Chronic inflammation and cancer

Prognostic impact of neutrophils and macrophages in solid tumors

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Prognostic impact of neutrophils and macrophages in solid tumors

PhD dissertation

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Preface

This thesis is based on research performed at the Department of Oncology and Department of Experimental Oncology, Aarhus University Hospital between 2009 and 2012. The thesis was partly based on collaboration with an international group of doctors in Sydney, Australia.

The following four original papers form the basis of the thesis:

I **Tumor-associated neutrophils and macrophages in non-small cell lung cancer: No immediate impact on patient outcome**
Andreas Carus , Morten Ladekarl, Henrik Hager, Hans Pilegaard, Patricia S Nielsen, Frede Donskov

Lung cancer 2013; **81**(1): 130-7.

II **Tumour-associated CD66b$^+$ neutrophil count is an independent prognostic factor for recurrence in localized cervical cancer**
Andreas Carus, Morten Ladekarl, Henrik Hager, Bettina S Nedergaard, Frede Donskov

British journal of cancer 2013; **108**(10): 2116-22.

III **Impact of baseline and nadir neutrophil index in non-small cell lung cancer and ovarian cancer patients: Assessment of chemotherapy for resolution of chronic inflammation**
Andreas Carus, Howard Gurney, Val Gebski, Paul Harnett, Rina Hui, Richard Kefford, Nicholas Wilcken, Morten Ladekarl, Hans von der Maase, Frede Donskov

Updated manuscript version, submitted 2013

IV **Validity and prognostic value of automatic digital image analysis (DIA) of tumor-associated leukocytes in localized cervical cancer**
Andreas Carus, Frede Donskov, Patricia S. Nielsen, Henrik Hager, Bettina S. Nedergaard, Torben Steiniche, Morten Ladekarl

Updated manuscript version, submitted 2013

List of abbreviations:

CTAD:	Chemotherapy toxicity-adjusted dosing
DIA	Digital image analysis
FIGO:	International Federation of Obstetrics and Gynaecology
G-CSF:	Granulocyte colony-stimulating factor
GM-CSF:	Granulocyte -macrophage colony-stimulating factor
IHC	Immunohistochemical
IL:	Interleukin
MMP-9:	Matrix Metalloproteinase-9 Enzyme
NSCLC:	Non-small cell lung cancer
OAS	Observer-assisted stereological sampling
OS:	Overall survival
RFS:	Recurrence-free survival
TA-NLR	Tumor-associated neutrophil to lymphocyte ratio
TAMs:	Tumor-associated macrophages
TANs:	Tumor-associated neutrophils
TMA:	Tissue micro array
TNF:	Tumor necrosis factor

Acknowledgements

This thesis was carried out from 2009 to 2012 during my employment as a research fellow at the Department of Oncology, Aarhus University Hospital. The field of tumor-associated inflammation is rapidly expanding, and it has been an exciting research area to explore and contribute to.

First, I wish to thank my supervisors for believing in me until the very end. Frede Donskov has opened my eyes to this exciting research field, has shown ever-lasting dedication, and put in long working hours when needed. Morten Ladekarl is an experienced researcher in the field of stereology and has aided me well in understanding these principles. I owe him great thanks for his work and contribution. I want to thank Henrik Hager for helping me understand pathology, a field where I was a novice when I entered, and his great help with the countless histologic sections. We have had some nice discussions on iPhones, Macs, and other nerdy stuff. Further, I wish to thank the Australian doctors that have aided me, especially Howard Gurney for his valuable contributions.

I am very grateful for my colleagues at the office, Lise and Hanna. We have shared our difficulties, frustrations and successes, as well as interesting discussions on all of life´s subjects, and I will be missing you. There are many in the department of Experimental Clinical Oncology (EKO) that I will miss as well, Charlotte, Anja P, Anja B, Stine, Christina, Trine, Louise, Torben, Nina, Line, Peter, Jimmi, Mohamed, and all the others. I will miss the sweet staff at EKO with whom I have shared many lunches. EKO is a dynamic scientific environment, and I have enjoyed the scientific input. I especially thank Professor Jens Overgaard for housing me and for his great support in the hours of need. Alex was usually there when IT help was needed. Likewise, Jan Alsner has always lend a hand when SPSS wasn´t behaving or when I needed to understand some obscure statistics.

I thank the laboratory personnel at the Department of Pathology for great help with the immunohistochemical stainings as well as support, in particular Tine Meyer and Helle Knakkergaard. I owe great thanks to Professor Torben Steiniche for his invaluable contributions. Patricia Nielsen has been a great help with my project and has always had time for me even when she was busy with her own projects, and I greatly respect her knowledge especially of digital image analysis.

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Andreas Carus, 2013

CONTENTS

The seventh hallmark of cancer – an inflammatory microenvironment

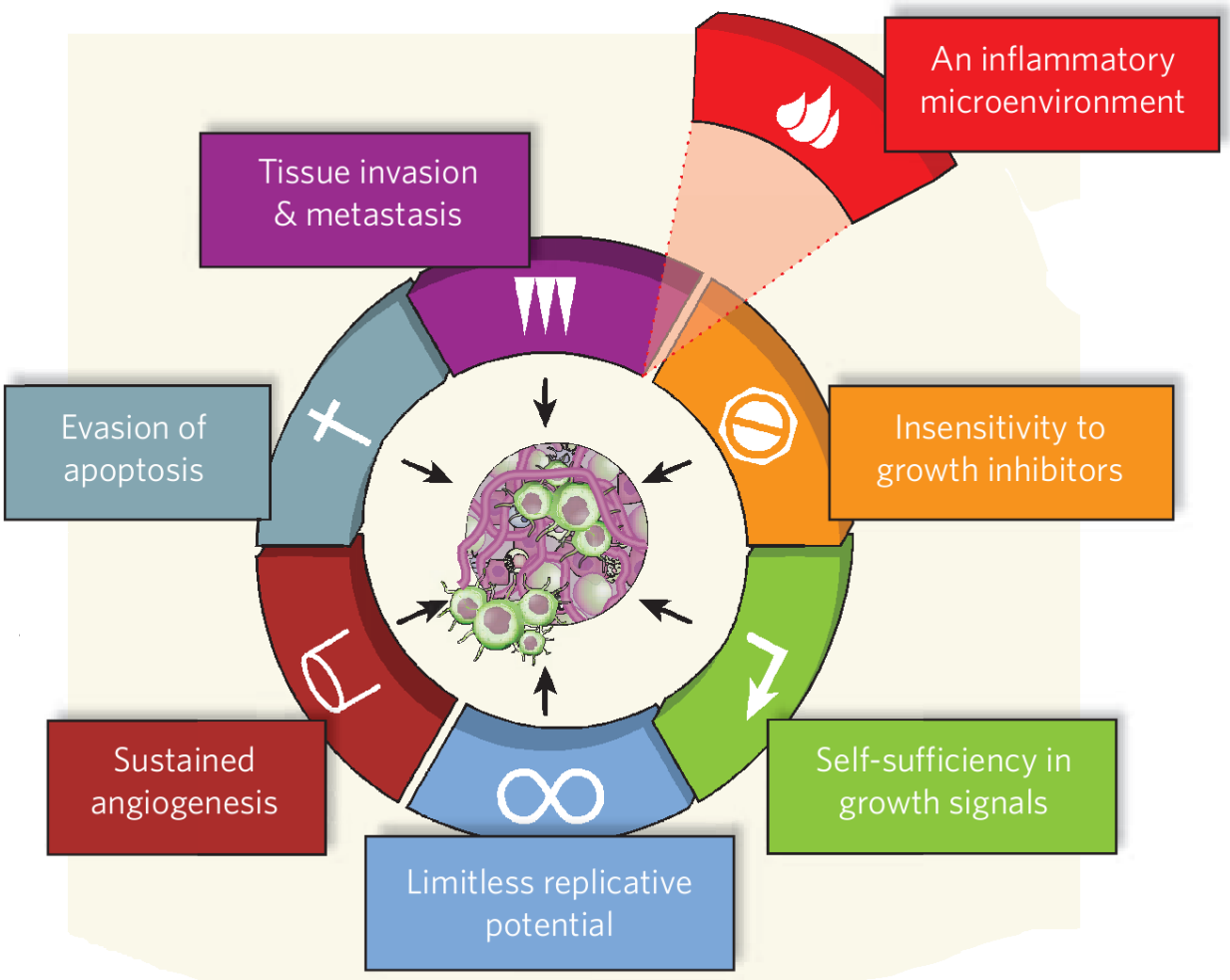

Figure 1. *Inflaming metastatasis (Mantovani et al[1])*
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1. Introduction

1.1 Inflammation and cancer

The link between cancer and inflammation is often attributed to the pathologist Rudolf Virchow who in 1863 noted that cancer originates at sites of chronic inflammation[2]. Pollutants, smoking, chemical or physical irritation, dietary factors, hormones, and chronic inflammatory disorders may induce an inflammatory state. Furthermore, it has been estimated that one in six cancers are caused by infections alone[3]. Gathering these factors it has become clear that cancers are not remote islands of tumor cells but rather an intricate assemblage of tumor cells, immune cells, and supportive tissue[4]. Notably, there is an interaction and collaboration between tumor cells and tumor environmental cells. Therefore, tumors have described as wounds that do not heal[5]. The process of tumor initiation, progression, and ultimately metastasing is decided by a delicate balance of the tumor and tumor microenvironment. Ample research has focused on approaches that induce an anti-tumor adaptive immune response and subsequently reverse the process of tumor escape. However, objective tumor response has only been achieved in patients with selected diagnoses, such as malignant melanoma and renal cell carcinoma patients[6]. In the last decade the recognition of tumor-promoting effects of innate immune cells has emerged. Although it is recognized that both tumor-promoting as well as anti-tumoral of both macrophages and neutrophils exist[7,8], the large bodies of studies in various human cancers demonstrate a detrimental impact of the presence of macrophages and neutrophils in the blood as well as in the tumor microenvironment (Table 1).

1.2 Neutrophils and macrophages

1.2.1 Neutrophils and macrophages polarize

Neutrophils and macrophages are part of the innate immune system and constitute the most abundant cells (approximately 70%) in the blood stream. It has been recognized in preclinical studies that dependent of the stimuli of cytokines and chemokines macrophages polarize to subtypes with different properties. Often, for simplification macrophages are described as either classically activated M1 anti-tumoral macrophages or alternatively activated M2 pro-tumoral macrophages, although many intermediate states exist[10] (Figure 2). Likewise, for neutrophils animal data indicate that tumor suppressing (N1) and tumor promoting (N2) differential states exists[11]. Whether this finding is similar in humans is currently being investigated (Albelda et al, "*Role of Tumor-Associated Neutrophils in Cancer", Keystone Symposia Dublin, 2012, unpublished data).*

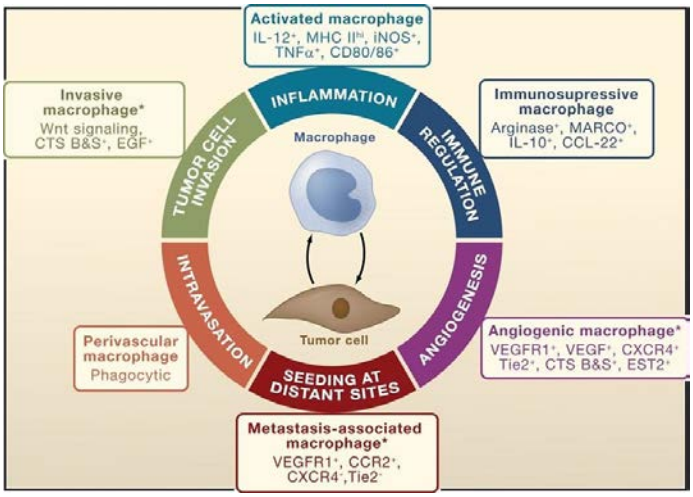

Figure 2. *Macrophage subtypes with distinct tumor promoting features (Qian et al[9])*
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1.2.2 Neutrophils effects in cancer

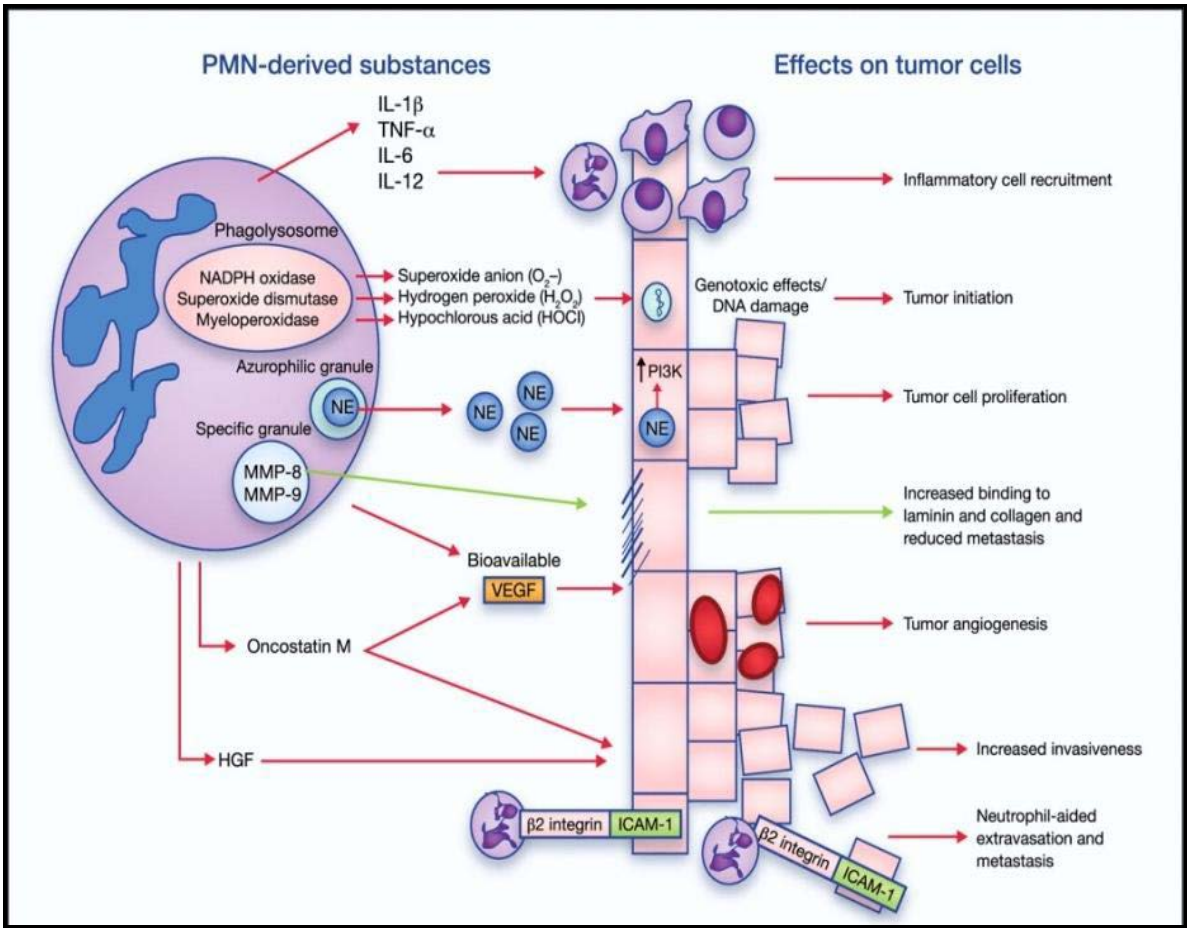

Figure 3.*Various tumor promoting effects of neutrophils on tumor cells by secretion of ROS, cytokines, and other factors (Gregory et al[12])*
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In the normal function of the body, neutrophils are the most common immune cells in the blood with up to 100.000 million cells produced every day[13] and they are usually considered short-lived cells (1-5 days depending on activation state[14]). Neutrophils patrol the blood stream and arrive at sites of infection and elicit acute inflammatory effects against invading pathogens. Still, in a tumor microenvironment the lifespan of neutrophils can be extended and allows for a chronic inflammatory state, possibly mediated by cytokines such as G-CSF, GM-CSF, TNF, interferon, and IL-6 and IL-8 [13,15,16]. G-CSF and GM-CSF are chemoattractants for neutrophils and support extravasation of neutrophils from the blood stream [17]. G-CSF induces elevated neutrophil levels in the blood[18] and consequently a link between neutrophil levels in the blood and presence in the tumor microenvironment may exist. Fossati et al has demonstrated a significant correlation between peripheral blood neutrophil count and infiltration of neutrophils in gliomas [19]. However, a range of other chemokines and cytokines that attract neutrophils to the tumor environment exists (TNF-α, IFN-Ɣ, ICAM-1, PECAM-1, and CXCL-1, CXCL-2, CXCL-6).

Essentially, it has been recognized that tumor-associated neutrophils (TANs) differ from neutrophils in the normal function of the body. TANs are involved in carcinogenesis (Figure 3) through for example mutagenic effects of reactive oxygen species (ROS) and myeloperoxidase (MPO), Matrix metallopeptidase 9 (MMP-9)[21]. In established tumors neutrophils play a pivotal role in angiogenesis, progression, and metastasing. TANs have been demonstrated as an important player in what is known as the angiogenic switch (Figure 4), which is the change from avascular tumor growth to angiogenesis, a necessary step for tumor growth beyond a size of 1-2mm diameter. This is likely mediated by secreted MMP-9, IL-8, and other chemokines[22-24]. Secreted factors like neutrophil elastase (NE) and neutrophil gelatinase-associated lipocalin (NGAL) have different pro-tumoral effect such as enabling the degradation of the extracellular matrix and increasing angiogenesis which can facilitate invasion, extravasation of tumor cells, and subsequently metastasing. Still, these factors have also been implicated in anti-tumoral effects highlighting the complexity of the tumor microenvironment[25,26]. Interestingly, neutrophils have been implicated in forming the "premetastatic niche". Before the arrival of metastatic tumor cells at predilection sites, increased infiltration of neutrophils, pro-inflammatory cytokines and MMP-9 can be seen which lead to vasculature remodelling and augmented metastatic spread [27,28]. In colorectal cancer, Rao et al found an adverse effect on OS of intratumoral CD66b$^+$ neutrophils and furthermore observed that increased neutrophil density was frequently observed in metastatic lymph nodes and was correlated with a metastatic phenotype[29].

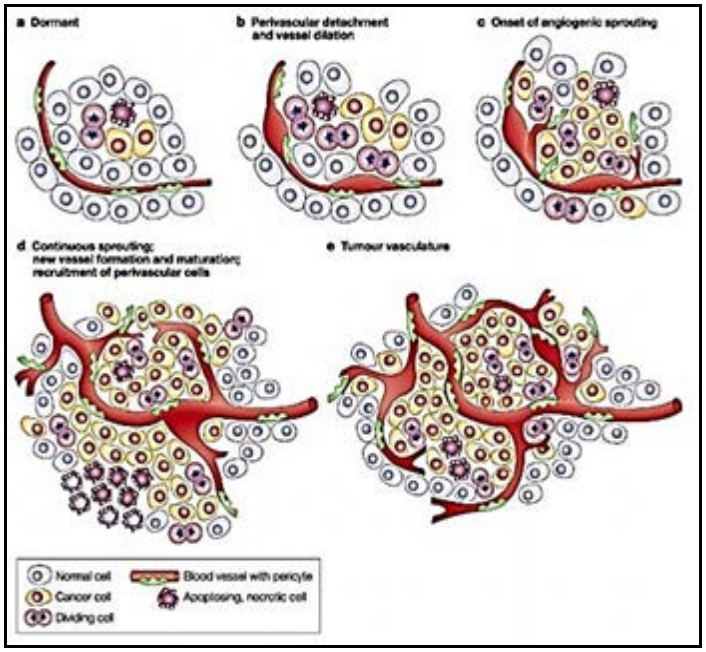

Figure 4. *The angiogenic switch and the different steps of tumor angiogenesis (Bergers et al[20])*
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1.2.3 Macrophages effects in cancer

The role of macrophages in the normal function of the body is to destruct and ingest cellular debris, foreign substances and invading microbes (collectively termed phagocytosis). They play an important role in presenting antigens of engulfed cells to T-lymphocytes which are part of the adaptive immune system. Furthermore macrophages are an important part of tissue healing after injury. Macrophages exert great plasticity as described before. Classically activated M1 macrophages are attributed with cytotoxic and debriding actions in inflammation[31] and the presence of macrophages with M1 immunohistochemical markers in solid tumors is usually associated with improved patient outcome (Table 1). However, in most cancers macrophage phenotype is skewed towards M2 macrophages which are involved in wound healing, angiogenesis, matrix regeneration, and cell proliferation[32] (Figure 5).

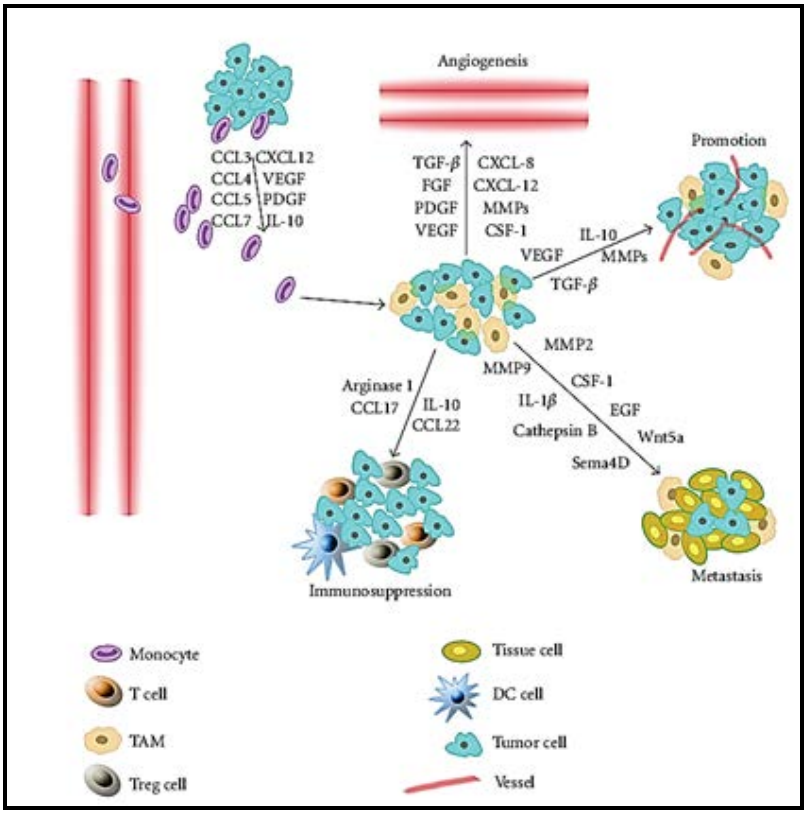

Figure 5. *Macrophages in tumor microenvironments and the progression of Tumors (Hao et al[30])*
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The presence of these predominantly wound healing macrophages in many cancers has coined the term "wounds that do not heal" [33], which implicates that the tumor microenvironment is dominated by inflammatory cells, cytokine and chemokines. This environment facilitates tumor growth, angiogenesis and remodelling of the tissue. Like neutrophils TAMs are involved in the angiogenic switch but also directly and indirectly (by mobilizing VEGF) support sustained angiogenesis [34]. Correspondingly, TAMs have been demonstrated to be associated with peritumoral lymphangiogenesis[35] and thereby possibly facilitating lymphatic metastatic spread. Matrix modelling by factors secreted by mainly TAMS allows tumor cells to locally invade tissue and ultimately penetrate vessels and permit blood stream metastasizing[36].

1.2.4 Crosstalk between neutrophils and macrophages

Neutrophils and macrophages originate from common myeloid progenitors (Figure 7), and in the normal function of the immune system it is advantageous with dual phagocytes with overlapping as well as complementing effects. Common secretion of some cytokines and chemokines and expression of target receptors creates various feedback loops[37]. In uncontained inflammation neutrophil-macrophage cooperation may lead to tissue damage forming the basis for chronic inflammation. In the tumor microenvironment neutrophils and macrophages share tumor promoting effects. In a cervical cancer mouse model it has been demonstrated that CCR2 inhibition blocks macrophage infiltration in

the stroma, but this blockade elicits a compensatory influx of neutrophils instead leading to uninterrupted tumor growth[38]. In tumor areas GM-CSF has been detected in monocytes/macrophages and has been demonstrated to inhibit neutrophil apoptosis in vitro prolonging neutrophil lifespan.
It has been suggested that the switch from M1 to M2 macrophage phenotype may be initiated predominantly by the phagocytosis of apoptotic cells (e.g., neutrophils)[31]. Upregulated chemokines in TANs play a role in recruiting macrophages (by CCL2 and CCL7) and T-regulatory cells (by CCL17) to the tumor[39].

