course of this study, you should write to Mr. J. Richardson, Academic Department of Anaesthesia, Critical Care, Pain and Resuscitation, Heart of England NHS Foundation Trust, Birmingham, B9 5SS.

Alternatively you may wish to contact the Patient Advice and Liaison Service (PALS) on (****) *** **** who will act independently to help you. If you wish to make a complaint via the NHS complaints mechanism the PALS service will assist you.

A2 Patient Consent Form for STARS-CRC

STAtins in Reduction of Septic complications after ColoRectal Cancer resection

REC: 13/WM/0485

PATIENT CONSENT FORM - CONFIDENTIAL

1. I confirm that I have read, understood and received a personal copy of the information sheet, dated 30th December 2013, for the above study and have had the opportunity to ask questions and discuss the study.
   YES / NO **Initials:**

2. I confirm that I have had sufficient time to consider whether or not to be included in this study requiring sample collection.
   YES / NO **Initials:**

3. I consent to the retention and subsequent testing of my blood and cells for this project.
   YES / NO **Initials:**

4. I understand that sections of my medical notes may be inspected by responsible individuals of research institutions or from regulatory authorities, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.
   YES / NO **Initials:**

5. I give permission that anonymous data may be made available to other groups for the purposes of improving clinical diagnosis and treatment of related conditions.
   YES/ NO **Initials:**

6. I give permission for any residual samples (blood and cells) to be stored following this research project, for use in future research projects aiming to improve understanding of related conditions.
   YES/ NO **Initials:**

7. I understand how the samples will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time, without giving any reason, without my medical care or legal rights being affected.
   YES / NO **Initials:**

8. I understand that in the unlikely event that I lose capacity (the ability to consent) during the study then I will be withdrawn from the study and no further research data or blood samples will be collected from me. The research team will retain data and blood samples already collected and continue to use it confidentially.
   YES / NO **Initials:**

_____	_____	_____
Name of Patient	**Date**	**Signature**
_____	_____	_____
Name of Person Taking Consent	**Date**	**Signature**

A3 Clavien-Dindo Classification of Surgical Complications (Clavien, 2009)

The Clavien-Dindo Classification of Surgical Complications is a validated classification used to rank surgical complications in an objective and reproducible manner. It consists of 7 grades (I, II, IIIa, IIIb, IVa, IVb and V). Complications that have the potential for long-lasting disability are highlighted in the present classification by a suffix ("d" for disability). This suffix indicates that a follow-up is required to comprehensively evaluate the outcome related to long-term quality of life.

Grades	*Definition*
Grade I	Any deviation from the normal post-operative course without the need for pharmacological treatment or surgical, endoscopic and radiological interventions Allowed therapeutic regimens are drugs such as anti-emetics, anti-pyretics, analgesics, diuretics, electrolytes and physiotherapy This grade also includes wound infections opened at the bedside
Grade II	Requiring pharmacological treatment with drugs other than those allowed for Grade I complications Blood transfusions and total parenteral nutrition are also included
Grade III	Requiring surgical, endoscopic or radiological intervention
III-a	Intervention not under general anaesthesia
III-b	Intervention under general anaesthesia
Grade IV	Life-threatening complication (including CNS complications ‡) requiring ICU management
IV-a	Single organ dysfunction (including dialysis)
IV-b	Multi organ dysfunction
Grade V	Death of a patient

‡ brain haemorrhage, ischaemic stroke, subarachnoid bleeding, but excluding transient ischemic attacks

A4 Surgical Recovery Scale Questionnaire (Paddison, 2011)

The Surgical Recovery Scale questionnaire is a validated, comprehensive multi-dimensional recovery scale consisting of 13 items adapted from the previously validated Identity-Consequence Fatigue Scale questionnaire which was designed specifically to assess post-operative fatigue and correlates with peritoneal inflammation and cytokine release following colorectal surgery. Higher scores indicate improved functional recovery.

During the last two days …..	*Not at all*	*Almost never*	*Some of the time*	*Fairly often*	*Very often*	*All of the time*
I have been feeling energetic						
I have been feeling worn out						
I have been feeling vigorous						
I have done very little with the day						
I have been feeling fatigued						
Physically, I have felt tired						
I have had to restrict how much I try and do in a day						
I have been feeling lively						
During the last two days I have been able to …..	*Not at all*	*Rarely*	*Some of the time*	*Nearly as often as usual*	*As often as usual*	*Not applicable to me*
Read a newspaper / book or watch TV						
Dress						
Visit or socialize with family and friends						
Engage in leisure or recreational activities						
Shop or do errands						

A5 Colorectal Physiological and Operative Severity Score (Tekkis, 2004)

POSSUM is a tool used to compare morbidity and mortality in a wide range of general surgical procedures in order to facilitate surgical audit and the comparison of performance, adjusting for the risk of a surgical procedure based on the patients physiological condition.