1.2.5 Neutrophil to lymphocyte ratio

Tumor-associated neutrophils can suppress the anti-tumor responses of for instance $CD8^+$ cytotoxic lymphocytes[11]. This may be exerted through the release of arginase I which inhibits T cell lymphocyte functions[41] as well as other effects (Figure 6). Elevated neutrophil/lymphocyte ratio in the blood has been correlated with poor outcome in a large range of cancers (Table 1). However, to our knowledge only one previous study in NSCLC has observed neutrophil to lymphocyte ratio in the tumor microenvironment as predictive for poor patient outcome[42]. Chua et al (Figure 6) suggested that various cytokines, chemokines, and other factors secreted by the tumor cells are responsible for blood neutrophilia and lymphocytopenia leading to a higher neutrophil-lymphocyte ratio.

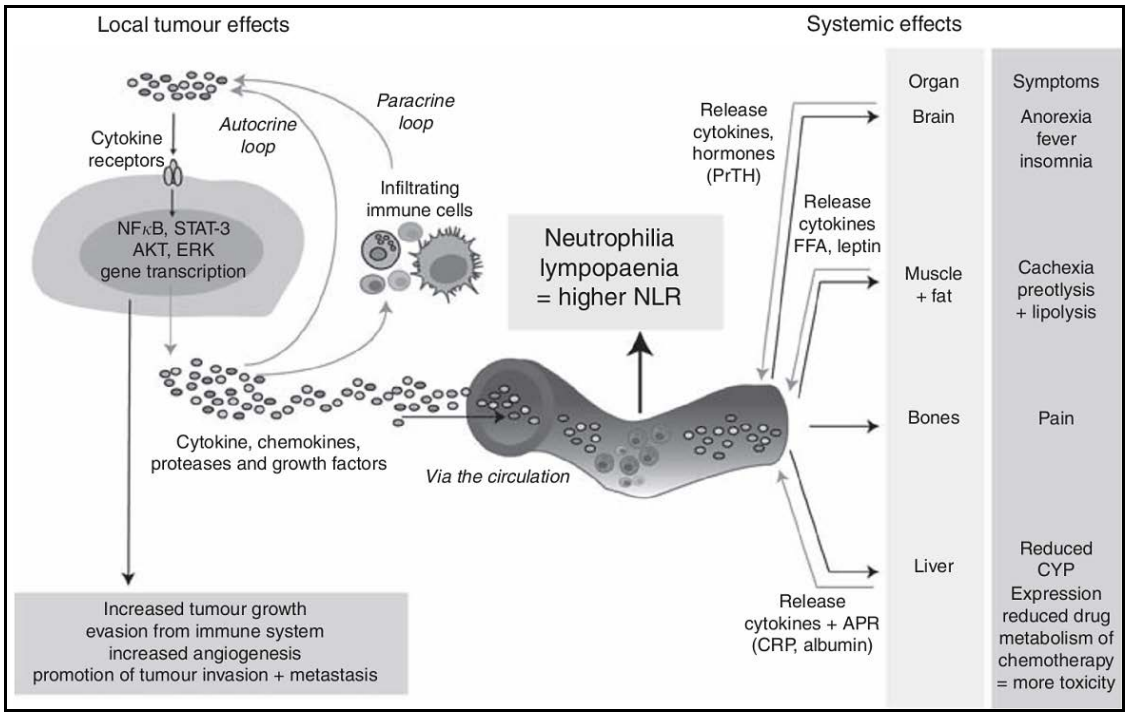

Figure 6. *Model for tumor-induced elevated neutrophil-lymphocyte ratio in the blood (Chua et al[40])*
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1.2.6 Immunohistochemical (IHC) parameters of neutrophils and macrophages

Various markers for macrophages and neutrophils exists (Figure 7). For the IHC studies in the present thesis CD66b and CD163 were chosen for neutrophil and macrophage staining, respectively.
CD66b (a member of the carcinoembryonic antigen family, CEACAM8) is a marker of activated neutrophils where CD66b is mobilized from secondary cytoplasmatic granules to the cell membrane. It

is a very specific marker of neutrophils, but expression on eosinophil granulocytes cannot be excluded[43]. CD66b has been linked with poor prognosis in various cancers and may be a marker for tumor-promoting neutrophils (Table 1).

CD163 is a marker belonging to the scavenger receptor cysteine-rich superfamily expressed on monocytes and resident tissue macrophages and is expressed only on a subpopulation of macrophages. CD163 also stains tissue specialized macrophages such as bone marrow macrophages, alveolar macrophages of the lung, Kupffer cells of the liver, and dermal macrophages of the skin[45]. CD163+ macrophages have been demonstrated as a poor prognostic factor in various solid tumors (Table 1) and are thought to be a marker of M2 macrophages[46].

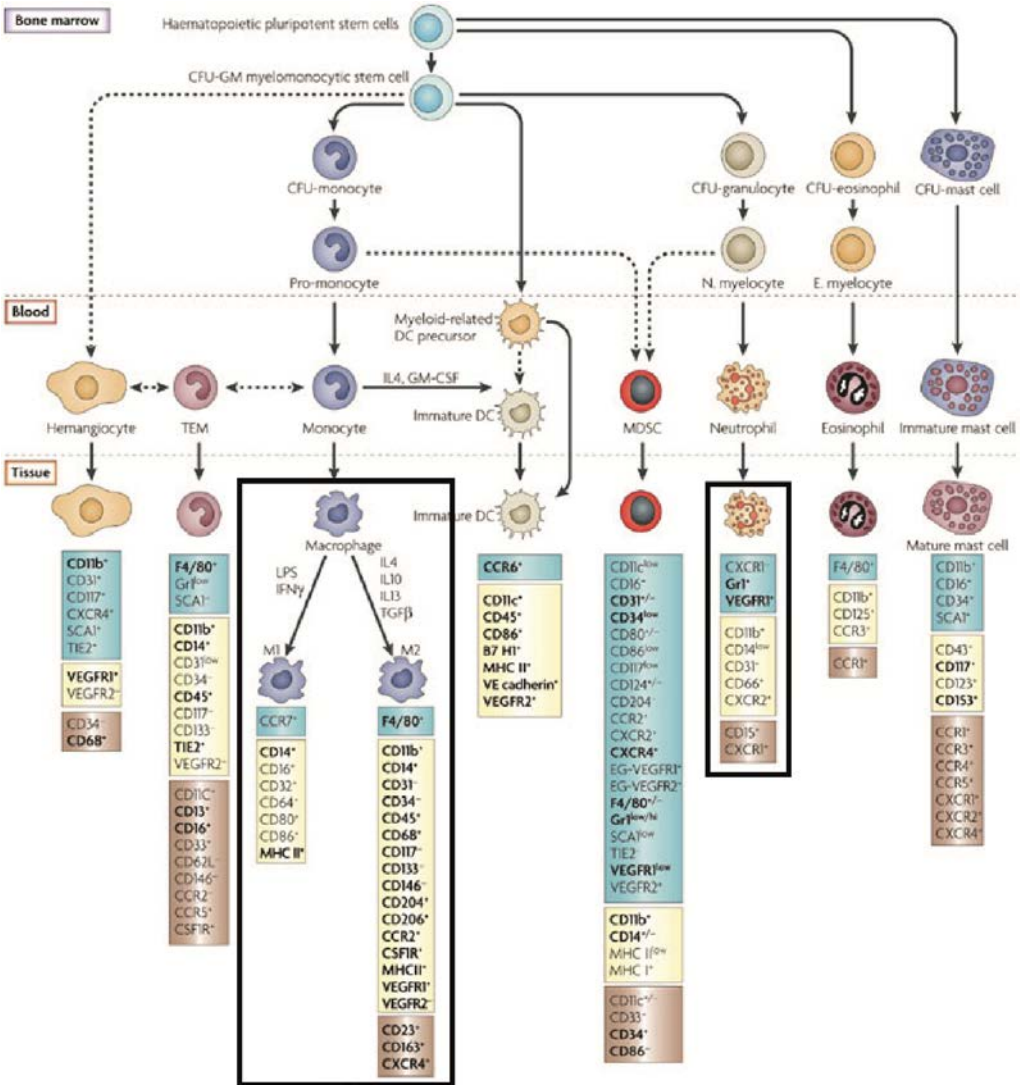

Figure 7. *Immunohistochemical markers for macrophages and neutrophils as well as other immune cell lineages* (Murdoch et al[44])
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1.3 *Epidemiology, treatment and prognosis of patient cohorts included in the thesis*

1.3.1 Non-small cell lung cancer

1.3.1.1 Epidemiology and treatment

Lung cancer is the most frequent cancer in the world[47]. In Denmark, approximately 4300 new cases are diagnosed per year and lung cancer accounts for almost 25% of all deaths to cancer. Lung cancer is vastly caused by smoking, although other substances and occupational and environmental exposures account for some cases. Overall, 5-years survival is around 10%[48]. Around 75% of cases are non-small cell cancers (NSCLC), and around 20% of NSCLC patients are surgically resected. Surgery is mainly reserved for TNM[49] stage IA through II cancers, although occasionally stage IIIA and IIIB cancers will receive resection (e.g. unexpected N2 stage). International figures for survival at 5 years after resection is for pathological stage IA 73%, whereas for stage III survival is only 25%[50].

1.3.1.2 Prognostic factors

A large range of prognostic factors have been investigated in NSCLC[51], In advanced diseases presence of brain or liver metastases are prognostically detrimental. In recent years, EGFR mutation and ALK mutation status has emerged as predictive factors for treatment effect of new biologically targeted drugs in advanced disease[52].

1.3.1.3 The role of neutrophils and macrophages in NSCLC

Various studies have established adverse effect of elevated peripheral blood WBC, neutrophils, and neutrophil/lymphocyte ratio in patients with operable as well as advanced NSCLC (Table 1A). A single study of tumor-associated neutrophils found an adverse impact of intratumoral neutrophils on RFS; however, this was only the case for late recurrences. No impact on OS was seen. The authors found in multivariate analysis an adverse impact on OS of intratumoral neutrophil to lymphocyte ratio. For tumor-associated macrophages results are conflicting since different markers (CD68, CD163, CD204, and HLA-DR) and different tumor compartments have been explored (Table 1B). Thus, in some studies an adverse impact has been demonstrated, others have found no impact, and some have found a favourable impact; the latter may be attributed to a M1 antitumor macrophage phenotype. It has been suggested that macrophages in the tumor nests exhibit an antitumor phenotype (M1) and in the peritumoral stroma are of a tumor-promoting phenotype (M2)[53].

1.3.2 Cervical cancer

1.3.1.1 Epidemiology and treatment

Cervical cancer is a frequent cancer worldwide [54]. In Denmark, however, cervical cancer only accounts for 2% of all cancer cases, mainly because of the screening programme introduced in the 1960's. Still, around 110 women succumb each year[48]. Human papilloma virus (HPV) is found in 99% of all cancers, which makes cervical cancer a good model for a virus-induced cancer associated with disturbances in the immunological system. The introduction of HPV-vaccination for teenage girls is expected to hinder 70% of all cases, but because of the *lag time* of about 20-30 years from primary infection to the development of invasive cancer, cervical cancer will still be a concern for years to come[55]. For FIGO[56] stage IA cancer, treatment is conisation or hysterectomy with possible pelvic lymph node dissection. For stage IB and IIA the main treatment is hysterectomy with possible pelvic lymph node dissection, but cervicetomy with lymph node dissection is offered in select cases.

1.3.2.2 Prognostic factors

Prognostic factors in clinical use are mainly disease stage, nodal status, tumor volume, depth of cervical stromal invasion, and lymphovascular space invasion[57]. HPV-status, histological subtype,

tumor proliferation rate (Ki-67), and in particular HPV-18 positivity may be of prognostic significance[58].

1.3.1.3 The role of neutrophils and macrophages in cervical cancer

Cervical cancer is generally a viral induced disease which elicits a cell mediated immune response and thus the vast majority of studies have focused on lymphocyte infiltration[58] Very few studies have studied the impact of neutrophils and monocytes in the blood (Table 1A) but have demonstrated an adverse impact of elevated WBC, neutrophils, neutrophil/lymphocyte-ratio, and monocytes. In the tumor-microenvironment adverse impacts on CIN progression, angiogenesis, and lymphangiogenesis have been demonstrated. In one older study of CIN it was suggested that the infiltration of macrophages was a first line of defence against the spread of HPV-virus infection[59]. However, Davidson et al found no correlation between HPV presence and $CD68^{+}$ macrophage infiltration[60].

1.3.3 Ovarian Cancer

Ovarian cancer remains the leading cause of death from gynaecological cancer with a 5-year survival rate for advanced ovarian cancer of 30%–40%[61]. In Denmark, less than 600 patients (3% of all cancers) are diagnosed each year [48]. Staging of patients is according to FIGO[62], and 70-80% of all cancers are advanced stage at time of diagnosis. Overall 5-yearsurvival is 83%, 62%, 23%, and 11% for stage I, II, III, and IV, respectively. Advanced stage II–IV ovarian cancer is usually treated with combination therapy with carboplatin, docetaxel, and bevacizumab, although in elderly or poor performance patients may be offered single agent carboplatin[63].

1.3.2.2 Prognostic factors

Established prognostic factors in ovarian cancer are FIGO-stage, histology, tumor-grade, presence of ascites, performance status, and extent of residual disease following debulking surgery[64]. The role of CA125 is unclear, but normalization of CA125 after chemotherapy is prognostic. Many other prognostic factors have been explored but are not in clinical use[65].

1.3.1.3 Role of neutrophils in ovarian cancer

A few studies have demonstrated an adverse effect of elevated neutrophil count, neutrophil/lymphocyte ratio, and monocytes in the blood (Table 1A). A single study have examined MPO-staining in the tumor tissue - indicative of neutrophil or monocyte infiltration - in gynaecological cancers including ovarian cancer, and found higher levels in cancers compared to normal tissue. No correlation with patient outcome was made.

1.4. *Table 1A and 1B*. Studies in humans of the impact on prognosis of white blood cells, neutrophils and macrophages in the blood and in the tumor compartment

Table 1A. Blood assessments

Marker	Year	N	Tumor	Impact	Endpoint(s)	Multivariate significance
WBC						
Gislason[1]	1985	258	NSCLC	Adverse	OS	Yes
Grimm[2]	1985	6222	All cancers	Adverse	CSS/OS	Yes
Thomson[3]	1986	75	Lung cancer	Adverse	Benign vs. malignancy	No
Engan[4]	1990	189	NSCLC	Adverse	OS	No
Hasenclever[5]	1998	4695	Hodgkin lymphoma	Adverse	PFS/OS	Yes
Herndon[6]	1998	337	Mesothelioma	Adverse	OS	No
Ray[7]	1998	301	NSCLC, SCLC	Adverse	Response	Yes
Kasuga[8]	2001	227	Lung cancer	Adverse	OS	No
Erlinger[9]	2004	7674	Lung cancer, all cancer	Adverse	Incidence/CSS	Yes
Jee[10]	2005	437454	All cancer	None	OS, not CSS	Yes
Lee[11]	2006	424419	Colon cancer	Adverse	CSS	Yes
Mandrekar[12]	2006	1053	St. IIIB/IV NSCLC	Adverse	TTP/OS	Yes
Shankar[13]	2006	3189	Lung cancer, all cancer	Adverse	Incidence/OS	Yes
Garcia-Arias[14]	2007	294	Advanced cervical cancer	Adverse	OS	Yes
Margolis[15]	2007	143748	Breast, CRC, endometrial, lung cancer	Adverse	Incidence/CSS	Yes
Ruggiero[16]	2007	2803	All cancer	Adverse	OS, not CSS	Yes
Schmidt[17]	2007	363	MM	Adverse	OS	Yes
Tibaldi[18]	2008	320	St. IIIB-IV NSCLC	Adverse	OS	Yes
Trujillo-Santos[19]	2008	3805	All cancers	Adverse	DSS, OS	Yes
Yamanaka[20]	2008	1220	St. IV gastric cancer	Adverse	OS	Yes
Granger[21]	2009	3770	Various	Adverse	OS	No
Johnson[22]	2009	926	Duke A-D rectal cancer	None	DSS	Yes
Park[23]	2009	358	St. IIIB/IV NSCLC	Adverse	OS, RFS	Yes
Tomita[24]	2009	289	St. I-III NSCLC	Adverse	OS	Yes
Connolly[25]	2010	4405	Various cancers	Adverse	OS	Yes
Dos Santos[26]	2010	19303	All cancers	Adverse	Risk of lung cancer	No
Qiu[27]	2010	318	Cervical cancer	Adverse	OS	Yes
Yang[28]	2010	4570	All cancers	Adverse	Risk of cancer	Yes
Mabuchi[29]	2011	536	St. I-IV cervical cancer	Adverse	OS	Yes
Holgersson[30]	2012	835	St. I-IV NSCLC	Adverse	OS	Yes
Lu[31]	2012	63	Cancer of unknown origin	Adverse	OS	No
Worley Jr.[32]	2012	1144	Endometrial cancer	Adverse	OS, not RFS	Yes
Neutrophil						
Paesmanns[33]	1995	1052	Advanced NSCLC	Adverse	OS	Yes
Lopez[34]	1996	215	mRCC	Adverse	OS	Yes
Fossati[35]	1999	105	Gliomas	Adverse	Tumor grade	Yes
Paesmanns[36]	2000	763	SCLC	Adverse	OS	Yes
Négrier[37]	2002	782	mRCC	Adverse	PFS/OS	Yes
Ferrigno[38]	2003	1201	St. I-IV lung cancer	Adverse	OS	Yes
Ferrigno[38]	2003	1201	St. 0-IV NSCLC	Adverse	OS	Yes
Schmidt[39]	2005	321	MM	Adverse	OS	Yes

Donskov[40]	2006	59	mRCC	Adverse	OS	No
Michael[41]	2006	134	mCRC	Adverse	PFS	Yes
Royston[42]	2006	425	mRCC	Adverse	OS	Yes
Schmidt[17]	2007	363	MM	Adverse	OS	Yes
Atzpodien[43]	2008	495	mRCC	Adverse	Response, OS	No
Teramukai[44]	2009	388	St. IIIB-IV NSCLC	Adverse	RFS/OS	Yes
Tavares-Murta[45]	2010	315	CIN-St. IV cervical cancer	Adverse	Recurrence	Yes
Hung[46]	2011	1040	St. II colon cancer	Adverse	OS, not DFS	Yes
Matsubara[47]	2011	30	mBC	Adverse	OS	Yes
Thavaramara[48]	2011	129	Ovarian cancer	Adverse	PFS, not OS	no
Azuma[49]	2012	84	mRCC (post-treatment)	Adverse	PFS, OS	Yes
Banerjee[50]	2012	964	St. IC-IV ovarian cancer	Adverse	PFS, not OS	Yes
Ishizuka[51]	2012	169	St. IV CRC	Adverse	OS	No
Neutrophil/ lymphocyte ratio						
Walsh[52]	2005	230	St. I-IV CRC	Adverse	CSS/OS	No
Halazun[53]	2007	440	mCRC	Adverse	RFS/OS	Yes
Gomez[54]	2008	96	Resected HCC	Adverse	DSS/OS	Yes
Gomez[55]	2008	705	Operated HCC	Adverse	Recurrence	Yes
Ong[56]	2008	113	Pancreatic cancer	Adverse	Resectable rate	Yes
Yamanaka[20]	2008	1220	St. IV gastric cancer	Adverse	OS	Yes
Cho[57]	2009	999	St. I-IV ovarian cancer	Adverse	OS	Yes
Guo[58]	2009	91	Operated HCC	Adverse	DFS, OS	Yes
Halazun[59]	2009	150	Liver transplanted HCC	Adverse	DFS/OS	Yes
Kishi[60]	2009	290	mCRC	Adverse	OS	Yes
Sarraf[61]	2009	178	St. I-IV NSCLC	Adverse	OS	Yes
Aliustaoglu[62]	2010	168	Locally advanced gastric cancer	Adverse	OS	No
An[63]	2010	95	St. III-IV pancreatic cancer	Adverse	OS	Yes
Bhatti[64]	2010	84	Resected pancreatic cancer	Adverse	OS	Yes
Ding[65]	2010	141	St. IIA colon cancer	Adverse	RFS	Yes
Kao[66]	2010	173	mMM	Adverse	OS	Yes
Kao[67]	2010	173	Mesothelioma	Adverse	OS	Yes
Kim[68]	2010	55	Sarcomas	Adverse	Recurrence	No
Liu[69]	2010	123	St. I-IV rectal cancer	Adverse	RFS	Yes
Ohno[70]	2010	192	T2/T3 RCC	Adverse	RFS	Yes
Porrata[71]	2010	255	Diffuse large B-cell lymphoma	Adverse	PFS/OS	Yes
Rashid[72]	2010	294	Resected esophageal cancer	None	DFS/OS	No
Shimada[73]	2010	1028	St. I-IV gastric cancer	Adverse	OS	Yes
Ubukata[74]	2010	157	St. I-IV gastric cancer	Adverse	OS	Yes
An[75]	2011	363	Nasopharyngeal cancer	Adverse	DSS, DMFS, LRFS	Yes
Bertuzzo[76]	2011	219	Liver transplanted HCC	Adverse	RFS/OS	Yes
Chua[77]	2011	349	mCRC	Adverse	OS	Yes
Garcea[78]	2011	74	Resected pancreatic cancer	Adverse	DSS	No
Huang[79]	2011	145	Unresectable HCC	Adverse/ favorable	OS	Yes
Jung[80]	2011	293	St. III/IV gastric cancer	Adverse	DFS, OS	Yes
Sharaiha[81]	2011	295	Resected esophageal	Adverse	DSS/OS	Yes

			cancer			
Tomita[82,83]	2011 2012	248	Resected NSCLC	Adverse	OS	Yes
Wang[84]	2011	101	Liver transplanted HCC	Adverse	DFS	Yes
Azab[85]	2012	316	St. I-IV BC	Adverse	OS	Yes
Chen[86]	2012	158	Early HCC	Adverse	OS	Yes
Gondo[87]	2012	189	Bladder cancer (cystectomy)	Adverse	DSS	Yes
Kaneko[88]	2012	50	Advanced CRC	Adverse	OS	Yes
Keizmann[89]	2012	133	mRCC	None	PFS/OS	Yes
Lee[90]	2012	1061	St. IB-IVA cervical cancer	Adverse	OS	Yes
Pinato[91]	2012	171	Mesothelioma	Adverse	OS	Yes
Proctor[92]	2012	12118	All cancers	Adverse	CSS/OS	Yes
Sato[93]	2012	83	Advanced esophageal cancer	Adverse	Response	Yes
Shafique[94]	2012	897	Prostate cancer	Adverse	OS	Yes
Young[95]	2012	142	HCC	Adverse	RFS	Yes
Zhang[96]	2012	92	CRC (RFA treated liver metastases)	Adverse	DSS, OS	Yes
Monocyte						
Schmidt[39]	2005	321	MM	Adverse	OS	Yes
Donskov[40]	2006	59	mRCC	Adverse	OS	No
Sasaki[97]	2006	235	Resected HCC	Adverse	DSS	Yes
Bishara[98]	2007	136	St. I-IV ovarian cancer	Adverse	RSS, not OS	Yes
Leitch[99]	2007	233	St. I-IV CRC	Adverse	DSS, OS	Yes
Sasaki[100]	2007	97	mCRC		CSS	Yes
Chen[101]	2009	278	Head and neck cancer	Adverse	OS	Yes
Cho[102]	2009	1818	St. I-IV cervix cancer	Adverse	DSS/OS	Yes
Burt[103]	2011	52	Mesothelioma	Adverse	OS	No
Wilcox[104]	2011	366	Diffuse large-B-cell lymphoma	Adverse	OS	Yes
Lee[105]	2012	788	St. I-IV cervical cancer	Adverse	RFS/OS	Yes
Porrata[106]	2012	476	Hodgkin lymphoma	Adverse	OS	Yes

Abbreviations: NSCLC=Non-small cell lung cancer; OS=Overall survival; CSS=Cancer specific survival; PFS=Progression-free survival; TTP=Time to progression; SCLC=Small-cell lung cancer; St.=Stage; DSS=Disease-free survival;

Table 1B. Tumor assessments

Cell	Year	N	Tumor	Marker	Place	Impact	Endpoint	Multivariate significance
Neutrophil								
Bellocq[107]	1998	29	BAC	Neutrophil elastase	BAL-fluid	Adverse	OS	Yes
Fossati[35]	1999	105	Glioma	CD15$^+$ MPO$^+$	T	Adverse	Tumor grade	No
Nielsen[108]	1999	584	St. I-IV colorectal cancer	H/E	P	Adverse	OS	No
Nagtegaal[109]	2001	160	St. I–III rectal cancer	Neutrophil elastase	P	Favorable	RFS	Yes
Song[110]	2001	261	Gynecological cancers	MPO$^+$	T	Adverse	Infiltration	No
Caruso[111]	2002	273	Stage IB-IV gastric cancer	H/E	S	None	OS	Yes
Coen[112]	2004	117	St. I-IV colorectal cancer	CD16$^+$	T	Favorable	OS	Yes
Klintrup[113]	2005	386	St. I-IV colorectal cancer	H/E	P	Favorable	OS	Yes
Donskov[114]	2006	120	mRCC	CD66b$^+$	T	Adverse	OS	Yes
Jensen[115]	2009	121	St. I-IV RCC	CD66b$^+$	T	Adverse	RFS/DSS/OS	Yes
Trellakis[116]	2010	99	HNSCC	CD66b$^+$ MPO$^+$	T	Adverse	Stage	No
Ilie[117]	2011	632	St. I-III NSCLC	CD66b$^+$	T	Adverse	RFS, not OS	No
Jensen[118]	2011	186	Stage I/II MM	CD66b$^+$	T	Adverse	OS	Yes
Kuang[119]	2011	238	Resected HCC	CD15$^+$	P	Adverse	DFS/OS	Yes
Li[120]	2011	197	HCC	CD66b$^+$	S	Adverse	RFS, OS	
Reid[121]	2011	517	Pancreatic cancer	H/E	T	Adverse	Aggressive tumortype	No
Gao[122]	2012	240	Resected HCC	CD66b$^+$ CXCR6$^+$	T	Adverse	RFS, not OS	Yes
Gu[123]	2012	123	Cholangiocarcinoma	CD66b	T	Adverse	OS	Yes
Rao	2012	229	St. I-IV CRC	CD66b$^+$	T	Adverse	OS	Yes
Zhao[124]	2012	212	St. I-IV Gastric cancer	CD15$^+$	T	Adverse	OS	Yes
Zhou[125]	2012	94	St. I-III HCC	CD66b$^+$ CXCL5$^+$	T	Adverse	TTR, OS	Yes
Macrophage								
Davidson[126]	1999	75	St. I – IV Cervical cancer	CD68$^+$	T	Reverse correlate stage	Correlates	No
Takanami[127]	1999	113	St. I-IV NSCLC	CD68$^+$	S	Adverse	OS	Yes
Fujimoto[128]	2000	80	St. I-IV Cervical cancer	CD68$^+$		Adverse	IL-8 level, OS	No
Nagtegaal[109]	2001	160	St. I–III rectal cancer	CD68$^+$	T	Favorable	RFS, OS	No
Nakayama[129]	2002	30	St. I-IV colorectal cancer	CD68$^+$	P	Favorable	RFS	No
Oberg[130]	2002	93	Stage III colorectal cancer	CD68$^+$	Ln	Favorable	OS	No
Schoppmann	2002	51	CIN,LSILSs,	CD68$^+$	P	Adverse	VEGF-C	No

[131]			Cervical cancer				expression, lymph vessel density	
Heller[132]	2003	24	Cervical cancer	CD68+	T	Reverse correlate stage	Correlations	No
Funada[133]	2003	97	St. I-III colorectal cancer	CD68+	P	Favorable	Stage, OS	No
Jørkov[134]	2003	13	St. IV MM	NCL-MACRO+	T	Favorable	Treatment response	No
Khorana[135]	2003	131	St. II-III colorectal cancer	CD68+	S	Favorable	OS	No
Baeten[136]	2004	117	St. I-IV colorectal cancer	CD68+	-	None	OS	No
Lackner[137]	2004	70	St. II-III colorectal cancer	CD68+	P	Favorable	OS	Yes
Marcus[138]	2004	102	Head and neck cancer	CD68+	T	Adverse	Lymph node metastases, stage	No
Inoue[139]	2005	22	St. I-III colorectal cancer	CD68+	-	None	OS	No
Klintrup[113]	2005	386	St. I-IV colorectal cancer	CD68+	P	Favorable	OS	Yes
Tan[140]	2005	60	St. I-IV colorectal cancer	CD68+	P	Favorable	OS	No
Welsh[141]	2005		St. I-IIIA NSCLC	CD68+	T, S	Favorable	OS	Yes
Donskov[40]	2006	63	mRCC	NCL-MACRO+	T	Not evaluated	Infiltration	No
Hansen[142]	2006	27	mMM	CD64+	T	Adverse	OS	No
Hillen[143]	2006	58	Resected melanoma	CD68+	P,T	Adverse	OS	No
Yang[144]	2006	61	Endometrial cancer	MMP-12+ CD68+	S	Adverse	Tumor grade	ND
Bailey[145]	2007	22	St. I-IV CRC	CCL2+	All	Trend	Increasing stage	ND
Forsell[146]	2007	446	St. I-IV colorectal cancer	CD68+	P	Favorable	CSS	Yes
Hammes[147]	2007	112	CIN/Cervical cancer	CD68+	T	Adverse	CIN Progression	No
Nagorsen[148]	2007	40	St. I-IV colorectal cancer	CD68+ CD163+	S	Favorable	OS	No
Zeni[149]	2007	50	NSCLC	IL-10+	P	Adverse	OS	Yes
Lee[150]	2008	51	Leiomyosarcoma	CD68+ CD163+	T,S	Adverse	OS	ND
Ryder[151]	2008	90	Thyroid cancer	CD68+ CD163+	T	Adverse	PFS/OS	No
Shabo[152]	2008	133	Stage II Breast cancer	CD68+ CD163+	T	Adverse	RFS/OS	Yes
Ding[153]	2009	137	HCC	CD68+	T	Adverse	DFS/OS	Yes
Jensen[154]	2009	227	St. I/II MM	CD163+ CD68+	P, S	Adverse	RFS/CSS/OS	Yes
Kawamura[155]	2009	40	Ovarian cancer	CD68+ CD163+	T,S	Adverse	Malignancy	ND

				CD204+ CSF-1+				
Kurahara[156]	2009	76	Resected pancreatic cancer	CD163+ CD68+ CD204+		Adverse	OS	No
Lu[157]	2009	92	St. I-IV oral squamous cancer	CD68+	T, S	Adverse	PFS/OS	yes
Ohri[158]	2009	40	St. I-IV NSCLC	CD163+ HLA-DR+ iNOS+ TNF-α+ MRP-8/14+	T	Favorable	OS	Yes
Shieh[159]	2009	41	MEC	CD68+	T	Adverse	Tumor grade, size	ND
Takayama[160]	2009	135	Prostate cancer	CD204+	T	Favorable	RFS	Yes
Utrera-Barillas[161]	2009	62	CIN/Cervical cancer	CD68+	T, P	Adverse	Lymph vessel density and angiogenesis	No
Van Dongen[162]	2009	47	GIST	CD68+ CD163+	T	Adverse	Metastasing	ND
Campbell[163]	2010	110	Breast cancer	CD68+/anti-PCNA	T	Adverse	TTP/OS	Yes
Clear[164]	2010	59	Follicular lymphoma	CD163+	T	Adverse	Vascular density	No
Dai[165]	2010	99	St. I-IV NSCLC	CD68+	T, S	Adverse	OS	Yes
Espinosa[166]	2010	64	Endometroid cancer	CD163+	S	Adverse	Myoinvasion	ND
Hasita[167]	2010	55	ICC	CD68+ CD163+	T,S	Adverse	DFS, not OS	Yes
Kang[168]	2010	118	CRC	CD68+ MMP+	T,S	Adverse	Clinical factors	ND
Ma[169]	2010	100	St. I-IV NSCLC	CD68+ CD163+ HLA-DR+	T	Favorable	OS (HLA-DR)	Yes
Niino[170]	2010	42	T-cell lymphoma	CD68+/ CD163+	T	Adverse	OS	Yes
Nonomura[171]	2010	71	Prostate cancer	CD68+	T	Adverse	OS	Yes
Ohtaki[172]	2010	170	St. I-IIIa NSCLC	CD68+ CD204+	S	Adverse	OS	No
Steidl[173]	2010	130	Hodgkin lymphoma	CD68+ MMP11+	T	Adverse	DFS, not OS	Yes
Zhou[174]	2010	160	St. IIIB-IV colorectal cancer	CD68+	P	Favorable	OS	Yes
Bronkhorst[175]	2011	43	Uveal MM	CD68+ CD163+	T	Adverse	CSS	No
Buddingh[176]	2011	53	Osteosarcoma	CD163+ HLA-DRα+ CD14+	T	Favorable	OS	Yes
Burt[103]	2011	52	Mesothelioma	CD68+	P	Adverse	OS	Yes
Caillou[177]	2011	27	Anaplastic thyroid carcinoma	CD68+ CD163+	T	-	-	ND
Komohara[178]	2011	79	Grade II-IV	CD68+	T	Adverse	Histological	ND

			glioma	CD163+ CD204+			grade	
Osinsky[179]	2011	105	Gastric cancer	CD68+	T	Adverse	OS	Yes
Algars[180]	2012	159	St. II-IV CRC	CD68+ CLEVER-1/ Stabilin-1+	T,P	Adverse /favorable	DSS	Yes
Azambuja[181]	2012	265	Hodgkin Lymphoma	CD68+ CD163+	T	None	PFS, DSS	No
Hirayama[182]	2012	208	Squamous NSCLC	CD204+	S	Adverse	OS	Yes
Ito[183]	2012	304	St. I NSCLC	CD204+	T	Adverse	RFP	Yes
Kryczek[184]	2012	103	FIGO I-IV Ovarian cancer	B7-H4+/ HAM56+	-	Adverse	OS	Yes
Medrek[185]	2012	144	Breast cancer	CD68+ CD163+	S	Adverse	CSS (CD68), not OS	Yes

Abbreviations: NSCLC=Non-small cell lung cancer; OS=Overall survival; CSS=Cancer specific survival; PFS=Progression-free survival; DSS=Disease-free survival; TTP=Time to progression; SCLC=Small-cell lung cancer; St.=Stage; CIN = Cervical intraepithelial neoplasia; HCC = Hepatocellular carcinoma;

2. Hypotheses and aims

The scope of the present PhD project was to assess the prognostic impact of tumor-associated neutrophils and macrophages in NSCLC and cervical cancer patients. Furthermore, we aimed at examining the prognostic impact of baseline and nadir neutrophils in NSCLC and ovarian cancer patients treated with chemotherapy. Finally, a review of the literature was planned.

The specific aims and hypotheses were:

Hypotheses:

- Increased densities of neutrophils, and macrophages in the tumor microenvironment are risk factors for patients with NSCLC and cervical cancer. Correlations with lymphocytes may add further prognostic information.
- Baseline neutrophil counts are poor prognostic factors in patients with NSCLC and ovarian cancer. Chemotherapy-induced neutropenia is a favourable prognostic factor.

Aims:

- To review research examining the prognostic impact of neutrophils, macrophages, and white blood cells in the blood as well as neutrophils and macrophages in the tumor microenvironment.
- To assess neutrophils and macrophages in the tumor microenvironment as risk factors for poor prognosis in patients with NSCLC and cervical cancer.
- To assess elevated neutrophil count in the blood as a risk factor for short overall survival in patients with NSCLC and ovarian cancer and evaluate if the possible detrimental effect could be intervened using a chemotherapy toxicity-adjusted dosing (CTAD) principle.
- To assess the validity and prognostic impact of digital imaging analysis (DIA) assessments of tumor-associated leukocytes

3. Patients and methods

3.1 Patient material

3.1.1 Cohort for non-small cell lung cancer (NSCLC) study

A total of 375 consecutive non-small cell lung cancer (NSCLC) patients who underwent a microscopically and macroscopically radical lobectomy or pneumonectomy at the Department of Thoracic Surgery, Aarhus University Hospital, Skejby, Denmark between January 2003 and December 2006 were included. Patients with pathological stage I–IIIA NSCLC were included (AJCC TNM 6th edition; squamous cell, adenocarcinoma, mixed adenosquamous, large cell, or undifferentiated NSCLC). A total of 40 patients were excluded either because of missing archive tissue blocks (N=19), tumor blocks with no or scarcely visible tumor (N=16), or because of poor IHC staining quality (N=5), leaving 335 patients for the final analysis (Figure 8). We detected no systematic differences between the 40 patients that were excluded and the 335 patients available for the analysis (Table 2).
Clinical data were collected from patient records as well as pre-operative peripheral blood C-reactive protein (CRP) values and white blood cell counts (WBC).

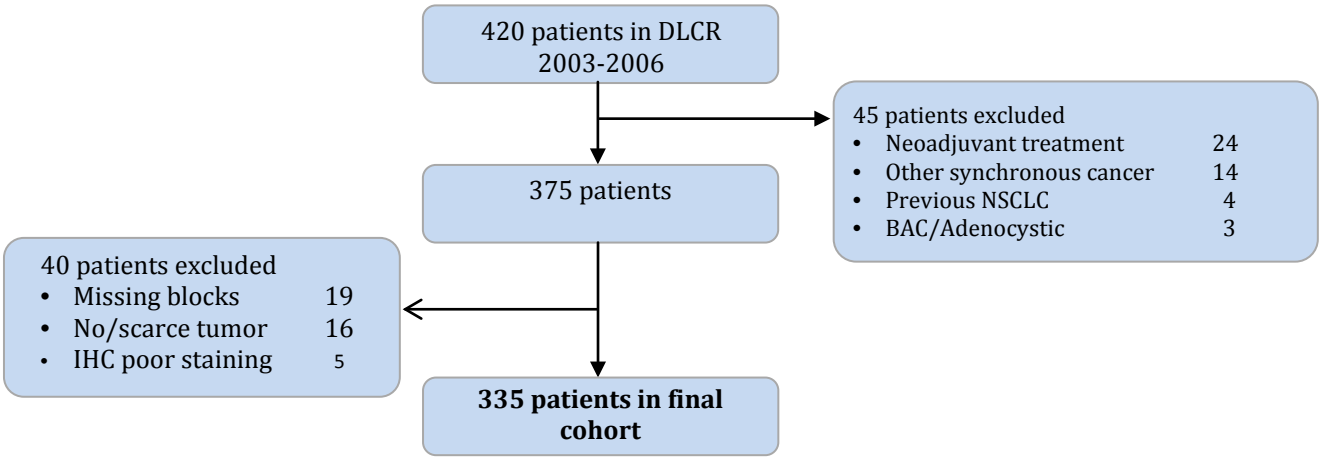

Figure 8. *Cohort definition for NSCLC patients in paper I*

Characteristic	Cohort (N=335)	%	*Excluded (N=40)*	%	*p*
Age					
< 65 years	135	*40*	*11*	*28*	*0.1*
≥ 65 years	200	*59*	*29*	*73*	
Sex					
Female	141	*42*	*17*	*43*	*1.0*
Male	194	*58*	*23*	*58*	
ECOG PS					
0	257	*77*	*30*	*75*	*0.8*
≥1	78	*23*	*10*	*25*	
Stage, pTNM					
IA/IB	86/133	*26/40*	*14/10*	*35/25*	*0.3*
II/IIIa	66/50	*20/15*	*8/8*	*20/20*	
N-stage					
N0	233	*70*	*26*	*65*	*0.8*
N1/N2	54/48	*16/14*	*7/7*	*18/18*	
Histology					
Adenocarcinoma	153	*46*	*14*	*35*	*0.4*
Squamous cell /all other	153/29	*46/8*	*23/3*	*58/8*	

Table 2. *Comparison of the 335 patients included in the NSCLC cohort for paper I and the 40 patients where tumor blocks were not eliaible.*

3.1.2 Cohort for cervical cancer studies

The study included 102 patients treated for FIGO stage IB and IIA cervical squamous cell carcinoma at Aalborg Hospital from 1990 to 2000. This cohort was previously used for assessment of the prognostic impact of tumor infiltrating lymphocytes, as described by Nedergaard et al (PhD dissertation[66]). However, one patient was excluded due to lack of tissue rendering 101 patients for the assessments. If operable, patients underwent hysterectomy with pelvic lymph node dissection and in cases of increased risk of relapse (assessed from depth of invasion, vascular invasion, spread to lymph nodes, invasion of the parametrium, and cancer close to resection margins) radiotherapy was added. Inoperable patients were treated by radiotherapy (both external radiotherapy and brachytherapy), and in such case the primary biopsy was available for analysis.

	N (%)
Number of patients	101
Age at diagnosis	44 [22–70]
Stage IB	91 (90%)
Stage IIA	10 (10%)
Lymph node metastases	
No	83 (82%)
Yes	18 (18%)
Primary treatment	
Surgery	87 (86%)
+ Adjuvant radiotherapy	23
Radiotherapy	14 (14%)

Table 3. Cervical cancer cohort

3.1.3 Cohorts for prognostic impact of chemotherapy-induced neutropenia

Patients with advanced non-small cell lung cancer (stage III-IV), and ovarian cancer (stage I-IV) treated with chemotherapy between 1997 and 2005 were **collected** from patient records at the Department of Medical Oncology at Crown Princess Mary Cancer Center Westmead in Sydney

Australia. Stage was graded according to TNM 2002 or FIGO 1998 where appropriate. Eligibility criteria were a complete medical record of the first 3 cycles of chemotherapy and a full set of baseline and nadir laboratory data. Patients routinely had a nadir blood count measured 10 to 17 days after chemotherapy or as appropriate according to schedule.

3.2 Immunohistochemistry

3.2.1 Immunohistochemical protocols

All antibodies used were obtained from commercial sources. The immunohistochemical stainings were performed at the Department of Pathology, Aarhus University Hospital NBG. FFPE specimens were sectioned at 2 μm. Primary antibodies were against CD66b (clone G10F5, 1:600, no. 555723, BD Biosciences, USA), CD34 (clone QBEND10, 1:400, Beckman Coulter, Immunotech PN IM0786, Prague, Czech Republic), and CD163 (clone EDHu-1, 1:100, MCA 1853, Abd Serotec, UK). Immunohistochemistry was performed using a Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Double stainings with CD66b and CD34 were visualized with the ultraVIEW Universal diaminobenzidine (DAB) detection system (Ventana Medical Systems, AZ, USA) and ultraVIEW Universal Alkaline Phosphatase Red Detection Kit (Fast red) detection system (Ventana Medical Systems, AZ, USA), respectively. Single staining with CD163 was visualized with ultraVIEW Universal Alkaline Phosphatase Red Detection Kit (Fast red) detection system (Ventana Medical Systems, AZ, USA). Positive signals of DAB were amplified using ultraVIEW copper. Sections were counterstained with hematoxylin and bluing reagent. Tonsillar, pancreatic, appendix, and liver sections were included as positive controls.

3.2.2 The immunohistochemical method

Immunohistochemistry was performed with standardized protocols on an automated staining platform using laboratory standard commercially available primary antibodies. This would minimize the run-to-run variance of the stainings. Positive and negative as well as internal control controls were to ensure specificity and quality. However, because of the retrospective design of the studies, tumor blocks from the archives of the pathological departments were used. This means that we could not control the fixation time which was dependent on the workflow in each laboratory and the processing between the surgery and pathology department. Further, NSCLC tumors (paper I) are oftentimes large which can prolong fixation time as well. Thus we cannot exclude some variance in tissue processing which can affect the immunohistochemistry result.

3.2.3 Interpretation of the immunohistochemical reactivity

Ten NSCLC patients with short survival and ten patients with long survival were selected for a pilot study. The primary antibody for CD66b was visualized with FastRed as well as DAB. DAB staining was selected as giving the best contrast. Consequently, for double staining with CD66b/CD34 was performed with DAB for CD66b and CD34 for FastRed for CD34.

Considering staining with $CD163^{+}$ antibody FastRed was selected for visualizing the primary antibody to improve the distinction of positive cells from macrophages with carbon black particles (inhaled from environmental pollution). Since macrophages are irregular in shape, with sometimes numerous, long dendrites, discriminating single cells would in many be cases be difficult as likewise noted by Bronkhorst et al [67] (Figure 14).

3.3 Stereological quantification

Stereological sampling methods allow efficient and unbiased estimation of e.g. numbers and densities of objects, such as immune cell profiles, in tissue sections[68]. Automated unbiased sampling was performed in a manually outlined area (region of interest, ROI) (Figure 10), using a stereological software on digital images of tumor sections. Whole tumor sections were scanned at a resolution of 20X using a whole slide scanner (NanoZoomer 2.0, Hamamatsu) (Figure 9). Digital images were then imported into newCAST for evaluation (Visiopharm, Denmark). A principle of systematic, uniformly random sampling of fields of view (FOV) was applied. The first field of vision was selected randomly by the software within the ROI, and each subsequent FOV was placed with a uniform step length which was calculated to obtain a chosen total number of FOV within the ROI (Figure 11).

Figure 9. *Whole slide scanner, NanoZoomer 2.0*

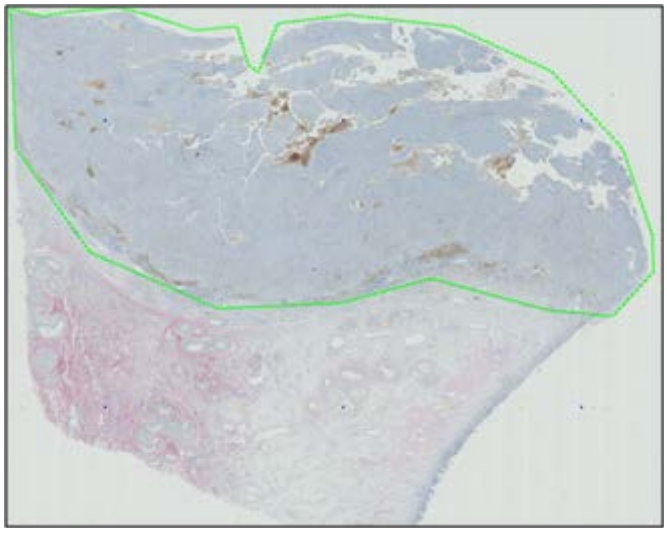

Figure 10. *The area containing tissue was delineated at low magnification as region of interest.*

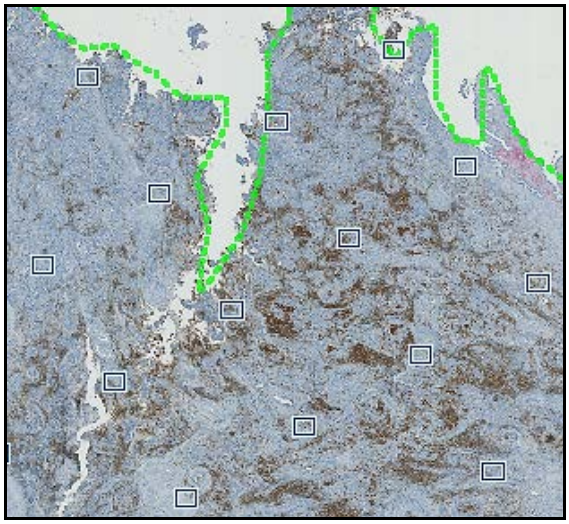

Figure 11. *Fields of vision were sampled in a systematic, random fashion.*

3.3.1 Definition of tumor compartments

We defined three compartments in the tumor microenvironment in the same manner as Nedergaard et al[69] (Figure 12). The tumor nest compartment was defined as within the tumor epithelium (T). An area containing tumor-associated stromal tissue was defined as peritumoral (P) if at least one tumor cell was observed inside the sampling frame of fixed dimensions. Finally, the stromal compartment was defined by containing only stromal cells and no malignant cells; but because the ROI was delineated closely around the area containing tumor tissue at low magnification (Figure 10), the stromal compartment was considered tumor-associated stroma.

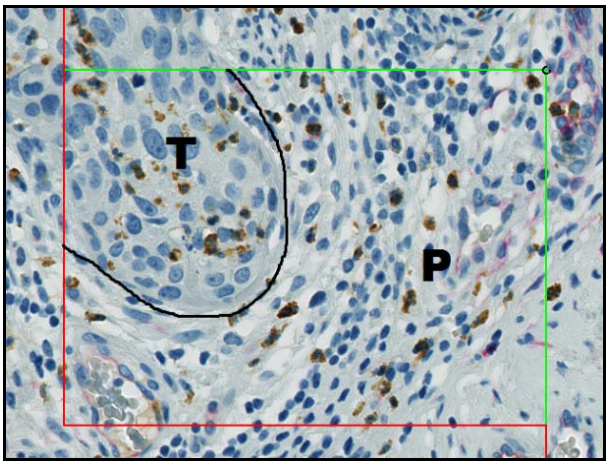

Figure 12. *Quantification by counting frame. Tumor nest and peritumoral compartment is visible All cell profiles inside the frame as well as cell profiles touching the green line with clearly visible nuclei are counted. Cell profiles touching the red line.* ***T*** *marks tumor nest and* ***P*** *marks peritumoral area*

3.3.2 Stereological analysis of CD66b$^+$ neutrophil stainings

In each field of views, sampled in a systematic random fashion, an unbiased counting frame (CF) was applied (Figure 12). Assessments of cells were done at a total magnification of 1556 (40X lens). An average number of 61 and 64 FOVs were sampled in NSCLC and cervical cancer, respectively. Each CF encompassed 40,480 µm^2. For CD66b$^+$ assessment a simple unbiased counting rule was applied (Figure 12). A cell profile staining for CD66b$^+$ (DAB brown) was counted, only if a nucleus (hematoxylin blue) was clearly visible (Figure 13). This principle ensures that the counting is applied in the same way and is in principle observer independent. The section thickness is of importance since more cell profiles will be superimposed in a thick section (Figure 13). In the studies in the present thesis sections were cut at only 2 µm (thin section) using a rotation microtome to ensure uniform thickness of the sections. A simple point count (upper right corner of CF) was used to estimate area fraction of each tumor compartment as defined above. Each counted cell was assigned to a certain compartment. The density of cells in selected compartments was calculated from the number of cells counted in each compartment divided by number of points hitting the specific compartment multiplied with the area of the counting frame in square millimetre.