Colorectal-POSSUM takes into account the following physiological and operative parameters and is calculated using the formula:

Ln R/1-R = -9.065 + (0.1692 x physiological score) + (0.1550 x operative severity score)

[R = predicted risk of mortality]

Physiological Parameters	
Age	<61 61-70 71-80 >81
Cardiac Failure	No / Mild Moderate Severe
Systolic Blood Pressure (mmHg)	100-170 >71 or 90-99 <89
Pulse Rate (bpm)	40-100 101-120 >121 or <39
Haemoglobin (g/dL)	13.0-16.0 10.0-12.9 or 16.1-18.0 <9.9 or >18.1
Urea (mmol/L)	<10 10.1-15.0 >15.1
Operative Parameters	
Operation Type	Minor Intermediate Major Complex Major
Peritoneal Contamination	None / Serous Local pus Free bowel content / pus / blood
Malignancy Status	No cancer / Dukes A/B Dukes C Dukes D
CEPOD	Elective Urgent Emergency

A6 Neutrophil Function Assay Results on Sequential Peri-operative Days

Neutrophil Extracellular Trap Production, Absolute Neutrophil Extracellular Trap Production, Stage of Neutrophil Apoptosis (4-hour and 24-hour incubation) and Neutrophil Phagocytosis Index (*E.Coli* and *S.Aureus*) on Sequential Peri-operative Days.

	Day-0	Day-1	Day-3	P-value * Day-0 - Day-1	P-value * Day-1 - Day-3	P-value $ Day-0 - Day-3
	NET Production AFU (Median)					
Unstimulated	11347	8654	10105	**0.0006**	0.1941	**0.0016**
PMA	39238	37638	33731	0.7929	0.1270	0.3351
IL-8	11925	9388	10829	**0.0003**	0.0914	**0.0045**
LPS	12473	9582	10057	**0.0001**	0.8456	**0.0025**
fMLP	12194	8680	10414	**<0.0001**	0.2989	**0.0014**
	Absolute NET Production (Median)					
Unstimulated	5.290	7.470	6.560	**0.0028**	**0.0113**	**0.0065**
PMA	16.530	33.320	22.350	**<0.0001**	**0.0002**	**<0.0001**
IL-8	5.330	7.445	7.345	**0.0002**	0.0597	**0.0009**
LPS	5.980	7.865	6.670	**0.0012**	**0.0178**	**0.0049**
fMLP	5.190	7.745	6.680	**0.0005**	**0.0064**	**0.0050**
	Stage of Neutrophil Apoptosis % (Median) at 4-hour incubation					
Alive	76.90	79.90	74.75	0.4679	**0.0203**	0.3169
Early Apoptosis	16.80	15.35	17.45	0.2355	0.0654	0.3959
Late Apoptosis	3.10	3.00	4.55	0.5530	0.0885	0.1917
Necrosis	1.40	1.35	1.75	0.8505	0.4552	0.5632
	Stage of Neutrophil Apoptosis % (Median) at 24-hour incubation					
Alive	14.05	36.90	18.10	**<0.0001**	**0.0093**	**<0.0001**
Early Apoptosis	71.85	51.80	76.79	**<0.0001**	**0.0210**	**0.0008**
Late Apoptosis	9.30	4.92	3.05	**0.0130**	0.7334	**0.0018**
Necrosis	0.30	0.60	0.40	0.0147	0.3223	0.0585
	Neutrophil Phagocytosis Index (Median) to E.Coli					
30 minutes	3446	4507	4545	0.0527	**0.0078**	0.1943
45 minutes	6615	7355	8686	0.0673	**0.0078**	0.2227
60 minutes	9777	10100	16130	0.1785	**0.0078**	0.1972
AUC	3255	4077	4756	0.0673	**0.0078**	0.1828
	Neutrophil Phagocytosis Index (Median) to S.Aureus					
30 minutes	4638	6079	8912	0.5184	0.2031	0.2617
45 minutes	8240	10260	11410	0.1700	0.1700	0.1455
60 minutes	14760	14330	16200	0.4196	0.4196	0.3111
AUC	4507	5119	6061	0.2260	0.2031	0.2313

* = p-value as determined by Wilcoxon Signed Rank test

$ = p-value as determined by Freidmann's test

AUC = Area Under Curve