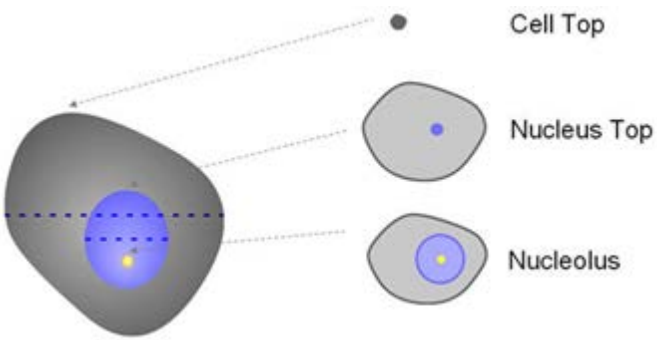

Figure 13. *Different characteristic points that can be selected for unbiased cell counting. We used a visible nucleus as the unique point (www.stereology.info)*

3.3.3 Stereological analysis of CD163+ macrophage stainings

The cell borders of individual macrophages could in some cases not be outlined with sufficient precision in routine sections and macrophages would often be elongated and fragmented (Figure 14). Therefore to quantify macrophages, the area of CD163+ macrophage immunostains relative to the tissue area, i.e. the macrophage area fraction (AF) or density was determined by point counting at a total magnification of 1556 (40X lens). To this end two point grids were applied (Figure 15) in each FOV with 100 points for estimation of the CD163+ macrophage immunostain area and 6 points for estimation of the area of tissue compartments. The area fraction was calculated as the number of points hitting an immunohistochemical signal (Fast red) in each tissue compartment divided by the number of points hitting the specific compartment and multiplied with 6/100. If two blocks from a patient were used for estimation, the final estimate was weighted according to sampling intensity in both blocks. As with other assessments, fields of views were sampled in a systematic random fashion. Each field of view covered an area of 67,477 μm².

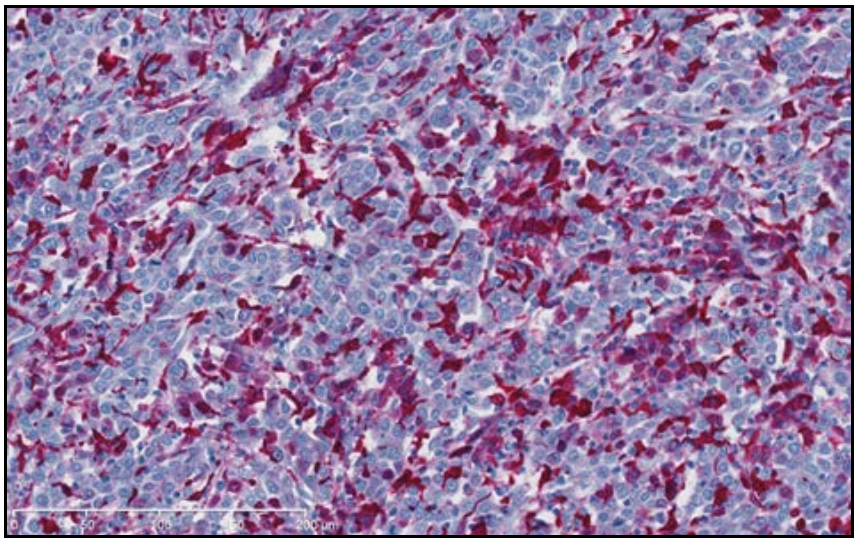

Figure 14. Macrophages would in some cases be elongated and fragmented and therefore hard to define by cell counting.

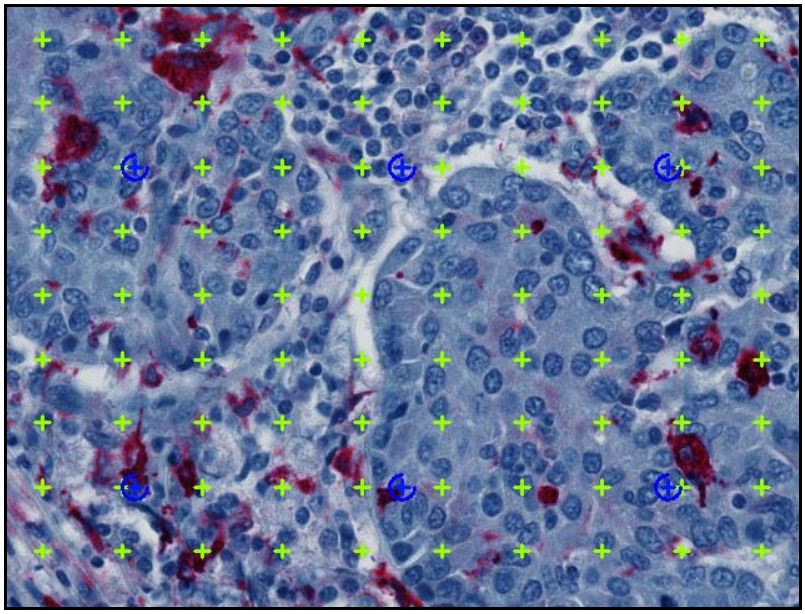

Figure 15. *CD163+ macrophage stain with double point grid. Tumor nest and peritumoral compartment is visible. Each green point hitting the red stain (macrophage) is counted if the stain covers the upper right corner of the cross.*

3.4 Reproducibility of tumor-associated neutrophils and macrophages assessments

For assessment of intraobserver reproducibility NSCLC and cervical cancer assessments were ranked according to density of immune cells and every tenth section was re-assessed. We observed a good correlation for all re-assessments (table 4). Generally, a higher reproducibility was observed for cervical cancer than for NSCLC. We observed no systematic differences between original assessments and re-assessments.

Assessment	Spearman ρ	*P*
Cervical cancer		
Neutrophils		
Tumor nest	0.80	0.001
Peritumoral	0.95	<0.0001
Stromal	0.96	<0.0001
Macrophages		
Tumor nest	0.81	0.004
Peritumoral	0.94	<0.0001
Stromal	0,79	0.01
NSCLC		
Neutrophils		
Tumor nest	0.80	<0.0001
Peritumoral	0.86	<0.0001
Stromal	0.52	0.005
Macrophages		
Tumor nest	0.66	<0.0001
Stromal	0.87	<0.0001

Table 4. *Reproducibility of assessments of CD66b⁺ neutrophils and CD163⁺ macrophages in cervical cancer and NSCLC*

3.5 Automated digital image analysis (DIA)

We applied automated DIA to the cervical IHC cohort as well as the NSCLC IHC cohort. Workflow for automated DIA consist of digitizing whole tissue sections as previously described using a NanoZoomer 2.0 scanner; scanned slides were imported to VisiomorphDP software (Visiopharm, Denmark). The region-of-interest (ROI) was then delineated encompassing the area of tumor tissue and immediate adjacent stroma at low resolution (2X). For DIA of cervical cancer, different automated protocols were developed for DAB CD66b stain, DAB CD8 stain, and FastRed CD163 stain. For the NSCLC cohort, we aimed at exploring the impact of assessing all CD66b DAB stain within the ROI, and thus only a protocol for CD66b DAB was developed. The protocols were devised based on 8-10 representative sections. The protocols encompassed a series of pre-processing steps (enhancement of red-green-blue colour levels and, if relevant, DAB deconvolution which is a computer algorithm to enhance tissue regions), segmentation (Bayesian classifier), and post-processing steps (mainly morphological operations). The software processes the whole ROI in large stepwise "blocks" and allows for processing the whole ROI within short time (2-15 minutes). All sections imported can be processed in batch mode and thus permits unmanned processing of a whole cohort once the ROIs are delineated and the protocol is set.

3.6 Statistical methods

The statistical analyses are described in details for each project in the individual papers.
Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA) statistical software. All tests were two-sided and P values less than .05 were considered statistically significant.

4. Results

A detailed presentation of the results is given in the enclosed manuscripts:

Paper I **Tumor-associated neutrophils and macrophages in non-small cell lung cancer: No immediate impact on patient outcome**
Andreas Carus , Morten Ladekarl, Henrik Hager, Hans Pilegaard, Patricia S Nielsen, Frede Donskov

Lung cancer 2013; **81**(1): 130-7.

Paper II **Tumour-associated CD66b$^+$ neutrophil count is an independent prognostic factor for recurrence in localized cervical cancer**
Andreas Carus, Morten Ladekarl, Henrik Hager, Bettina S Nedergaard, Frede Donskov

British journal of cancer 2013; **108**(10): 2116-22.

Paper III **Impact of baseline and nadir neutrophil index in non-small cell lung cancer and ovarian cancer patients: Assessment of chemotherapy for resolution of chronic inflammation**
Andreas Carus, Howard Gurney, Val Gebski, Paul Harnett, Rina Hui, Richard Kefford, Nicholas Wilcken, Morten Ladekarl, Hans von der Maase, Frede Donskov

Updated manuscript version, submitted 2013

Paper IV **Validity and prognostic value of automatic digital image analysis (DIA) of tumor-associated leukocytes in localized cervical cancer**
Andreas Carus, Frede Donskov, Patricia S. Nielsen, Henrik Hager, Bettina S. Nedergaard, Torben Steiniche, Morten Ladekarl

Updated manuscript version, submitted 2013

Paper I

Andreas Carus, Morten Ladekarl, Henrik Hager, Hans Pilegaard, Patricia S Nielsen, Frede Donskov

Tumor-associated neutrophils and macrophages in non-small cell lung cancer: No immediate impact on patient outcome

Lung cancer 2013; **81**(1): 130-7.

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Tumor-associated neutrophils and macrophages in non-small cell lung cancer: No immediate impact on patient outcome

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ABSTRACT

Introduction: A tumor-promoting impact of neutrophils and macrophages has been demonstrated in some cancers. However, the prognostic significance of innate immune cells in patients with non-small cell lung cancer (NSCLC) is unclear.
Methods: A total of 335 consecutive patients resected for stage I–IIIA NSCLC were assessed for $CD66b^+$ neutrophils and $CD163^+$ macrophages in the tumor nests and adjacent stromal tissue by immunohistochemistry in whole tissue sections using stereology as well as automatic computerized quantification. Findings were correlated with clinical and histopathological parameters, baseline blood inflammatory markers (C-reactive protein (CRP) and white blood cell count (WBC)). Endpoints were recurrence-free survival (RFS) and overall survival (OS).
Results: Elevated CRP above median (101 nmol/l) and WBC above median (8.6×10^9cells/l) were associated with poor RFS ($p \leq 0.002$) and poor OS ($p \leq 0.01$). Higher density of $CD66b^+$ in tumor nests and stroma was associated with elevated CRP and WBC, squamous cell histology, tumor size, and necrosis ($p \leq 0.01$). Higher density of $CD163^+$ macrophages in tumor nests and stroma was associated with elevated CRP and lymph node metastases ($p \leq 0.049$). The densities of tumor nest $CD66b^+$ neutrophils and $CD163^+$ macrophages were not significantly correlated with RFS or OS, irrespective of assessment method.
Conclusions: The densities of tumor-associated $CD66b^+$ neutrophils and $CD163^+$ macrophages in NSCLC were correlated with adverse prognostic factors and systemic blood inflammation markers, but not directly correlated with RFS or OS. Further research of chronic inflammation in NSCLC is warranted.

1. Introduction

Lung cancer is the most common cause of cancer-related deaths in the world with a low survival even in resectable cases [1]. Tumor inflammation has emerged as a hallmark of neoplasia [2] and a multitude of studies have demonstrated a negative effect of systemic inflammatory markers [3]. In surgically managed non-small cell lung cancer (NSCLC) elevated white blood cell count has been correlated with poor survival for patients with resectable disease [4,5]. Furthermore elevated differential counts of blood neutrophils and neutrophil to lymphocyte ratio [6,7] and other systemic inflammation markers such as C-reactive protein [8,9] have been correlated with impaired outcome. Collectively these results point to a detrimental impact of inflammation in resectable NSCLC.

However, data on the role of immune cells in the tumor microenvironment are limited. Tumor associated neutrophils (TANs) are involved in tumor initiation, angiogenesis, invasion, progression, and dissemination of cancer [10–16]. TANs have been correlated with poor patient outcome in a range of cancers [17], including glioma [18], renal cell cancer [19,20], malignant melanoma [21], head-and-neck cancer [22], gastric carcinoma [23], hepatocellular cancer [24], colon cancer [25] and cervical cancer [26]. In NSCLC the information on TANs is scarce with only a single study demonstrating impact of intratumoral neutrophil to lymphocyte ratio on patient outcome [27]. Tumor-associated macrophages (TAMs) [28,29] have been shown to possess a number of cancer-promoting features [30,31], and have been associated with accelerated lymphangiogenesis [32] and lymph node

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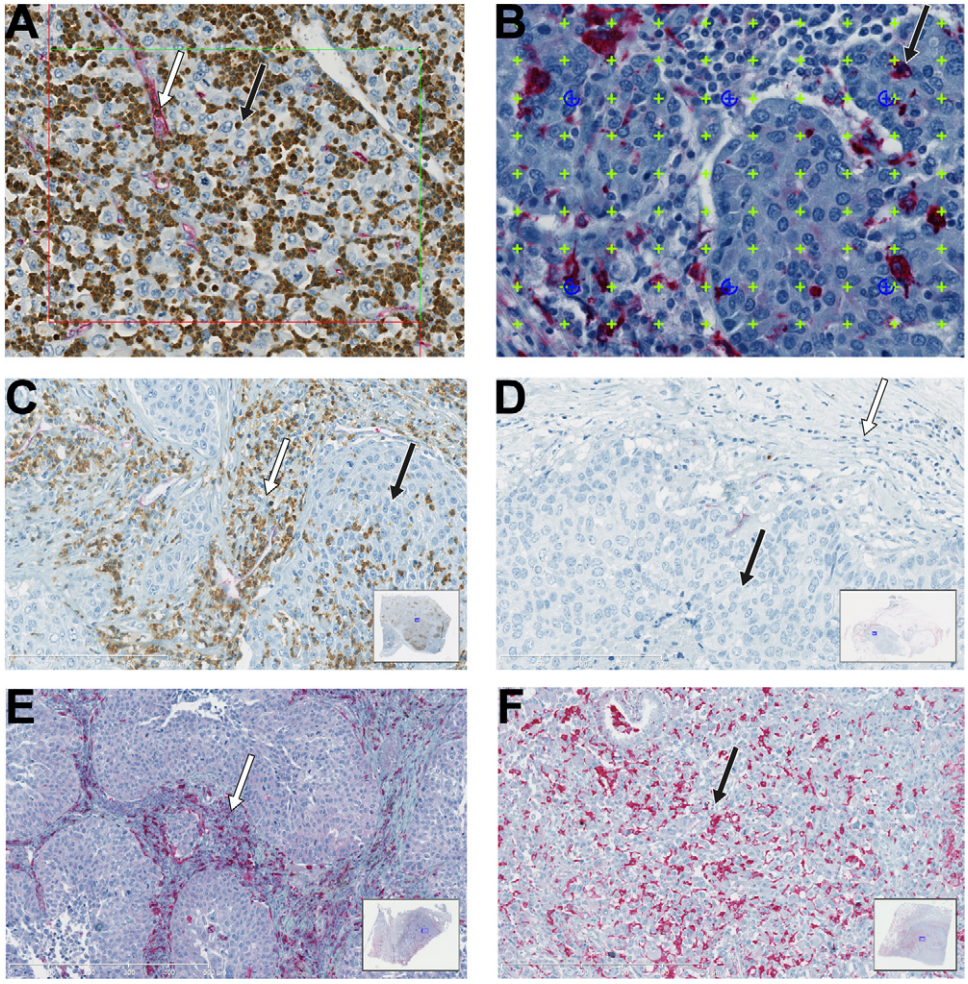

Fig. 1. (A) Representative example of CD66b$^+$/CD34$^+$ staining (40×) with tumor nest neutrophil (black arrow) and intravascular neutrophil (white arrow). Unbiased counting frame is applied. (B) CD163$^+$ immunostaining (40×) illustrating tumor nest macrophage (black arrow). Counting grid is applied. (C) Squamous cell carcinoma (20×) with high density of CD66b$^+$neutrophils in stroma (white arrow) and tumor nests (black arrow). (D) Adenocarcinoma (20×) with no infiltration of CD66b$^+$ neutrophils in stroma (white arrow) or tumor nests (black arrow). (E) Squamous cell carcinoma (10×) with high density of CD163$^+$ macrophages in stroma (white arrow) and little infiltration in tumor nests. (F) Adenocarcinoma (10×) with high density of CD163$^+$ macrophages in tumor nests (black arrow).

metastasing [33,34]. Consequently, TAMs have been correlated with short survival in a large range of cancers [35]; nonetheless, in NSCLC the role of TAMs is conflicting [36–40].

Neutrophils and macrophages have significant collaboration and crosstalk in the normal function of the immune system [41]. In the tumor setting TAMs and TANs exhibit considerable plasticity where impairing macrophage recruitment induces a compensatory neutrophil influx and allows for sustained paracrine induction of angiogenesis and progression [42]. Thus the copresence of TAMs and TANs or the relative balance between them may impact tumor promotion.

CD66b is a marker of neutrophil activation and has been associated with poor patient outcome in some cancers [19,21,26,27] and may be a marker of tumor-promoting neutrophils and possible N2 neutrophil polarization [10]. CD163 is specific for pro-inflammatory macrophages and is highly expressed on M2-polarized macrophages [43,44]. CD163$^+$ macrophages have been associated with poor patient outcome in a range of cancers [45–51].

In the present study we hypothesized that tumor-associated CD163$^+$ macrophages and tumor-associated CD66b$^+$ neutrophils would negatively impact patient outcome for NSCLC patients with primary resectable disease. Furthermore, we hypothesized that systemic inflammatory markers in the blood could be indicative of infiltration of TAMs or TANs in the tumor microenvironment.

2. Patients and methods

A total of 375 consecutive patients with non-small cell lung cancer (NSCLC) underwent a microscopically and macroscopically radical lobectomy or pneumonectomy at the Department of Thoracic Surgery, Aarhus University Hospital, Skejby, Denmark between January 2003 and December 2006. Patients with pathological stage I–IIIA NSCLC were included (AJCC TNM 6th edition; squamous cell, adenocarcinoma, mixed adenosquamous, large cell, or undifferentiated NSCLC). Patients who were offered neoadjuvant treatment, patients with previous lung cancer, or other synchronous cancers were excluded. Clinical data and blood test were collected from patient records. Formalin-fixed paraffin-embedded tumor (FFPE) blocks used for routine pathologic evaluation were used. A total of 40 patients were excluded either because of missing archive tissue blocks ($N = 19$), tumor blocks with no or scarcely visible tumor ($N = 16$), or because of poor IHC staining quality ($N = 5$), leaving 335 patients for the final analysis.

We aimed to analyze two sections from separate tissue blocks of each tumor. However, in 34 (10%) only one section was available.

The study was approved by the local Ethics Committee.

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Table 1
Clinical, pathologic and baseline blood values predicting RFS and OS in univariate analysis ($N = 335$).

Characteristic	No.	RFS HR (95% CI)	*p*	OS HR (95% CI)	*p*
Age			*0.6*		*0.01*
<65 years	135	1		1	
≥65 years	200	1.1 (0.8–1.6)		1.5 (1.1–2.0)	
Sex			*0.005*		*<0.0001*
Female	141	1		1	
Male	194	1.7 (1.2–2.4)		1.8 (1.3–2.5)	
ECOG performance status			*0.7*		*0.09*
0	257	1		1	
≥1	78	1.1 (0.7–1.6)		1.3 (1.0–1.9)	
Stage, pTNM			*<0.0001*		*<0.0001*
IA/IB	86/133	1		1	
II/IIIa	66/50	3.0 (2.2–4.3)		2.8 (2.1–3.7)	
N-stage			*<0.0001*		*<0.0001*
N0	233	1		1	
N1/N2	54/48	3.3 (2.4–4.6)		2.9 (2.2–3.9)	
Histology			*0.03*		*0.08*
Adenocarcinoma	153	1		1	
Squamous cell carcinoma/all other	153/29	1.5 (1.0–2.1)		1.3 (1.0–1.8)	
Smoking			*0.1*		*0.5*
Current	146	1			
Former	175	1.3 (0.9–1.8)		1.0 (0.7–1.4)	
Never	14	0.5 (0.2–1.7)		0.6 (0.2–1.5)	

2.1. Immunohistochemistry (IHC)

FFPE specimens were sectioned at 2 μm and mounted on glass slides. Primary antibodies were against CD66b (clone G10F5, diluted 1:600, no. 555723, BD Biosciences, USA), CD34 (clone QBEND10, diluted 1:400, Beckman Coulter, Immunotech PN IM0786, Prague, Czech Republic), and CD163 (clone EDHu-1, diluted 1:100, MCA 1853, Abd Serotec, UK). Immunohistochemistry was performed using a Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Deparaffinization, epitope retrieval, and immunostaining were performed according to the instructions in the pathological department by using cell-conditioning solutions. Double stainings with CD66b and CD34 were visualized with the ultraVIEW Universal diaminobenzidine (DAB) detection system (Ventana Medical Systems, AZ, USA) and ultraVIEW Universal Alkaline Phosphatase Red Detection Kit (Fast red) detection system (Ventana Medical Systems, AZ, USA), respectively. Single staining with CD163 was visualized with ultraVIEW Universal Alkaline Phosphatase Red Detection Kit (Fast red) detection system (Ventana Medical Systems, AZ, USA). Positive signals were amplified using ultraVIEW copper, and sections were counterstained with hematoxylin and bluing reagent. Tonsillar, pancreatic, appendix, and liver sections were included as positive controls.

2.2. Quantitative evaluation of immunostaining

Double staining with CD66b and CD34 allowed distinguishing between intra- and extravascular neutrophil localization as previously described [19,21]. Neutrophils were further classified according to tumor compartment as located in tumor nests, peritumoral (i.e. in the stroma at the migrating border of tumor nests), or stromal as previously described by Nedergaard et al. [52]. CD163-positive macrophages were classified as localized either in tumor nests or in the stroma. CD163 has been suggested to be expressed on blood monocytes [53], but evaluation of whole tissue sections revealed none or very weak staining of CD163 of the scarce population of monocytes within vessels in the cancer tissue. Furthermore, vessel monocytes were easily distinguishable by shape and size from tissue macrophages. Thus double staining with CD34 for macrophage evaluation was considered redundant. The evaluation was done using computer-assisted sampling (newCAST software, Visiopharm, Denmark) applying statistically unbiased stereological sampling and counting techniques [54,55]. Whole tissue sections were scanned at a maximum resolution of 20× using a whole slide scanner (NanoZoomer 2.0, Hamamatsu, Japan). Digital images were then imported into newCAST. The total tumor area visible at low resolution was outlined as region of interest and the tumor nest and stromal compartments were defined at high resolution by a senior pathologist. The quantitative histological analysis was done by one observer blinded to all clinical features. The first counting frame was sampled by the software at random for estimation of the density of $CD66b^+$ neutrophils (Fig. 1A) and subsequently a mean of 61 counting frames (each 40,480 μm^2) were sampled in the total tumor area in a systematic fashion [54] at a magnification of 1556 (40× lens). A mean of 3.3% (range 0.7–52%) of the total outlined area was sampled. Individual neutrophils were counted when a neutrophil nucleus was clearly visible along with a surrounding positive IHC signal. Clearly confluent necrotic areas were often heavily positively stained with CD66b, and surrounded by many viable neutrophils in the adjacent tumor epithelium. As necrotic areas impacted the estimates, we performed three separate estimations of $CD66b^+$ neutrophils; the first was performed excluding areas of necrosis and peri-necrotic areas; the second with inclusion of peri-necrotic areas, but ignoring obvious necrotic areas without viable cells; and the third with an automated digital image analysis quantification method including the entire tumor and necrotic areas using a fixed protocol in the Visiomorph DP module in VIS (Visiopharm, Denmark).

For the assessment of $CD163^+$ macrophages (Fig. 1B), point-counting was applied to estimate the area fraction of $CD163^+$ immunostains per viable tumor area [55] at a magnification of 1556 (40× lens). In concordance with a method applied by Kamper et al. [56], point counting of immunostains was chosen as the cell borders of individual macrophages could not be outlined with sufficient precision. A mean of 21 fields of visions were assessed per tumor. Only a clear stain of CD163 was counted as a positive point to avoid assessment of any weak background staining. Areas of necrosis or artifacts were ignored.

The degree of necrosis in each section was scored semi-quantitatively as either none (0%), light (<25%), moderate (<50%), or massive (≥50%).

2.3. Heterogeneity and reproducibility

Heterogeneity between paired sections from the same patient was modest, as a significant concordance was observed for

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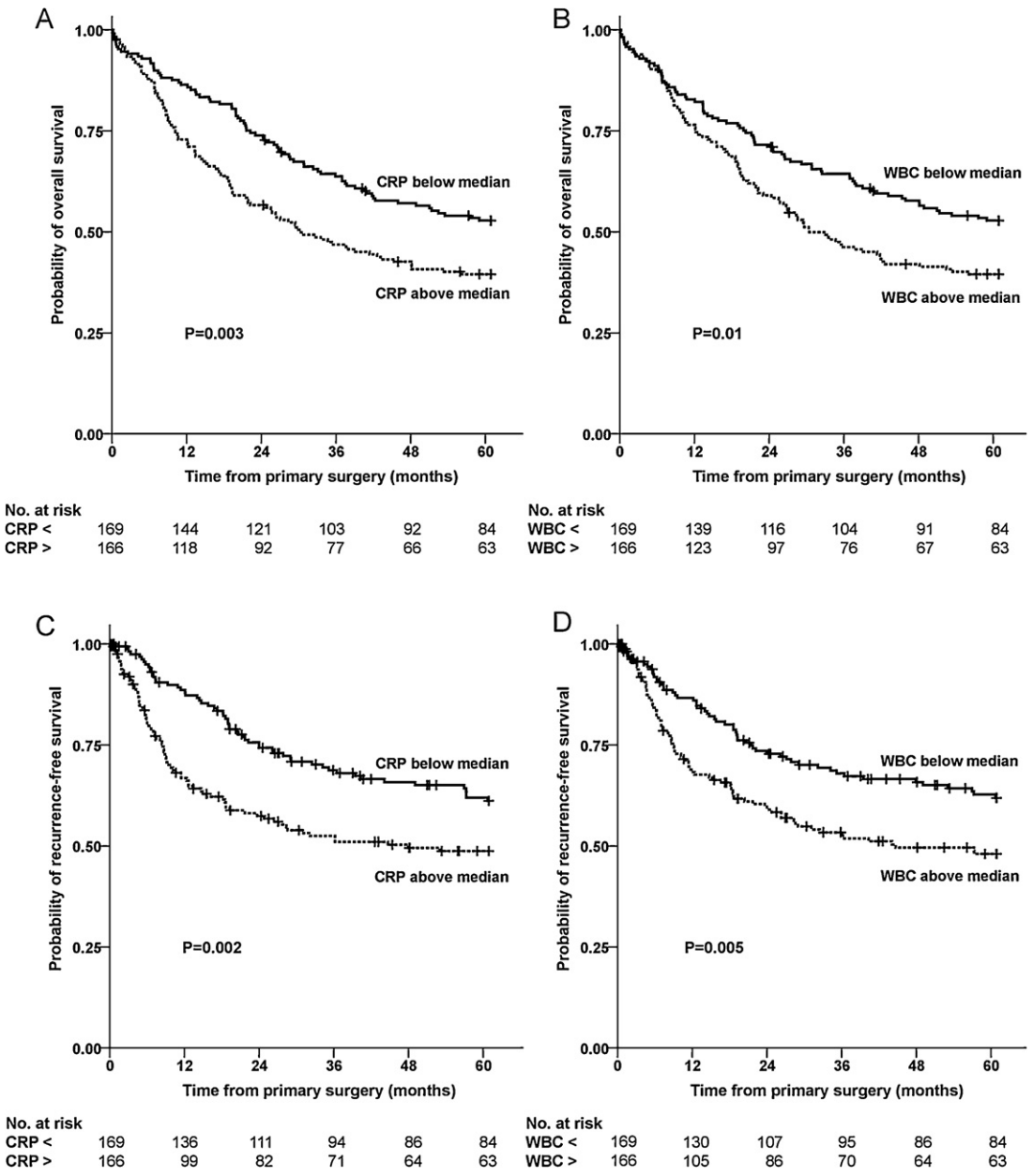

Fig. 2. CRP and WBC as prognostic factors. High levels of CRP were correlated with short overall survival (A) and short recurrence-free survival (C). High levels of WBC were correlated with short overall survival (B) and short recurrence-free survival (D).

peritumoral neutrophil count (paired samples *T*-test correlation = 0.6; $p = 0.0001$) with no significant differences between samples ($p = 0.7$). Likewise, for peritumoral macrophages we observed a paired samples *T*-test correlation of 0.7 with no significant differences between samples ($p = 0.24$). For testing the intraobserver reproducibility $CD66b^+$ and $CD163^+$ sections were ranked according to their density of immune cells and every eleventh case ($N = 32$) was reassessed. An adequate reproducibility was observed for both neutrophils (Spearman $\rho = 0.81$; $p = 0.0001$) and macrophages (Spearman $\rho = 0.75$; $p = 0.0001$).

3. Statistics

Maximum follow-up was set to 5 years. Cox's proportional hazards regression modeling was applied to estimate the hazard rates for each parameter in a univariate analysis. The relationship between survival and each parameter was estimated by the Kaplan–Meier method. Assessments of tumor-associated immune cells did not follow a Gaussian distribution and non-parametric test were performed. Subgroups of patients with respect to density of tumor-associated immune cells were compared with either the Wilcoxon rank-sum (Mann–Whitney *U*) test, or the Kruskal–Wallis equality-of-populations rank test. Correlation between paired sections was tested with paired samples *T*-test.

All tests were two-sided and *p* values less than .05 were considered statistically significant. Survival data were updated on May 2012. Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA) statistical software.

4. Results

4.1. Patient characteristics

Characteristics of the 335 surgically resected NSCLC patients are listed in Table 1. Overall 5-year recurrence-free survival rate (RFS) were 55% (95% CI, 49–60%), and for stage IA, IB, II, IIIA, 79%, 59%, 39%,

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Table 2
Median densities of tumor-associated CD66b⁺ neutrophils and CD163⁺ macrophages.

Characteristic	Neutrophils, tumor nests (cells/mm²)	Neutrophils, stroma (cells/mm²)	Neutrophils, peritumoral (cells/mm²)	Macrophages, tumor nests % area fraction	Macrophages, stroma % area fraction
CRP	*	***	*	*	*
<median	5.6	15.9	16.5	0.18	1.9
>median	11.8	29.9	25.0	0.24	2.5
WBC	*	***	*	ns	ns
<median	5.6	16.9	16.8	0.20	2.0
>median	10.5	28.8	24.4	0.24	2.7
CRP+WBC	**	***	*	ns	ns
Not elevated	4.7	13.1	16.0	0.19	1.9
One elevated	8.3	20.9	18.7	0.18	2.1
Both elevated	12.4	33.1	25.7	0.27	2.9
Necrosis	***	***	***	***	***
None	2.1	8.9	11.1	0.11	1.1
Little	8.2	15.8	17.4	0.36	2.6
Moderate	15.8	27.4	27.8	0.27	2.9
Massive	17.2	31.6	24.9	0.16	2.0
T-stage	*	ns	ns	ns	ns
T1	4.8	18.3	17.6	0.17	2.1
T2/T3	33.6	34.4	30.0	0.03	1.5
N-stage	ns	ns	ns	*	**
N0	8.9	20.6	20.3	0.16	1.9
N1/N2	8.5	24.3	23.1	0.34	2.7
Histology	*	**	ns	ns	***
Adeno	6.2	15.8	16.3	0.25	1.6
Squamous or other	10.0	25.1	24.7	0.18	2.7
Age	*	ns	Ns	ns	ns
<65	12.4	23.6	23.6	0.25	2.0
≥65	7.1	20.4	20.8	0.18	2.4
Smoking	ns	ns	ns	ns	ns
Never	3.7	21.4	22.5	0.07	1.2
Former	6.4	20.6	23.5	0.17	2.1
Current	11.8	21.8	19.2	0.31	2.5

* $p < 0.05$.
** $p < 0.001$.
*** $p < 0.0001$.

and 19%, respectively. Correspondingly, 5-year overall survival (OS) rate was 46% (95% CI, 41–51%), and for stage IA, IB, II, IIIA, 67%, 53%, 30%, and 14%, respectively.

4.2. Clinical variables and correlation with patient outcome

Factors associated with short RFS were sex, stage, lymph node metastasis, histology, CRP above median (101 nmol/l), and white blood cell count (WBC) above median (8.6×10^9cells/l), $p \leq 0.03$ (Table 1). Features associated with short OS were age, sex, stage, lymph node metastasis, histology, elevated CRP, and elevated WBC, all $p \leq 0.04$ whereas smoking status at time of operation was not significant The association between CRP and WBC, and outcome is shown in Fig. 2.

4.3. Distribution of tumor-associated neutrophils and macrophages

CD66b⁺ neutrophils were distributed in all tumor compartments with the highest density in the stromal compartments. Median density of neutrophils localized in the tumor nests was 8.7 cells/mm² tumor area (range 0–2216). Median density of cells in the stroma was 21.0 cells/mm² stroma (range 0–830). Intravascular CD66b⁺ neutrophils in the tumor nests were generally scarce with a mean density of 1.4 cells/mm² (median 0 cells/mm²; range 0–62). Neutrophils in the tumor nests were not observed in 34 patients (10%).

The median area fraction of macrophages in the tumor nests was 0.21% (range 0–11.7%) and in the stroma 2.19% (range 0–19.0%). Macrophages in the tumor nests were not found in 79 patients (24%). A weak association between the density of neutrophils and the area fraction of macrophages was observed (Spearman $\rho = 0.19$; $p < 0.0001$).

4.4. Tumor-associated neutrophils and macrophages correlated with prognostic factors

The number of neutrophils in tumor nest, peritumorally, and in the stroma, as well as the density of macrophages in tumor nest and stroma correlated with the degree of necrosis in a section (Table 2). There was an increase in median infiltration of neutrophils and macrophages in all compartments from sections with no necrosis to sections with heavy necrosis ($p < 0.0001$). Tumor nest neutrophil density was significantly higher with increasing T-stage ($p = 0.025$). Tumor nest macrophage, and stromal macrophage density was significantly higher in patients with metastases to regional lymph nodes ($p \leq 0.05$). Tumor nest and stromal neutrophil densities, as well as stromal macrophages density was significantly lower in patients with adenocarcinoma as compared to squamous cell histology. Additionally, the density of tumor nest neutrophils was significantly lower in patients above 65 years. We observed no significant correlation between smoking history (current, former, or never smoker) and neutrophil or macrophage infiltration (Table 2).

The subgroup of patients with elevated CRP above median had a statistically significant higher density of neutrophils, as well as macrophages in all compartments compared with patients with CRP below median (Table 2). The subgroup of patients with elevated blood WBC above the median value had a statistically significant higher number of neutrophils in all compartments compared with patients with WBC below the median, whereas tumor nest and stromal macrophages were not significantly different.

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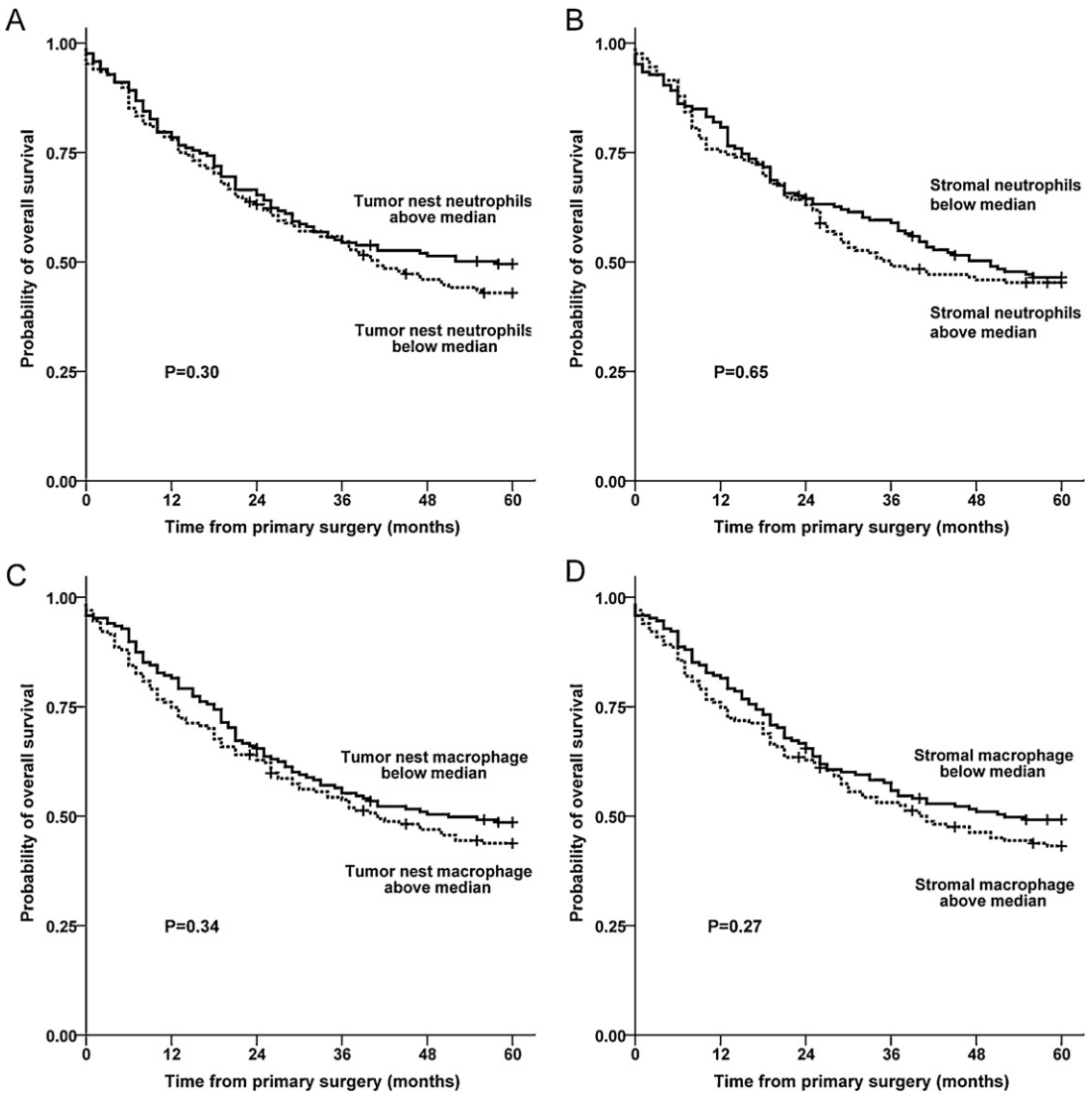

Fig. 3. No detectable prognostic impact of tumor-associated CD66b$^+$ neutrophils and CD163$^+$ macrophages stratified at median values. Levels of tumor nest (A) and stromal (B) neutrophils correlated with overall survival. Levels of tumor nest (C) and stromal (D) macrophages correlated with overall survival.

4.5. Tumor-associated neutrophils and macrophages and relation to patient outcome

No significant prognostic impact of tumor nest, stromal, or intravascular CD66b$^+$ neutrophils was associated with RFS or OS ($p \geq 0.25$) (Fig. 3). This finding was irrespective of whether areas of necrosis were included or excluded from region of interest for sampling. Using an automated digital image analysis we likewise observed no significant impact of CD66b$^+$ staining cells on patient outcome.

Similarly, the degree of infiltration of tumor nest and stromal CD163$^+$ macrophages were not correlated with RFS or OS ($p \geq 0.27$) (Fig. 3). Moreover, restricting analyses to patient subgroups (i.e. T-stage, CRP-level, and WBC-level) did not impact RFS or OS. Subgroup analyses of patients with squamous cell carcinoma or adenocarcinoma likewise revealed no significant impact of CD66b$^+$ neutrophils or CD163$^+$ macrophages on RFS or OS (all $p > 0.18$).

5. Discussion

To the best of our knowledge this is the first study in primary resected NSCLC to assess the collective role of two key tumor-promoting innate immune cells, CD66b$^+$ neutrophils and CD163$^+$ macrophages, in the tumor microenvironment. We demonstrated increased densities of tumor-associated CD66b$^+$ neutrophils and CD163$^+$ macrophages were correlated with other adverse prognostic factors, but not directly correlated with RFS or OS in NSCLC.

We observed a prominent heterogeneity of infiltration of CD66b$^+$ neutrophils as well as CD163$^+$ macrophages with some tumors without infiltration, and others with massive infiltration; this finding for neutrophil infiltration in NSCLC has also been reported by Fridlender et al. [57]. Interestingly, the density of CD66b$^+$ neutrophils was clearly correlated to the extent of necrosis in a section. Necrosis in itself has been shown to correlate with poor outcome in resected NSCLC [58,59]. One explanation for the lack of statistically significant association with RFS and OS in our study could be due to our efforts to avoid the large clearly necrotic areas seen in many sections, thereby possibly underestimating the true number of infiltrating neutrophils and macrophages in the tissue. We selected the whole tissue sections that contained as little necrosis as possible, but despite our efforts many sections still contained areas of necrosis and these necrotic areas would stain heavily for CD66b. However, estimating neutrophils located within necrotic areas is technically challenging. We therefore applied an automated quantification method of computerized digital analysis to estimate CD66b$^+$ staining in all compartments also including necrotic

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areas attempting to avoid underestimating neutrophil quantification. Still, we found no impact of CD66b$^+$ staining cells on patient outcome. When restricting analyses to subgroups (i.e. stage and histology) we were still unable to detect any statistically significant prognostic impact of CD66b$^+$ neutrophils or CD163$^+$ macrophages. We saw no significant correlation between smoking status and density of neutrophils or macrophages, although there was a trend toward lower densities in never smokers.

We observed that elevated blood WBC and CRP were strongly associated with a poor outcome in NSCLC as shown previously [60]. Both parameters correlated with an increased density of CD66b$^+$ in the tumor, and elevated CRP also correlated with increased intratumoral CD163$^+$ density. Tumor-related leukocytosis has been associated with increased levels of G-CSF, GM-CSF, and IL-6 [60]. G-CSF is implicated in extravasation of neutrophils, and GM-CSF has been shown to be a chemoattractant for neutrophils, whereas IL-6 among many other effects can induce tumor promoting properties of neutrophils [57].

To the best of our knowledge only one previous study in NSCLC has explored the significance of tumor nest neutrophils on patient outcome [27]. The authors applied semiautomatic quantification of CD66b$^+$ cell densities in tissue-micro array (TMA) cores and observed an association among incidence of late relapse and elevated tumor nest neutrophil density, however, no statistically significant association was observed with overall survival. This is in line with our findings of no significant association between tumor nest neutrophils in various compartments and OS or RFS.

Assessments of the role of TAMs in NSCLC are hampered by the use of various macrophage markers, varying techniques, and the lack of understanding of the spectrum of polarization between M1 and M2 macrophages, or other subtypes. CD163$^+$ macrophages in NSCLC have been assessed by two research groups. Ohri et al. demonstrated tumor nest CD163$^+$ in tumor nests to be correlated with decreased survival [40], whereas Ma et al. was unable to demonstrate an association between tumor nest CD163$^+$ macrophages and survival [39]. Our results are in agreement with Ma et al.

We demonstrated that the density of TAMs in tumor nests as well as stroma was statistically significant elevated in patients with regional lymph node metastases compared with patients without. TAMs have shown to be related to accelerated lymphangiogenesis in NSCLC which is perceived as a necessary step for lymph node metastasing [61], a strong predictor of recurrence, distant metastases, and overall survival in NSCLC [62]. Limitations to our study are the retrospective design, the non-random selection of tumor blocks, and the heterogeneity of the tumors.

In conclusion, increased densities of tumor-associated CD66b$^+$ neutrophils and CD163$^+$ macrophages were correlated with adverse prognostic factors, but not directly correlated with RFS or OS in NSCLC. Our data suggest that tumor-associated macrophages can facilitate regional lymph node spread. Further research of chronic inflammation in NSCLC is warranted.

Conflicts of interest

None declared.

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Paper II

A Carus, M Ladekarl, H Hager, B S Nedergaard, F Donskov

Tumour-associated CD66b+ neutrophil count is an independent prognostic factor for recurrence in localised cervical cancer

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Keywords: tumour-associated neutrophils; tumour-associated macrophages; stereology; cancer inflammation; cervix carcinoma

Tumour-associated CD66b$^+$ neutrophil count is an independent prognostic factor for recurrence in localised cervical cancer

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Background: The prognostic impact of tumour-promoting immune cells in cervical cancer is unclear.

Methods: Federation of Gynaecology and Obstetrics (FIGO) stage IB and IIA cervical cancer patients ($N = 101$) were assessed for tumour-associated CD66b$^+$ neutrophils and CD163$^+$ macrophages by immunohistochemistry in whole tissue sections using stereology. Results were correlated with previous results on tumour-infiltrating CD3$^+$, CD4$^+$, and CD8$^+$ lymphocytes in the same cohort with recurrence-free survival (RFS) as end point.

Results: The highest densities of CD66b$^+$ neutrophils and CD163$^+$ macrophages were observed in the peritumoural compartment (median 53.1 cells mm^{-2} and 1.3% area fraction, respectively). Above median peritumoural and stromal CD66b$^+$ neutrophils and peritumoural CD163$^+$ macrophages were significantly associated with short RFS. Multivariate analysis identified high peritumoural neutrophils (HR 2.27; 95% CI 1.09–4.75; $P = 0.03$), low peritumoural CD8$^+$ lymphocytes (HR 3.67; 95% CI 1.63–8.25; $P = 0.002$), and lymph node metastases (HR 2.70; 95% CI 1.26–5.76; $P = 0.01$) as independent prognostic factors for short RFS, whereas CD163$^+$ macrophages were not significant. An index of combined intratumoral and peritumoral CD66b$^+$ neutrophils to CD8$^+$ lymphocytes had good discriminatory power for each quartile with 5-year RFS of 92%, 80%, 62%, and 44% ($P = 0.001$).

Conclusion: Tumour-associated neutrophil count is an independent prognostic factor for short RFS in localised cervical cancer. Combining CD66b and CD8 may further improve prognostic stratification. These findings require prospective validation.

Squamous cell cervical cancer is a frequent cancer worldwide (Bray *et al*, 2013). The favourable prognostic impact of lymphocyte infiltration is well documented (Rahir and Moser, 2012), however, the role of tumour-promoting immune cells is largely unknown. Cancer inflammation has emerged as an enabling characteristic for the hallmarks of cancer, and the detrimental effect of tumour-associated innate immune cells has been suggested in a number of cancer types (Mantovani, 2009; Hanahan and Weinberg, 2011), although conflicting results have been published (Souto *et al*, 2011). In cervical cancer, studies have demonstrated a negative prognostic effect of elevated blood neutrophil and monocyte counts have been demonstrated (Cho and Kim, 2009; Lee *et al*, 2012b). In addition, blood neutrophil-to-lymphocyte ratio (NLR) has been identified as a poor prognostic factor (Lee *et al*, 2012a). Suppression of lymphocyte function has been documented for neutrophils (Schmielau and Finn, 2001) and tumour-associated macrophages (TAMs) (Lepique *et al*, 2009). Matrix metalloproteinase-9 secreted by TAMs, and in particular tumour-associated neutrophils (TANs), has been correlated with poor survival (Ardi *et al*, 2009). Tumour-associated macrophages have been correlated with accelerated lymphangiogenesis (Schoppmann *et al*, 2002; Utrera-Barillas *et al*, 2010), which is perceived as a necessary step

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for lymph node metastasing. Collectively, these data from cervical cancer indicate a potential harmful role of TANs and TAMs.

Our institution has previously reported poor prognosis for patients with the presence of intratumoural CD66b$^+$ neutrophils in primary (Jensen *et al*, 2009a) and metastatic renal cell carcinoma (Donskov *et al*, 2006), as well as poor prognosis for patients with primary melanoma with the presence of intratumoural neutrophils (Jensen *et al*, 2012) and CD163$^+$ macrophages (Jensen *et al*, 2009b). In the present study, we assessed CD66b$^+$ neutrophils and CD163$^+$ macrophages in the tumour nests and adjacent stromal tissue in patients with localised cervical cancer. We correlated the assessments with previous findings of tumour-associated CD4$^+$ and CD8$^+$ lymphocyte counts with recurrence-free survival (RFS) as end point.

MATERIAL AND METHODS

The study included 102 patients treated with surgery or radiotherapy for cervical squamous cell carcinoma for the International Federation of Gynaecology and Obstetrics (FIGO) stage IB and IIA (Benedet *et al*, 2000), at Aalborg Hospital from 1990 to 2000 (Table 1). Retrospective clinical sub-classification in stage IB1 and IB2 was not possible. All patients had squamous cell carcinoma. The cohort is identical to the cohort published by Nedergaard *et al* (2007a); however, one patient was excluded due to the lack of tissue rendering 101 patients for the assessments. Tumour samples were collected from the tumour tissue blocks used for routine pathologic evaluation. We aimed at analysing two sections from separate tissue blocks of each tumour. A previous study in renal cell cancer has shown that analysis of two sections is superior to analysis of a single section, as it significantly decreases the coefficient of variance when taking the variance between tumours and the variation between sections into account (Jensen, Donskov *et al*, unpublished data). However, in 16 patients (16%) only one section was available. The study was approved by the local Ethics Committee.

Immunohistochemistry. Formalin-fixed paraffin-embedded tumour specimens were sectioned at 2 μm and mounted on glass slides. Primary antibodies were against CD66b (clone G10F5, 1 : 600, no. 555723, BD Biosciences, San Jose, CA, USA), CD34 (clone QBEND10, 1 : 400, Beckman Coulter, Immunotech PN IM0786, Prague, Czech Republic), and CD163 (clone EDHu-1, 1 : 100, MCA 1853, Abd Serotec, Oxford, UK). Immunohistochemistry was performed using a Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Deparaffinisation, epitope retrieval, and immunostaining were performed according to the instructions of the pathological department by using cell conditioning solutions. Double stainings with CD66b and CD34 were visualised with the ultraVIEW Universal diaminobenzidine detection system (Ventana Medical Systems) and ultraVIEW Universal Alkaline Phosphatase Red Detection Kit (Fast red) detection system (Ventana Medical Systems), respectively. Single staining with CD163 was visualised with ultraVIEW Universal Alkaline Phosphatase Red Detection Kit (Fast red) detection system (Ventana Medical Systems). Positive signals were amplified using ultraVIEW copper, and sections were counterstained with haematoxylin and bluing reagent.

The stainings for CD3$^+$, CD4$^+$, and CD8$^+$ lymphocytes have been described in detail previously (Nedergaard *et al*, 2007a).

Table 1. Patient characteristics ($N = 101$)

	Without recurrence	Recurrence
Number of patients	70 (69%)	31 (31%)
Age at diagnosis (range), years	45 (26–68)	41 (22–70)
Stage IB	63 (90%)	28 (91%)
Stage IIA	7 (10%)	3 (9%)
Lymph node metastases		
No	62 (89%)	21 (68%)
Yes	8 (11%)	10 (32%)
Primary treatment		
Surgery	61 (87%)	26 (85%)
+ Adjuvant radiotherapy	13 (21%)	10 (38%)
Radiotherapy	9 (13%)	5 (15%)

Quantitative evaluation of immunostaining. The evaluation was done using computer-assisted sampling (newCAST software, Visiopharm, Hoersholm, Denmark) applying statistically unbiased stereological sampling of fields of view (Gundersen *et al*, 1988a, b). Whole slides were scanned at a maximum resolution of $\times 20$ using a whole slide scanner (NanoZoomer 2.0, Hamamatsu Photonics, Hamamatsu City, Japan). Digital images were then imported into newCAST. The total tumour area visible and adjacent tumour-associated stroma at low resolution was outlined as region-of-interest. Areas of necrosis or artefacts were ignored. The quantitative histological analysis was done by one observer blinded to all clinical features and previous lymphocyte counts.

Double staining of CD66b and CD34 allowed for the distinction between intra- and extravascular neutrophil localisation as previously described (Jensen *et al*, 2009a, 2012). Neutrophils and macrophages were further classified according to tumour compartment as located in tumour nests, peritumoural (i.e., in the stroma at the migrating border of tumour nests) (Figure 1), or stromal as previously described by Nedergaard *et al* (2007b). An area of stroma was denoted peritumoural, if at least one epithelial malignant cell was observed in the field of view inside a sampling frame. If no tumour cells were observed the area was denoted as stromal. For estimation of the density of CD66b$^+$ neutrophils (Figure 1A) the first counting frame was sampled by the software at random, and subsequently a mean of 64 counting frames (each 40 480 μm^2) were sampled in the total tumour area in a systematic manner (Gundersen *et al*, 1988a) at a total magnification of 1556 ($\times 40$ lens). Individual neutrophil profiles were counted when a neutrophil nucleus was clearly visible along with a surrounding positive IHC signal. For assessment of CD163$^+$ macrophages (Figure 1B), point counting (Kamper *et al*, 2011) was applied to estimate the area fraction of CD163$^+$ immunostains per viable tumour area at a magnification of 1556 ($\times 40$ lens). Point counting of CD163 immunostains was chosen as the cell borders of individual macrophages could not be outlined with sufficient precision. A mean of 28 fields of visions were assessed per tumour section. Areas of necrosis or artefacts were ignored. This stereological approach with a single section from each tumour tissue block only allows for estimation of two-dimensional cell profiles either as number or area fraction. Assessment of number of CD3$^+$, CD4$^+$, and CD8$^+$ lymphocytes were performed with identical stereological approach as for CD66b$^+$ neutrophil estimation and has been described in detail previously (Nedergaard *et al*, 2007a).

Heterogeneity and reproducibility. Heterogeneity between paired sections from the same patient was modest, as a significant concordance was observed for neutrophil count peritumourally (Paired T-test correlation = 0.6; $P = 0.0001$) with the paired t-test revealing no significant differences between samples ($P = 0.7$). Likewise, for macrophages peritumourally we observed a paired T-test correlation of 0.69 with no significant differences between samples ($P = 0.08$). Intraobserver reproducibility of variables was tested in a systematic random sample of every 10th case ($N = 10$), ranked according to neutrophil density. A high reproducibility was

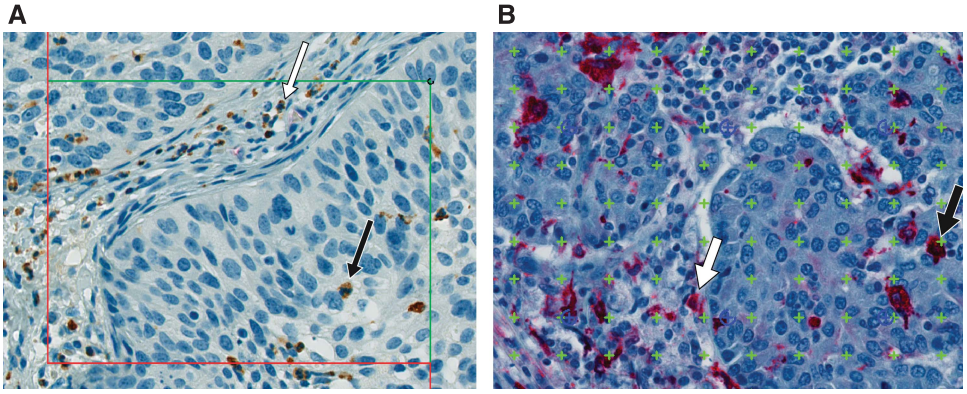

Figure 1. (**A**) Representative example of CD66b$^+$/CD34$^+$ staining (20x lens) with tumour nest neutrophil (black thin arrow) and peritumoural neutrophil (white thin arrow). An unbiased counting frame is applied. (**B**) CD163$^+$ immunostaining (20x lens) illustrating tumour nest macrophage (black fat arrow) and peritumoural macrophages (white fat arrow). A counting grid is applied.

observed for neutrophils in all compartments (Spearman $\rho > 0.80$; $P < 0.0001$).

Statistics. Follow-up was median 9.8 years and clinical features (Table 1) were as previously published (Nedergaard *et al*, 2007a). Correlation between paired sections was tested with paired samples *T*-test. Intraobserver reproducibility of variables was tested with Spearman rank test. For all cell assessments the median was used as cutoff point. Survival distributions were estimated using the Kaplan–Meier method and the relationship between survival and each parameter was analysed with the log-rank test. A Cox proportional hazards model was created to identify independent predictors of RFS, including factors with a *P*-value <0.01 in univariate analyses. On the basis of the number of patients in the cohort and the number of events we included a maximum of five variables in the multivariate analysis (Vittinghoff and McCulloch, 2007): peritumoral CD66b$^+$ neutrophils above median, peritumoral CD163$^+$ macrophages above median, peritumoral CD8$^+$ lymphocytes below median, peritumoral CD4$^+$ lymphocytes below median, and presence of lymph node metastases. Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA) statistical software. All tests were two-sided and *P*-values <0.05 were considered statistically significant.

RESULTS

Patient characteristics. Characteristics of the 101 cervical cancer patients are listed in Table 1. For patients alive minimum follow-up was at least 5 years. Overall, 10-year RFS rate was 67% for stage IB and 70% for stage IIA.

Tumour-infiltrating neutrophils, macrophages, and lymphocytes correlated with RFS. The number of intravascular neutrophils was negligible (mean absolute count 0). The median densities of neutrophils in tumour nests, peritumoural, and stromal were 23.2 cells mm^{-2} (range 0–939), 53.1 cells mm^{-2} (range 1.7–677), and 28.3 cells mm^{-2} (range 0–780), respectively. The median area fraction of macrophages in tumour nests, peritumoural, and stromal were 0.22% (0–12%), 1.3% (0–17%), and 0.73% (0–9.4%), respectively.

A density of CD66b$^+$ neutrophils above median in the peritumoural compartment and stromal compartment was significantly associated with short RFS ($P = 0.039$ and $P = 0.011$, respectively) (Figure 2), whereas neutrophils within the tumour nests were not ($P = 0.23$). For CD163$^+$ macrophages assessments, a density above median in the peritumoural compartment was significantly associated with short RFS ($P = 0.042$) (Figure 2). The density of CD163$^+$ macrophages in the stroma or intratumoural compartments was not significantly associated with RFS. The highest rate of recurrence was noted for patients with the highest density (quartile 4) of neutrophils and macrophages in all three compartments (Table 2). The 5-year RFS was 52% for patients with peritumoural neutrophil in the fourth quartile compared with 76% for patients with peritumoral neutrophils in the first quartile. Likewise, the 5-year RFS was 60% for patients with peritumoural macrophages density in the fourth quartile compared with 80% for patients with peritumoral macrophages in the first quartile. Higher median densities of peritumoral macrophages were observed in patients with lymph node metastasing with a median area fraction of 2.7% for patients with lymph node metastases *vs* 1.1% for patients without lymph node metastases (Mann–Whitney $P = 0.004$). However, the two groups were overlapping and no optimal cutpoint could be established. The results of tumour-infiltrating lymphocytes in these patients and the correlation with RFS have previously been published (Nedergaard *et al*, 2007a). Low peritumoural CD4$^+$ and CD8$^+$ lymphocyte counts stratified at median values were statistically significantly correlated with short RFS ($P \leqslant 0.03$).

Multivariate analysis. A multivariate Cox proportional-hazard regression model was used to analyse the relative strength and potential independence of CD4$^+$ lymphocytes, CD8$^+$ lymphocytes, CD66b$^+$ neutrophils, CD163$^+$ macrophages, and lymph node metastases. For comparability, estimates from the peritumoural compartment were chosen for the model. Federation of Gynaecology and Obstetrics stage or age had no significant impact on RFS in univariate analysis in this material and thus were not included in multivariate analysis. High density of peritumoural CD66b$^+$ neutrophils, low density of CD8$^+$ lymphocytes, and lymph node metastases were significant independent predictors of poor RFS (Table 3), whereas low density of CD4$^+$ lymphocytes and high densities of CD163$^+$ macrophages were not significant.

Neutrophil-to-lymphocyte ratio. The prognostic information provided by the peritumoral CD66b$^+$ neutrophil to peritumoral CD8$^+$ lymphocyte ratio was not significantly different from that of individual cell counts ($P = 0.05$). However, as the whole tumor area is much easier definable than individual compartments, we further constructed a ratio based on combined intratumoral and peritumoral counts of CD66b$^+$ neutrophils and CD8$^+$ lymphocytes (i.e., the Tumour Associated-Neutrophil to Lymphocyte Ratio (TA-NLR)). The TA-NLR had excellent discriminatory power for each quartile with 5-year RFS for TA-NLR quartile I (<0.065), II (0.07–0.16), III (0.17–0.36), and IV (>0.364) of 92%, 80%, 62%, and 44%, respectively ($P = 0.001$) (Figure 3).

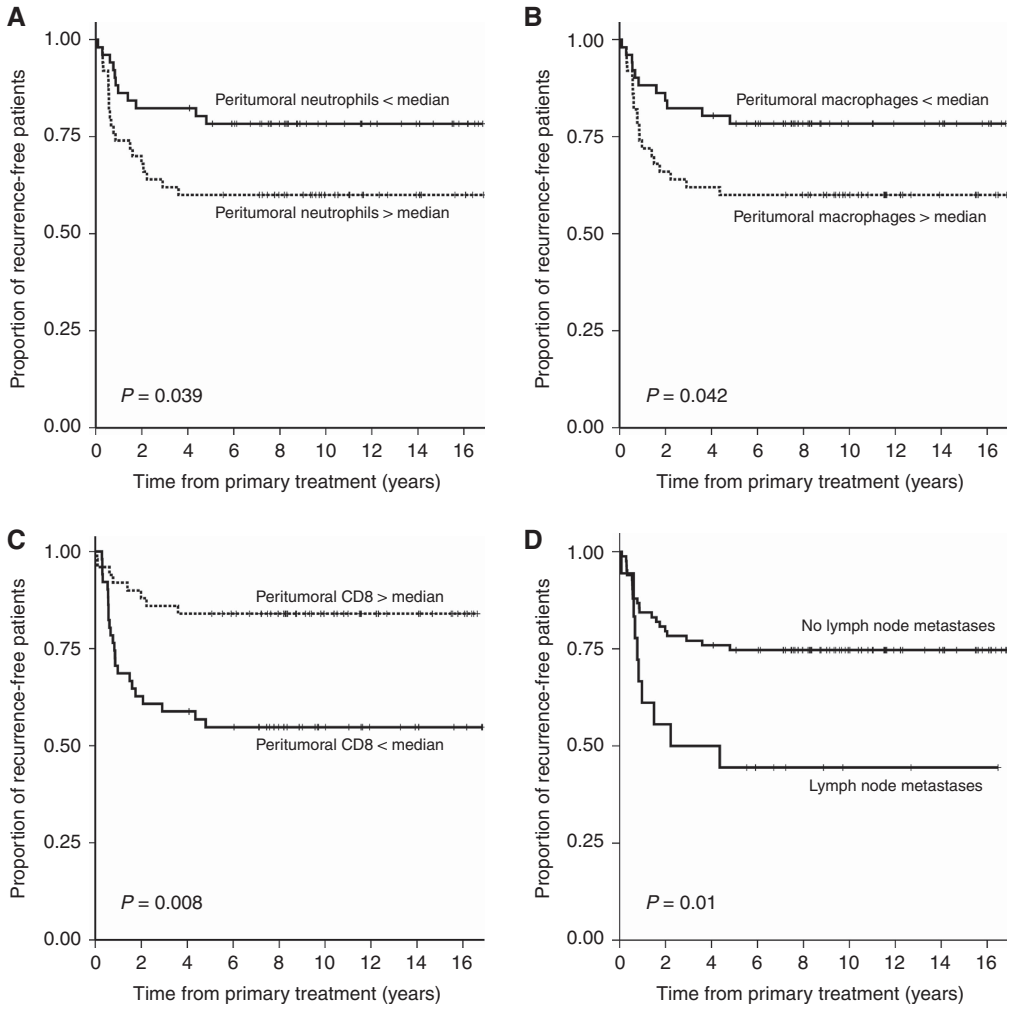

Figure 2. Kaplan–Meier RFS curves according to peritumoural CD66b$^+$ neutrophils (**A**), peritumoural CD163$^+$ macrophages (**B**), and peritumoral CD8$^+$ lymphocytes (**C**), all stratified by medians, and according to presence or absence of lymph node metastases (**D**). *P*-values obtained from log-rank tests.

DISCUSSION

To our knowledge, this is the first study to identify tumour-associated CD66b$^+$ neutrophils as an independent poor prognostic factor for RFS in patients with early-stage cervical cancer. Peritumoural neutrophil counts had good discriminatory power, identifying subgroups with 5-year RFS of 52% and 76%, respectively. Moreover, a simple combined prognostic score incorporating both intratumoral and peritumoral CD66b$^+$ neutrophils to intratumoral and peritumoral CD8$^+$ lymphocytes, that is, TA-NLR, was able to further discriminate patients with particularly poor and good risk (5-year RFS of 44% *vs* 92%, respectively). Elevated densities of CD163$^+$ macrophages were significantly correlated with the presence of lymph node metastases, but were not independently associated with RFS. Thus, patients with high densities of peritumoral neutrophils, low densities of CD8$^+$ lymphocytes, or high TA-NLR should be considered for closer follow-up or intensified adjuvant treatment because of the higher risk of recurrence. These intratumoral features may also serve as stratification factors in adjuvant trials. However, the results require validation in an independent and larger population.

The interplay between immune cells and tumour microenvironment is an area of intense research. Tumours are composed of an assemblage of various cell types, including cancer cells, cancer stem cells, endothelial cells, fibroblasts, and immune cells, that communicate and collaborate (Hanahan and Weinberg, 2011). A delicate interplay between these cells determines whether a tumour progresses or regresses. Tumour-infiltrating lymphocytes have been demonstrated to be a favourable prognostic feature in cervical cancer and other tumour types (Rahir and Moser, 2012). In the present study, we likewise demonstrated that high numbers of CD8$^+$ T-lymphocytes were independently associated with long RFS. However, a novel hallmark of cancer is the capability to avoid tumour destruction (Hanahan and Weinberg, 2011), and TANs as well as TAMs have the potential to suppress cytotoxic lymphocyte function (Schmielau and Finn, 2001; Lepique *et al*, 2009). High blood neutrophils as well as elevated blood neutrophil-to-lymphocyte ratio have been correlated with poor patient outcome in cervical cancer and other cancers (Yamanaka *et al*, 2007; Tomita *et al*, 2012; Lee *et al*, 2012a). Our observations of high peritumoural CD66b$^+$ neutrophils and low peritumoural CD8$^+$ lymphocytes as independent prognostic factors for short RFS, as well as the TA-NLR demonstrating a strong discriminatory power, are in line with this and suggest a link between peripheral immune cells and the immune cells of the tumour microenvironment, as well as a delicate balance between these immune cells.

Tumour-associated neutrophils and TAMs functionally contribute to multiple hallmarks of cancer (Hanahan and Weinberg,

Table 2. Percentage of patients with recurrence within 5 years from diagnosis of cervical cancer stratified by quartiles of CD66b$^+$ neutrophil count and CD163$^+$ macrophage cell density

Quartiles	Cells per mm^2 or area fraction	% Of patients recurred
Neutrophils tumour nests		
I	0–8.5	28
II	8.6–23.1	20
III	23.2–68.9	31
IV	69.0–939.3	44
Neutrophils peritumoural		
I	2–18.1	24
II	18.2–53.1	16
III	53.2–125.2	35
IV	125.3–677.1	48
Neutrophils stroma		
I	0–8.5	16
II	8.6–28.3	24
III	28.4–66.8	27
IV	66.7–780	56
Macrophages tumour nests		
I	0–0.021	21
II	0.022–0.18	29
III	0.22–0.99	23
IV	1.0–12	52
Macrophages peritumoural		
I	0–0.46	20
II	0.47–1.27	24
III	1.28–3.0	39
IV	3.1–16.6	40
Macrophages stroma		
I	0–0.26	20
II	0.27–0.73	32
III	0.74–2.1	23
IV	2.2–9.4	48

Table 3. Multivariate analysis with RFS as end point ($N = 101$)

Risk factor	Categories compared	HR	95% CI	*P*-value
High CD66b$^+$ neutrophils peritumoural	$>$ *vs* $\leqslant$	2.27	1.09–4.75	0.03
Low CD8$^+$ lymphocytes peritumoural	$\leqslant$ *vs* $>$	3.67	1.63–8.25	0.002
Lymph node metastases	Yes *vs* no	2.70	1.26–5.76	0.01

Abbreviations: CI = confidence interval; HR = hazard ratio; RFS = recurrence-free survival. Cox regression analysis.

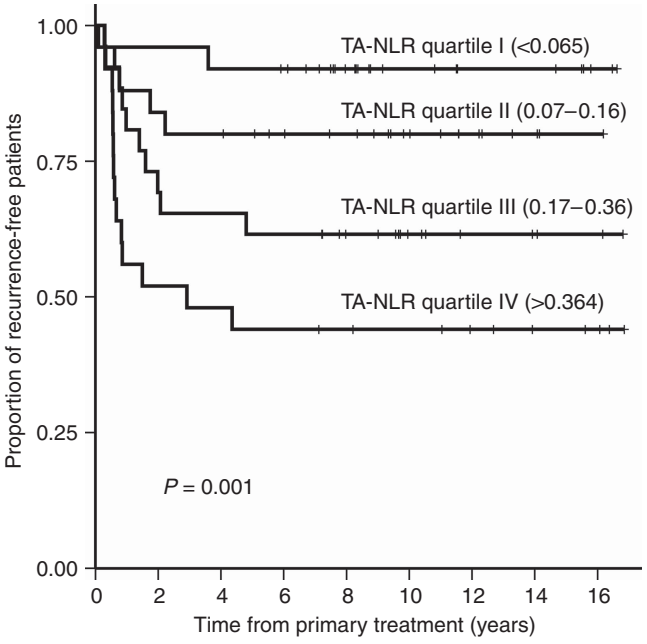

Figure 3. Kaplan–Meier recurrence-free survival curves according to the TA-NLR stratified at quartiles from lower quartile I to highest quartile IV. *P*-value obtained from log-rank test.

2011); TANs are involved in tumour initiation, angiogenesis, invasion, progression, and dissemination of cancer (Coussens and Werb, 2001; Di Carlo *et al*, 2001; Wu *et al*, 2001; Ishikawa *et al*, 2008; Fridlender *et al*, 2009; Tazzyman *et al*, 2009; Hofman, 2010; Donskov, 2013).

Wu *et al* (2011) assessed neutrophils localised in the tumour nests, peritumoural, and in the stroma in cervical cancer patients and likewise observed the highest density of neutrophils in the peritumoural area, however, the authors did not correlate their findings with patient outcome. A major novelty of our study is the systematic assessment of the innate immune cells in the different compartments, i.e., tumour, peritumoral and stroma, and the correlation of these cell subsets with recurrence. We confirmed the findings of the highest densities of CD66b$^+$ neutrophils and CD163$^+$ macrophages in the peritumoural compartment, but were only able to demonstrate an independent prognostic capacity for peritumoral CD66b$^+$ neutrophils. This may reflect the importance of neutrophil activity in the migrating tumour border and should be further studied. However, our TA-NLR data suggest that the compartment distribution of immune cells is less important for recurrence assessment and a simple estimate of numbers of CD66b$^+$ neutrophils relative to numbers of CD8$^+$ lymphocytes in the global tumour area may be clinically translatable. We observed higher densities of peritumoral macrophages in patients with lymph node metastases, which suggest an association between macrophage infiltration and lymph node metastasing. Tumour-associated macrophages in the tumour-associated stroma has been shown to relate to the secretion of endothelial growth factors, as well as other factors, which accelerates lymphangiogenesis and can lead to lymph node metastasing (Schoppmann *et al*, 2002; Utrera-Barillas *et al*, 2010).

The CD163$^+$ macrophage marker is considered specific for M2-polarised macrophages (Lau *et al*, 2004; Ambarus *et al*, 2012). The less specific CD68$^+$ pan-macrophage marker (which may also be expressed by other cell types) have previously been explored in a feasibility study of cervical cancer patients by Nedergaard *et al* (2007b) and was likewise found not to be significantly associated with tumour recurrence. This supports our results of no independent correlation between macrophages and RFS.

In this study, we employed stereological systematic random sampling methods, and although manual cell counting is labour intensive, the sampling technique is unbiased, efficient and reproducible, and also allows for discrimination of individual cells

belonging to the different tumour and stromal compartments. However, limitations to our study are the retrospective design, the small sample size, the lack of some clinical prognostic factors due to the clinical standard between 1990 and 2000, and the lack of HPV subtyping.

In conclusion, elevated $CD66b^+$ TAN count is an independent prognostic factor for short RFS in early-stage cervical cancer. Combining assessments of $CD66b^+$ neutrophils and $CD8^+$ lymphocytes may further improve prognostic stratification. Prospective, larger studies to validate and further elucidate this finding are required.

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Paper III

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Impact of baseline and nadir neutrophil index in non-small cell lung cancer and ovarian cancer patients: Assessment of chemotherapy for resolution of chronic inflammation

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Impact of baseline and nadir neutrophil index in non-small cell lung cancer and ovarian cancer patients: Assessment of chemotherapy for resolution of chronic inflammation

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ABSTRACT

Background
Chronic inflammation has been recognized to foster tumour development. Whether chemotherapy can be used to neutralize chronic inflammation is unclear.
Methods
We evaluated baseline and nadir neutrophils in 111 patients (pts.) with non-small cell lung cancer (NSCLC) and 118 pts. with ovarian cancer (OC) treated with chemotherapy administered with dose-individualization to achieve nadir neutropenia of 1.5. We used predefined baseline neutrophil cut-offs $4.5x10^9$/L (NSCLC) and $3.9x10^9$/L (OC).
Results
Absence of chemotherapy-induced nadir neutropenia (CTCAE grade 0, neutrophils ≥LLN) was seen in 23% of OC and 25% of NSCLC pts. Absence of nadir neutropenia was associated with decreased overall survival (OS) compared with presence (>grade 0) of neutropenia (9 vs. 14 months, *P*=0.004 for NSCLC and 23 vs. 56 months; *P*=0.01 for OC). Obtaining grade 3/4 neutropenia did not improve survival compared with grade 1/2 neutropenia. In multivariate analyses baseline neutrophils ≥$4.5x10^9$/L HR: 2.0 (95% CI: 1.11-3.44) and absence of nadir neutropenia HR: 1.6 (95% CI: 1.02-2.65) for NSCLC and absence of nadir neutropenia HR: 1.9 (95% CI: 1.1-3.1) for OC were independently associated with short OS.
Three prognostic neutrophil index (NI) groups were defined. Favourable NI; low baseline neutrophils and presence of nadir neutropenia (>grade 0), Intermediate NI; elevated baseline neutrophils and presence of nadir neutropenia (>grade 0), and Poor NI; elevated baseline neutrophils and absence of nadir neutropenia (grade 0). For NSCLC patients, the median OS was 18.0, 13.4, and 8.8 months for favourable, intermediate and poor NI, respectively (fav vs. poor *P*=0.002; fav vs. intermed *P*=0.04; and intermed vs. poor *P*=0.03). For OC patients, median OS was 69, 52 and 23 months for favourable, intermediate and poor NI, respectively (fav vs. poor *P*=0.03; fav vs. intermed *P*=0.3; and intermed vs. poor *P*=0.02). Interestingly, survival rates in the intermediate NI groups indicated that individualised dose of chemotherapy to induce neutropenia may partly overcome the negative impact of elevated baseline neutrophils.
Conclusions
A neutrophil index comprising elevated baseline neutrophils and absence of neutropenia identified a high risk group of NSCLC and ovarian cancer patients with only modest effect of chemotherapy. New treatment options for this subset of patients are required.

KEYWORDS: Lung cancer; ovarian cancer; neutrophils, tumour microenvironment; prognostic factor

BACKGROUND

Although cancer begins as a single cell and initially develops as a clone, by the time a tumour is clinically detectable, it is no longer a mass of isolated, identical, neoplastic cells [1]. It has been realized that tumours are composed of an assemblage of cell types that communicate and collaborate, including cancer cells, cancer stem cells, endothelial cells, pericytes, fibroblasts and tumour-promoting inflammatory cells [2]. Thus, multiple non-malignant cell types are recruited to become components of the tumour and contribute to the hallmarks of cancer [3].

Among inflammatory cells, the realisation of the negative effect of neutrophils has recently begun to emerge [4]. Several studies have demonstrated that tumours stimulate neutrophils to promote angiogenesis and immunosuppression, as well as migration, invasion, and metastasis [5]. In clinical trials, the prognostic role of tumour-infiltrating neutrophils, elevated blood neutrophils, and elevated blood neutrophil/lymphocyte ratio has been clearly associated with poor clinical outcome in several human cancers, most notably in renal cell carcinoma, melanoma, colorectal cancer, hepatocellular carcinoma, cholangiocarcinoma, glioblastoma, GIST, gastric, esophageal, lung, ovarian, head and neck, and cervical cancer [6-8]. A striking finding is the notion that high baseline neutrophil count hinder benefit from surgery, chemoradiotherapy, radiofrequency ablation, and chemotherapy [6]. Consequently, neutrophils, in addition to tumour cells, are potential targets for cancer therapy [9,10].

Traditionally, neutropenia in relation to chemotherapy has been regarded as a harmful side effect that should be avoided. However, several retrospective studies have suggested an inferior outcome for patients failing to achieve mild neutropenia during chemotherapy for breast, ovarian, and non-small cell lung cancers (NSCLC) as well as Hodgkin's lymphoma [11-17] and for targeted therapy with sunitinib, cetuximab and imatinib [18-21]. Nevertheless, it is unclear whether baseline and nadir neutrophils are linked in the individual patient.

In the present study, we evaluated the prognostic impact of combined baseline and nadir neutrophils in an institution with a standard practise of individualizing chemotherapy dosing upwards or downwards to achieve target nadir neutropenia of 1.5 x 10^9/L [22,23]. We chose patients with NSCLC and ovarian cancer (OC) as predefined baseline neutrophil cutoff values of 4.5x10^9/L and 3.9x10^9/L, respectively, have been determined from previous studies [24] [25]. We identified a new prognostic neutrophil index by combining baseline and nadir neutrophil values in patients with NSCLC and ovarian cancer.

METHODS

Patient population

Data from patients diagnosed with non-small cell lung cancer (stage III-IV) and advanced ovarian cancer (stage I-IV) treated with chemotherapy between 1997 and 2005 were collected from patient records at the Department of Medical Oncology at Crown Princess Mary Cancer Centre Westmead in Sydney Australia.

Eligibility criteria were a complete medical record within three cycles of chemotherapy and a full set of baseline and nadir laboratory data. All available medical files were reviewed and a number of files were excluded due to lack of essential data. Patients routinely had a nadir blood count measured 10 to 17 days after chemotherapy or as appropriate according to schedule. The highest grade of myelosuppression at nadir was recorded. Demographics, type of chemotherapy, use of G-CSF, clinical,

laboratory, and survival data were collected. Stage was graded according to TNM 2002 or FIGO 1998 as appropriate. No upfront G-CSF was used. Toxicity and laboratory data were graded according to CTCAE v.3.0. Survival data were updated May 2010.
The study has received institutional review board and Ethics Committee approval.

Treatment and toxicity-adjusted dose modification

A protocol of chemotherapy-toxicity adjusted dosing (CTAD) was implemented as clinical standard practice in the mid-1990s at the institution [22]. After an initial administration of chemotherapy based on standard body surface area, subsequent doses were adjusted upwards or downwards in each patient to yield target nadir neutrophil count of 1.5 x 10^9/L (i.e. CTCAE grade 1 neutropenia). If target neutropenia was not reached and other haematological or non-haematological toxicity was ≤Grade 2, the subsequent dose of chemotherapy was *increased* by 15-20%. In contrast, if the patient experienced significant non-haematological toxicity >Grade 2 toxicity or severe neutropenia, the dose was *reduced* by 15-20%. Otherwise the dose remained unchanged [22].

Statistical analysis

Summary statistics were performed to estimate relevant baseline patient demographic and disease characteristics. Relative chemotherapy intensity was calculated as the actual cumulated dose of chemotherapy divided by the standardized cumulated dose according to expected number of chemotherapy cycles. The impact on outcome was explored for all patients in each tumour type as well as in the subgroups of patients receiving the most frequent chemotherapy regimen.
Based on previous studies identifying baseline neutrophil count as independent prognostic factors in NSCLC [24] and ovarian cancer [25] baseline neutrophil counts were dichotomized according to the pre-defined cutoff values of 4.5x10^9/L for NSCLC and 3.9x10^9/L for ovarian cancer. Patients who developed various CTCAE version 3 grades of myelosuppression were compared with those who did not. The relationship between assessed parameters and overall survival (OS) was evaluated using the method of Kaplan–Meier and log-rank tests. Multivariate Cox regression models were constructed to report hazard ratios (HRs) for OS. Factors with $P<0.1$ in univariate analysis were included in the multivariate model.
All reported *P*-values were two-tailed; an alpha-value below 0.05 was considered statistically significant. The analyses were performed using SPSS version 20.0.0 (IBM Corporation, NY, USA).

RESULTS

A total of 111 patients with non-small cell lung cancer (NSCLC) and 118 patients with ovarian cancer (OC) fulfilled inclusion criteria for the present study. Patient characteristics are shown in Table 1 and 2. For NSCLC patients, 71 (64%) achieved ≥ grade 2 nadir neutropenia (i.e., neutrophils <1.5 x 10^9/L), 15 (14%) achieved grade 1 nadir neutropenia (i.e., neutrophils <LLN to 1.5 x 10^9/L), and 25 (23%) had no chemotherapy-induced neutropenia (grade 0, i.e., neutrophils ≥ LLN). For ovarian cancer patients 64 patients (54%) achieved ≥ grade 2 neutropenia, 24 (20%) achieved grade 1 neutropenia, and 30 (25%) had no neutropenia (grade 0). One ovarian cancer patient received G-CSF due to neutropenia. Dose increase due to absence of target neutropenia occurred in 19% of NSCLC and 23% of OC patients. Dose reduction due to haematological or non-haematological toxicity occurred in 36% of NSCLC patients and 25% of OC patients.

Impact of baseline and nadir neutrophils in univariate analyses

Non-small cell lung cancer

Overall median survival was 13.0 months for NSCLC patients with a median follow-up of 6.7 years. Patients failing to achieve any grade of nadir neutropenia (i.e., neutrophils ≥ LLN) after chemotherapy had decreased survival rate compared with patients who obtained any grade of neutropenia (i.e. >grade 0 neutropenia), median survival 9 vs. 14 months; (*P*=0.004). Obtaining grade 3 or 4 neutropenia did not improve survival rate compared with grade 1 or 2 neutropenia (Figure 1A).

Patients with baseline elevated blood neutrophils above or equal to the predefined cutoff of $4.5x10^9$/L had decreased OS compared with patients with baseline neutrophil counts below 4.5, median survival 11.6 vs. 18.0 months; (*P*=0.02). The impact of relative dose intensity of chemotherapy was significantly associated with overall survival (*P*=0.003). Other factors associated with poor overall survival were performance status >0 (*P*=0.08), presence of bone metastases (*P*=0.003), Stage IV cancer (*P*=0.001), and haemoglobin count below lower limit of normal (*P*=0.02).

Ovarian cancer

Median survival was 50 months for all ovarian cancer patients with a median follow-up time of 9.0 years. Patients who failed to achieve neutropenia (i.e., neutrophils ≥LLN, grade 0 neutropenia) had less than half the median survival compared to patients achieving any grade of neutropenia (23 vs. 56 months; *P*=0.01). Obtaining grade 3 or 4 neutropenia did not improve survival rate compared with grade 1 or 2 neutropenia (Figure 1B). We observed no statistically significant survival impact of baseline elevated neutrophils above or below the predefined cutoff of $3.9x10^9$/L (*P*=0.3). Relative chemotherapy dose did not impact overall survival (HR 1.00; 95% CI 0.98–1.03; *P*=0.6). Other factors significantly associated with poor overall survival were performance status >0 (*P*=0.001), residual disease >1cm (*P*=0.0001), presence of ascites (*P*=0.0001), and failure to normalize serum CA125 (*P*=0.003).

Multivariate analyses

For NSCLC patients factors independently associated with short survival in multivariate analysis were failure to achieve neutropenia with chemotherapy (i.e., grade 0 neutropenia), baseline neutrophil count above or equal to $4.5x10^9$/L, relative chemotherapy intensity <100%, and TNM stage IV (Table 3).

For ovarian cancer patients the following factors were independently associated with short survival in multivariate analysis: failure to achieve neutropenia with chemotherapy (i.e., grade 0 neutropenia), minimal residual disease >1 cm, presence of ascites, and failure to normalize CA125 after chemotherapy (Table 3).

Impact of baseline and nadir neutrophil index

To evaluate the combined prognostic impact of both baseline and nadir neutrophils we performed a four-group analyses. Based on predefined baseline neutrophil cutoff values and nadir neutropenia grade (0 *vs.* >0), we identified a favourable neutrophil index prognostic group (comprising patients with low baseline neutrophils and presence (>grade 0) of nadir neutropenia), an intermediate neutrophil index prognostic group (comprising patients with elevated baseline neutrophils and presence (>grade 0) of nadir neutropenia), and a poor neutrophil index prognostic group (comprising patients with elevated baseline neutrophils and absence of (grade 0) nadir neutropenia). The fourth potential group of low baseline neutrophils and grade 0 nadir neutropenia comprised only 4 patients

with ovarian cancer and no NSCLC patients, and was therefore not classified. For NSCLC patients, the median OS was 18.0, 13.4, and 8.8 months for favourable, intermediate and poor neutrophil index prognostic group, respectively (Figure 2A): (favourable vs. poor *P*=0.002, favourable vs. intermediate *P*=0.04, intermediate vs. poor *P*=0.03). Number of dose increase and dose reduction in the intermediate group was not statistically different from the poor prognostic group. For ovarian cancer patients, median OS was 69, 52 and 23 months for favourable, intermediate and poor neutrophil index prognostic group, respectively (Figure 2B): favourable vs. poor *P*=0.03, favourable vs. intermediate *P*=0.3, intermediate vs. poor *P*=0.02. A significantly higher number of patients in the intermediate group had dose reduction compared with the poor prognostic group (*P*=0.017) whereas no difference in dose increase or relative chemotherapy dose intensity was observed between the intermediate and poor prognostic group.

DISCUSSION

To our knowledge, this is the first study to identify a prognostic neutrophil index in non-small cell lung cancer (NSCLC) and ovarian cancer patients taking into account both pre-treatment and post-treatment neutrophils. Using baseline and nadir neutrophils in a combined prognostic index we were able to identify a subgroup of patients - with baseline neutrophils above the pre-defined cutoffs and failure to achieve neutropenia following chemotherapy - who had a dismal prognosis comprising approximately one quarter of the patient population. In this poor neutrophil index group it appears that chemotherapy had minimal impact for resolution or neutralization of the negative effect of neutrophils despite the use of a protocol designed to induce neutropenia. It is unknown whether further dose escalation in those individuals would have had a positive benefit. It might be that the effect of chemotherapy in these patients has reached its ceiling and other means of therapy are required [3]. In contrast, patients with baseline neutrophils below the pre-defined cutoff and who obtained neutropenia below lover limit of normal (>grade 0) did benefit from chemotherapy and had a two to three-fold better overall survival.

High baseline neutrophil count hinder benefit from surgery, chemoradiotherapy, radiofrequency ablation, and chemotherapy in several human cancers (reviewed in [6]). Our findings validate the cutoff baseline neutrophil count above or equal to $4.5x10^9$/L in NSCLC patients previously identified by Teramukai *et al* [24] as an independent prognostic factor for poor outcome. Other studies in NSCLC have also demonstrated an adverse prognostic effect of high baseline neutrophil count [26-30] Data from the Teramukai study also suggested a link between high pre-treatment neutrophil count and increased treatment-related non-haematological toxicity. The incidence of grade 3 or 4 non-haematological toxicity within the first three cycles of treatment was significantly higher in the high-neutrophil group compared to the low neutrophil group. Moreover, none of the patients in the high-neutrophil group who experienced grade 3 or 4 non-haematological toxicity within the first three cycles completed the planned six cycles [24]. This data suggests that simply increasing the dose of chemotherapy to induce neutropenia may be difficult to achieve in those patients with a high neutrophil count.

High neutrophil count has been shown to be negative prognositc factor for other tumour types. In colorectal cancer patients treated with anti-VEGF containing regimens, elevated neutrophil count predicted poor OS and RFS [31]. In gastrointestinal stromal tumours (GIST) elevated baseline neutrophil count correlated with initial as well as late resistance to imatinib treatment [32]. In metastatic renal cell carcinoma, elevated baseline blood neutrophil count has been integrated as a

validated prognostic factor in the Heng criteria for patients treated with targeted therapy [33] and both elevated blood neutrophils as well as presence of intratumoral neutrophils were independently correlated with poor survival in patients treated with cytokines [34]. Taken together, elevated neutrophils have serious clinical implications. However, we were unable to confirm the cutoff baseline neutrophils count above $3.9x10^9$/L in ovarian cancer patients identified by Banerjee *et al* [25], probable due to small sample size in our series.

Treatment-induced neutropenia was found to be an independent favourable variable in our population. Approximately one quarter of the patients failed to achieve any neutropenia despite the use of a chemotherapy toxicity adjusted protocol, where the goal was to induce neutropenia, and these patients had a worse survival. Moreover, we observed that severe neutropenia (i.e., grade 3-4) was no better than mild neutropenia (i.e., grade 1-2) but that both were better than no neutropenia (i.e., grade 0) in terms of improved median survival. In other words, it is the presence, but not the severity, of neutropenia that is prognostic. Similar to our results, Di Maio et al [13] and Kishida et al [15] have previously shown an inferior survival in NSCLC patients who experienced no treatment-related neutropenia (grade 0) but that no apparent additional benefit was seen with higher than grade 1 neutropenia. If validated prospectively, these findings may impact future routine clinical practice.

The novel, important finding from our data, that may have practical implications, is that among patients with higher baseline neutrophils, approximately 75% obtained neutropenia >grade 0 following chemotherapy and subsequently had a significantly improved overall survival compared to those 25% who experienced no neutropenia. These figures were almost identical in NSCLC and OC patients. Similar assessments have been done in colorectal cancer using neutrophil/lymphocyte ratio (NLR) as a marker [35]. Baseline blood NLR (>5) was shown to independently predict poor OS. Importantly, normalization of the NLR after one cycle of chemotherapy was observed in a subset of patients, which resulted in a 2-month PFS improvement (5.8 vs. 3.7 months) compared with patients without NLR normalization [35]. However, normalization of the NLR after one cycle of chemotherapy did not result in a statistically significant improvement in OS compared with patients without NLR normalization. No chemotherapy dose individualization was done in these patients based on toxicity or nadir neutrophil count, as was done in our study. Thus, these data imply that not all patients with elevated baseline neutrophil are "protected from the benefits of chemotherapy" as suggested by Maione et al [36]. In our series both the higher baseline count and absence of treatment-induced neutropenia were independent adverse prognostic factors and thus were not linked. Moreover, the relative dose intensity of chemotherapy was an independent prognostic factor in NSCLC. This implies that the dose of chemotherapy matters and may partially overcome the negative effects of an elevated baseline neutrophil count. The results in our intermediate prognosis group patients in both cancer groups seem to support this concept. Patients with a relatively high baseline neutrophil count who developed neutropenia from chemotherapy (i.e., the intermediate prognostic group) had a statistically significantly better survival than those patients who did not achieve treatment-induced neutropenia (i.e., the poor prognostic group). However, survival was still lower compared to patients with relatively low neutrophil count at baseline (i.e., the favorable group).

Inflammatory cells, including neutrophils, influence many aspects of cancer initiation, progression and metastatic potential in the tumour microenvironment [37]. Recruitment of neutrophils from the bone marrow to sites of inflammation is a well-documented process guided by chemochine-, lipid-, complement- and N-formylated peptide chemoattractant mediators [5]. However, human studies evaluating at the same point in time peripheral blood inflammatory cells and intratumoral

inflammatory cells are scarce. Recently, in resected stage 1-IIIA NSCLC patients [8] we demonstrated densities of tumour-associated CD66+ neutrophils and CD163+ macrophages were correlated with adverse clinical prognostic factors as well as CRP and white blood cell (WBC) systemic inflammatory markers. We also demonstrated that elevated blood CRP and WBC were associated with short recurrence free interval (RFS) and overall survival but tumour-associated neutrophils and macrophages were not directly correlated with RFS or overall survival [8]. In patients with metastatic renal cell carcinoma, elevated blood neutrophils as well as presence of CD66b + intratumoral neutrophils were independently correlated with poor OS in multivariate analysis [34]. This suggests that the tumour microenvironment may have two compartments, a local and a systemic and that both compartments may be important targets for therapy. Assessment of chemotherapy for resolution of chronic inflammation is a new paradigm and should be evaluated further in randomized trials incorporating the neutrophil index in the study design. However, the tumour may render refractory to chemotherapy and anti-VEGF treatment [38,39]. Further research in the area of chronic inflammation and cancer is encouraged.

Limitations of our study are the low sample size, the retrospective design, inhomogeneous chemotherapy regimens, and patient accrual over a long period of time. Additionally, the requirement for data for three cycles of treatment excludes patients who died early, potentially skewing the survival analysis. However, the present study was conducted in two independent, different tumour types with achievement of almost identical results.

CONCLUSIONS

In conclusion, absence of chemotherapy-induced neutropenia was an independent adverse prognostic factor in NSCLC and ovarian cancer patients. By combining baseline elevated neutrophil count and absence of neutropenia, we identified a poor prognostic group who appeared to have little benefit from chemotherapy despite a dose escalation protocol. New treatment options for this subset of patients are required. Importantly, we found an intermediate prognostic group where the induction of neutropenia by chemotherapy may have partially overcome the negative impact of elevated baseline neutrophils leading to a better survival. This has implications for dose individualisation in this subgroup. The combined prognostic neutrophil index comprising both baseline and nadir neutrophil count is a potentially new and important finding that requires validation in larger, prospective studies.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
FD collected patient data and planned the study with HG and HvdM. AC and FD made an equal contribution in data analysis, manuscript planning, and writing. HG participated in manuscript writing and data analysis. VG provided statistical analysis and advice. PH, RH, HG, RK and NW provided patients and revised the manuscript critically. All authors read and approved the final manuscript.

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Table 1. Patient characteristics for NSCLC patients (N=111)

Characteristic	**N**	**%**
Median age, years	61	
Age range, years	32–84	
Sex		
Male	53	48
Female	58	52
NSCLC stage		
3A	23	21
3B	36	32
4	52	47
ECOG performance		
0	51	46
1	52	47
2	7	6
Missing	1	1
Baseline neutrophils ≥ 4.5		
Yes	90	81
No	21	19
Tumour Histology		
Adenocarcinoma	58	52
All other	53	48
Bone metastases		
Yes	17	15
No	90	81
Missing	4	4
Chemotherapy regimens		
Carboplatin based	87	78
Cisplatin based	4	4
Other	20	18

Abbreviations: NSCLC=non-small-cell lung cancer; ECOG=Eastern Cooperative Oncology Group;

Table 2. Patient characteristics for ovarian cancer patients (N=118)

Characteristic	**N**	**%**
Median age, years	58	
Age range, years	33–82	
FIGO stage		
1	16	14
2	17	14
3	71	60
4	14	12
ECOG performance		
0	70	59
1	41	35
2+	6	5
Missing	1	1
Baseline neutrophils > 3.9		
Yes	98	83
No	20	17
Optimal debulking		
No	36	31
Yes	80	68
Missing	2	<2
Ascites at surgery		
No	32	27
yes	79	67
Missing	7	6
Normalized CA125		
No	16	14
yes	96	81
Missing	6	5
Chemotherapy regimens		
Carbo + Tax	73	62
Carbo monotherapy	33	28
Carbo + other	12	10

Abbreviations: FIGO= International Federation of Gynaecology and Obstetrics; ECOG=Eastern Cooperative Oncology Group; CA125=Cancer Antigen 125; Carbo=Carboplatin; Tax=Taxol (Paclitaxel)

Table 3. Multivariate analysis of association between on-treatment neutropenia and OS for non-small cell lung cancer and ovarian cancer

Tumour type	HR for death (95% CI)	P-value
NSCLC		
Neutropenia grade 0	1.6 (1.02;2.65)	0.04
Baseline neutrophils≥4.5	2.0 (1.11;3.44)	0.02
Stage IV cancer	1.8 (1.18;2.71)	0.006
Relative chemo intensity<100%	1.7 (1.11;2.60)	0.01
Ovarian cancer		
Neutropenia grade 0	1.9 (1.1;3.1)	0.02
Residual disease>1cm	2.9 (1.7;4.9)	0.0001
Ascites present	2.4 (1.3;4.7)	0.009
CA125 not normalized	3.3 (1.8;6.1)	0.0001

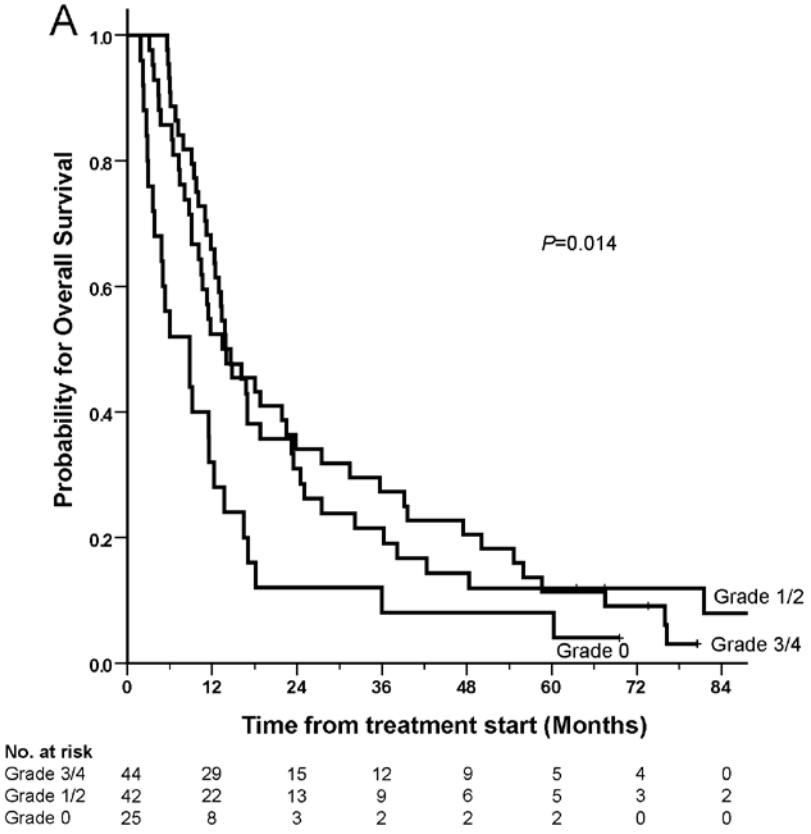
Abbreviations: OS = overall survival; NSCLC = Non-Small Cell Lung Cancer; Grade 0 = neutropenia at nadir ≥ 2.0x10^9/L; CA125 = Cancer antigen 125;

Figure legends

Fig.1 Kaplan-Meier plot of overall survival for NSCLC patients (A) (N=111) and ovarian cancer patients (B) (N=118) stratified for neutropenia grade at nadir. Grade 0, neutrophils ≥ LLN; grade 1, neutrophils <LLN to 1.5 x 10^9/L; grade 2, <1.5 - 1.0 x10^9/L; grade 3, <1.0 – 0.5 x10^9/L; grade 4, < 0.5x 0^9/L

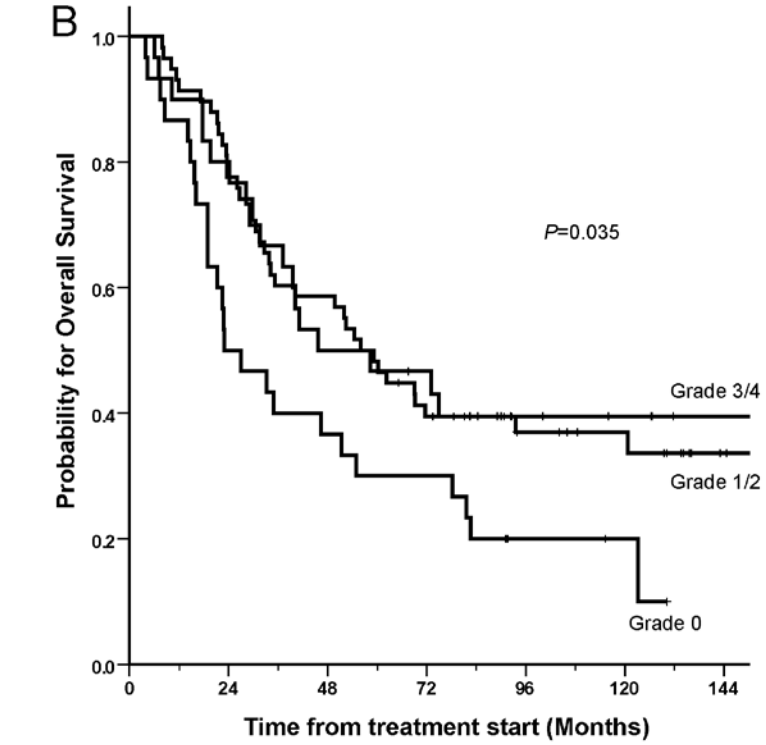
Fig.2 Kaplan-Meier plot of overall survival for NSCLC patients (A) (N=111) and ovarian cancer patients (B) (N=118) stratified for *favourable neutrophil index (NI)* (low baseline neutrophils and presence of nadir neutropenia), *intermediate neutrophil index* (elevated baseline neutrophils and presence of nadir neutropenia), and *poor neutrophil index* (elevated baseline neutrophils and absence of nadir neutropenia) prognostic group. A fourth group with low baseline neutrophils and absence of nadir neutropenia comprised only 4 patients with ovarian cancer and no NSCLC, and was not classified.

Figure 1



No. at risk

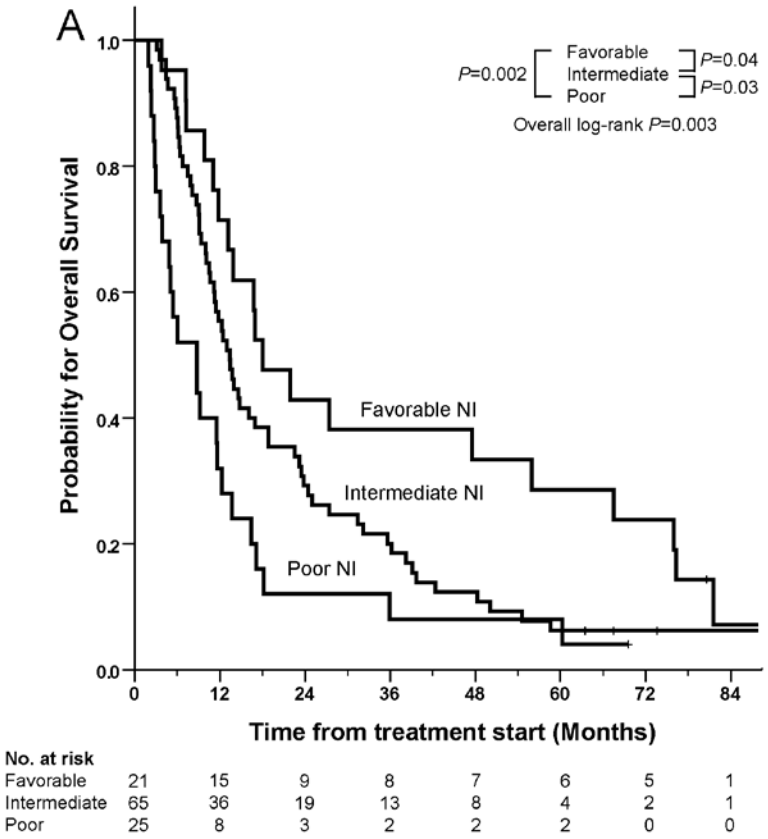
Grade 3/4	44	29	15	12	9	5	4	0
Grade 1/2	42	22	13	9	6	5	3	2
Grade 0	25	8	3	2	2	2	0	0



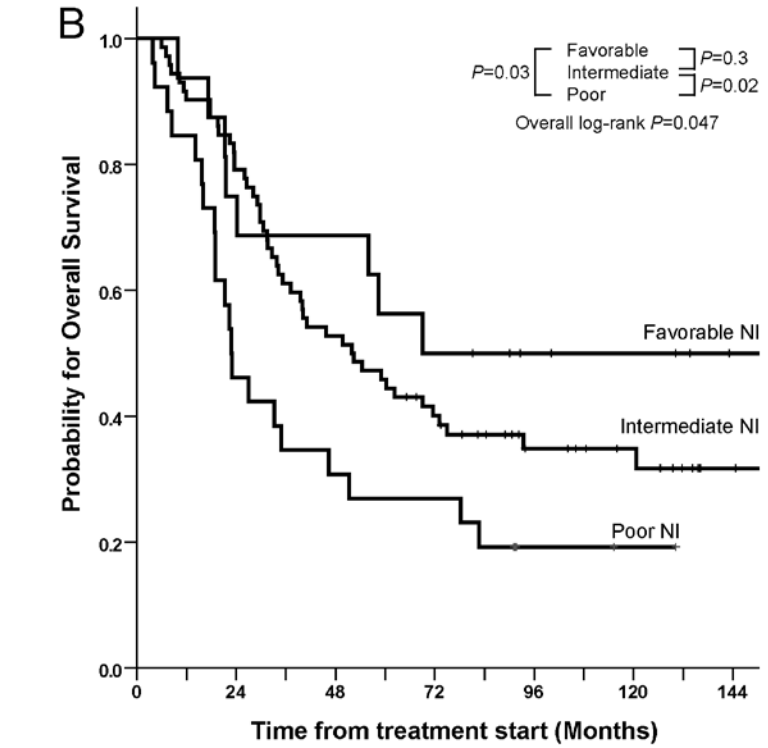
No. at risk

Grade 3/4	30	24	15	13	6	4	1
Grade 1/2	58	45	34	22	14	11	3
Grade 0	30	15	11	9	3	2	0

Figure 2



No. at risk								
Favorable	21	15	9	8	7	6	5	1
Intermediate	65	36	19	13	8	4	2	1
Poor	25	8	3	2	2	2	0	0



No. at risk							
Favorable	16	12	11	8	5	4	1
Intermediate	72	57	38	27	15	11	3
Poor	26	12	8	7	2	1	0

Paper IV

Andreas Carus, Frede Donskov, Patricia S. Nielsen, Henrik Hager, Bettina S. Nedergaard, Torben Steiniche, Morten Ladekarl

Strong prognostic value and high efficacy of automated digital image analysis of tumour-associated leukocytes in localized cervical cancer: A comparison with a manual cell counting method

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Strong prognostic value and high efficacy of automated digital image analysis of tumour-associated leukocytes in localized cervical cancer: A comparison with a manual cell counting method

Running title: Digital image analysis of leukocytes in cervical cancer

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KEYWORDS: Digital image analysis, stereology, $CD66b^+$ neutrophils, $CD163^+$ macrophages, $CD8^+$ lymphocytes, cervical cancer, prognosis.

ABSTRACT

AIMS

We compared the prognostic impact, efficacy and validity of tumour-associated leukocyte assessments obtained by automated digital image analysis (DIA) and manual observer assisted stereology (OAS).

METHODS AND RESULTS

Visiomorph software was used to obtain densities of immunostains for CD66b, CD163, and CD8 paraffin-embedded tumour sections from 101 patients with FIGO stage IB/IIA cervical cancer.

DIA required much less human resources than OAS assessments. We observed high correlations between DIA and OAS variables; spearman ρ was 0.79 ($P<0.0001$) for $CD8^+$ lymphocytes, 0.85 ($P<0.0001$) for $CD66b^+$ neutrophils, and 0.92 ($P<0.0001$) for $CD163^+$ macrophages. Hazard rates for recurrence of DIA assessments in the global tumour area were comparable with the prognostically strongest OAS assessments in the peritumoural compartment. In multivariate analysis, CD66b and CD8 immunostain densities, assessed by DIA, and regional lymph node metastases were independent predictors of RFS, while CD163 immunostain density and FIGO stage were not. The CD66b/CD8 immunostain index accurately predicted the risk of relapse, ranging from 8% to 52% ($P=0.001$).

CONLUSIONS

DIA variables of tumour-infiltrating leukocytes are highly correlated with corresponding variables obtained by OAS. The $CD66b^+$ neutrophil/$CD8^+$ lymphocyte immunostain index obtained by DIA is a strong and efficient prognostic variable with potential for routine application.

INTRODUCTION

A multitude of histological and immunohistochemical (IHC) biomarkers for outcome of various cancers have been explored over time, but only few have found a place in the clinical setting and are largely based on qualitative assays of lesser reproducibility [1,2]. Compared with qualitative techniques, unbiased stereological random sampling methods are objective and offer greater reproducibility [3,4]. Observer-assisted methods allow for association of immunostains to specific morphological structures including specific cell types, and for assigning the observations to defined compartments, such as "hot spots" or the tumour invasion front. However, the methods are relatively labour intensive and require specialized training [4,5].

Digital image analysis (DIA) is an emerging, high-throughput method for automated quantitative assessments of immunostained sections. Next-generation whole slide scanners can digitize IHC slides at pixel resolutions close to high-quality optical microscopes. Software for automated DIA of whole slide images (WSI) has evolved rapidly, and, based on mathematical algorithms, allows for fast quantification of the distribution of IHC stained cells or structures within a large area in less time and with reduced workload compared with observer-assisted methods [4]. Care has to be taken as DIA protocols are still sensitive to variation by for instance tissue processing, immunohistochemical protocols, unspecific staining, and definition of region-of-interest (ROI) [1,5,6].

In previous studies published in we showed the prognostic impact on recurrence-free survival (RFS) of tumour-associated CD66b$^+$ neutrophils and CD8$^+$ lymphocytes in patients with early-stage cervical cancer, assessed by the observer assisted stereology (OAS) methods [7,8]. A simple prognostic score incorporating both intra- and peritumoural CD66b$^+$ neutrophils to intra- and peritumoural CD8$^+$ lymphocytes, that is, the tumour-associated neutrophil-lymphocyte ratio (TA-NLR), was able to discriminate patients with particularly poor and good risk (5-year RFS of 44% vs. 92%, respectively). However, these OAS assessments were considerably cumbersome [7]. For a prognostic marker to gain foothold in the clinic the feature must be strongly predictive and the assessment has to be practical, fast, and preferably observer independent [9].

In the present study, we developed automated DIA protocols for quantifying CD66b neutrophil, CD163 macrophage and CD8 lymphocyte immunostains in the same cohort of early-stage cervical cancer patients. We assessed the validity of the DIA-estimates by comparing with OAS results of corresponding variables. Furthermore, we tested the prognostic impact of the TA-NLR obtained by DIA in univariate and multivariate analyses of recurrence-free survival and demonstrated this as a strong, efficient prognostic variable with potential for routine application.

MATERIAL AND METHODS

The study included 101 consecutive patients treated with surgery (N=87) or definitive radiotherapy (N=14) for cervical squamous cell type carcinoma of International Federation of Gynaecology and Obstetrics (FIGO) [10] stage IB (N=91) and IIA (N=10) at Aalborg Hospital from 1990 to 2000 [7]. Clinical sub-classification in stage IB1 and IB2 was not available. Mean age was 44 years (22–70 years). At the time of operation 18 patients had lymph node metastases. Adjuvant therapy was given to 23 patients. During the follow-up (median 9.8 years) 31 patients relapsed. Tumour samples were collected from the blocks used for routine pathologic evaluation.

The study was approved by the local Ethics Committee (Case number M-20100011; date 09-02-2010).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumour specimens were sectioned at 2 µm and mounted on glass slides. Primary antibodies were against CD66b (clone G10F5, 1:600, no. 555723, BD Biosciences, USA), CD163 (clone EDHu-1, 1:100, MCA 1853, Abd Serotec, UK), and CD8 (Clone C8/144B, 1:250, M 7103, Dako). Immunohistochemistry was performed using a Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Sections were counterstained with hematoxylin and bluing reagent. The IHC protocols have previously been described in detail [7,8].

Quantitative evaluation of immunostaining

Whole slide images were captured by NanoZoomer 2.0 (Hamamatsu Phototonics K.K., Hamamatsu City, Japan) using the 20X magnification mode. The total tumour area visible and adjacent tumour-associated stroma, excluding areas of large necroses, was outlined as region of interest (ROI) by the observer at low screen magnification (Figure 1A). For assessment of $CD66b^+$ immunostains the DIA protocol was setup to exclude intravascular neutrophils [11] (Figure 1C). For DIA, three automated protocols were developed using Visiomorph DP software (Visiopharm, Denmark) for assessing the densities of CD66b (Figure 1B), CD163, and CD8 immunostain, respectively (i.e. the area of immunostain divided by the tissue area within the ROI). The protocols were devised by assessing 8-10 representative sections and encompassed a series of pre-processing steps (enhancement of red-green-blue colour levels and, if relevant, DAB deconvolution), segmentation (Bayesian classifier), and post-processing steps (mainly morphological operations).

OAS assessments of immune cells in three, non-overlapping tumour compartments (tumour nests, peritumoural and stromal compartments, respectively), were obtained using computer-aided, unbiased stereological sampling techniques (newCAST software, Visiopharm, Denmark), as described in detail previously [7]. The OAS assessment of $CD163^+$ macrophages was performed by point-counting yielding a density of $CD163^+$ immunostain per area of tissue, as macrophages are often elongated and fragmented rendering individual cell profile identification impossible. In contrast, OAS assessments of $CD66b^+$ neutrophil and $CD8^+$ lymphocyte were performed as cell profile counts per area of tissue. For comparison with DIA, results of OAS variables obtained in the global tumour area were calculated from the sum of estimates in all compartments of the tumour, weighted according to sampling intensity.

Statistics

The DIA assessments of CD66b, CD163, and CD8 immunostains were positively skewed and some assessments had a value of 0. Therefore, log-transformation after adding a constant of 1 was applied to achieve approximate normality [12]. Non-parametric spearman rank test was performed to investigate correlations between non-transformed DIA assessments and OAS assessments. Pearson correlation tests were also performed on the log-transformed values, but yielded similar results (data not shown). A linear regression analysis was performed to compare log-transformed values of OAS and DIA assessments of $CD163^+$ macrophage immunostain. A non-parametric Mann-Whitney test was performed to compare variables obtained in patients with and without lymph node metastases. For prognostic analyses, variables were dichotomized at medians or quartiles. Univariate cox regression models were used to assess the hazard ratio (HR) of the DIA and OAS variables. OAS assessments performed in the peritumoural compartment, which in our previous study generally had the highest

discriminatory power [7], were included in the prognostic models for comparison. A Cox proportional hazards model was created to identify independent predictors of RFS, including DIA immunostain densities of CD66b+ neutrophils, CD163+ macrophages, and CD8+ lymphocytes, as well as clinical prognostic variables.
Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA) statistical software. All tests were two-sided and P values less than .05 were considered statistically significant.

RESULTS

The time spend by an experienced observer to obtain OAS densities of immune cells by manual cell counting, using a sampling frame with associated sampling points in automatically selected fields of visions, was approximately 2 min. for delineation of the sampling area and roughly 15 min. per cell variable per patient. In contrast, total observer time spend for DIA was only approximately 2 min. per patient, used for delineation of the ROI. Computation runtime for a single slide was 2–15 min, and the analysis could be performed at any time, with no request of observer assistance.

We observed high correlations between DIA and OAS variables of corresponding parameters (Figure 2A-C). For CD8+ lymphocytes spearman ρ was 0.79 (95% CI 0.68–0.87; P<0.0001); for CD66b+ neutrophils spearman ρ was 0.85 (95% CI 0.75–0.91; P<0.0001); and for CD163+ macrophages spearman ρ was 0.92 (95% CI 0.88–0.95; P<0.0001). The CD8+ lymphocyte and CD66b+ neutrophil assessments are not directly comparable, since the DIA yields an immunostain area fraction, whereas the OAS counts produces a numerical density of immunostained cells per tissue area [7]. Hence the correlation between these variables is not expected to be linear, best illustrated in Figure 2A for neutrophils. Linear regression analysis of the similar DIA obtained and OAS obtained CD163+ macrophage immunostain densities revealed an excellent correlation (R^2=0.86; p<0.0001).

The DIA CD163+ macrophage immunostain area fraction was significantly lower in patients with lymph node metastases (median 0.9% (95% CI 0.6–1.6%)) compared to those without (median 2.0% (95% CI 0.9–3.3%)) (P=0.007), but the two groups were overlapping. No significant correlation among CD66b+ and CD8+ immunostain densities and lymph node metastases was observed.

The prognostic significance of immune cells assessed by DIA in the global tumour area and of previously obtained OAS measurements assessed in the peritumoural compartment [7] is shown for comparison in Table 1. The analysis showed approximate similar predictive effect on outcome of similar parameters.

A multivariate Cox proportional-hazard regression model was used to analyse the relative strength of DIA assessments of CD66b+ neutrophils, CD163+ macrophages, and CD8+ lymphocytes in addition to FIGO stage and lymph node status. High density of CD66b immunostain (HR 2.6; 95% CI 1.2–5.7; P=0.02), low density of CD8 immunostain (HR 2.3; 95% CI 1.1–4.9; P=0.03), and presence of lymph node metastases (HR 2.6; 95% CI 1.2–5.5; P=0.02) were significant independent predictors of poor RFS, whereas clinical stage and CD163 immunostain density were not.

The CD66b/CD8 immunostain index obtained by DIA had excellent discriminatory power for each quartile with 5-year RFS of 92%, 80%, 65%, and 48% for quartile I (<0.019), II (0.02-0.05), III (0.06-0.24), and IV (>0.25), respectively (P=0.001) (Figure 3).

DISCUSSION

In the present study we demonstrated that automated digital image analysis (DIA) assessments of CD66b$^+$ neutrophils, CD163$^+$ macrophages, and CD8$^+$ lymphocytes correlate well with previous results of observer-assisted stereological (OAS) assessments [7,8]. For an experienced observer the observer time consumption was reduced substantially with DIA compared with the OAS method. Variables obtained by DIA and by the OAS techniques had similar ability to identify subgroups of patients with poor and favourable prognosis. In particular, the CD66b/CD8 immunostain index (comparable to the manually assessed TA-NLR) obtained by DIA had excellent discriminatory power, identifying very precisely the risk of cancer recurrence in each quartile of the index. DIA therefore was the most efficient.

Lymphocytes infiltrating cervical cancers have been recognized as prognostically favourable for decades [13-16], and the efficacy of the host immune response to virus-associated antigens and oncogenes seems reflected by the composition, localization and numbers of specific types of lymphocytes *in situ*. For example, in CIN 2-3 lesions HPV-16 infection correlated with low numbers of CD8$^+$ lymphocytes, and stromal CD8$^+$ lymphocyte numbers were independent regression predictors [17]. In contrast, high numbers of tumour-associated CD66b$^+$ neutrophils and CD163$^+$ M2 macrophages are correlated with poor outcome of many malignant diseases [18-20], although some results have been conflicting [21,22]. Neutrophils are involved in crucial steps for cancer development, including increased carcinogenesis, initiation of the angiogenic switch, extravasation of tumour cells, and formation of the "premetastatic niche" [18]. Tumour-promoting M2 macrophages have likewise been implicated in essential steps such as increased inflammation, tumour growth, angiogenesis, and lymph node metastasing [23,24]. The number of infiltrating, tumour-promoting neutrophils relative to tumour-antigen-responsive mononuclear cells may further predict patient outcome as suggested by studies of these cells in the peripheral circulation of cancer patients [18,20,25]. Our results are in line with this hypothesis and to our knowledge, this is the first study to validate and demonstrate a prognostic impact of an intratumoral CD66b/CD8 immunostain index.

Variables obtained by DIA in the global tumour area and by the OAS technique in the peritumoural compartment had similar power to identify subgroups of patients with poor and favourable prognosis. Because OAS assessments of tumour-associated leukocytes performed at the "invasion front" or peritumoural compartment in general were the prognostically most informative, DIA observations obtained exclusively in this tumour compartment may perform better. However, for routine application in a clinical setting the global tumour area is more easily defined, in contrast to the peritumoural compartment that must be defined at higher magnification and is segmentated [26]. Compartment information in DIA may possibly be obtained by specific staining of tumour cells by cytokeratin (KL1) or other tumour-specific markers [27,28], or by more advanced software algorithms [29-31].

An important bias of DIA analysis of IHC stains is non-specific or irrelevant immunostaining. In particular, we observed some CD66b$^+$ immunostaining of necrotic areas, which therefore were excluded from the ROI. Likewise, the CD163$^+$ antibody also stains some tumour cells. However, in the present study we found no impact of this potential bias, as we obtained almost identical results of observer-assisted and DIA assessments of macrophage immunostaining. Software solutions associating immunostains to individual cells of specific morphological types are in some instances available [4], but the robustness of automated morphological cell identification is debatable and must be

validated for specific cell types and protocols. Double staining methods may be applicable in some instances as has been shown in malignant melanoma for DIA assessments of the frequency of Ki67- and phosphohistone-expression in MART1-positive tumour cells [32].

In the present study, we found that DIA assessments of the global tumour density of neutrophil immunostain relative to that of cytotoxic lymphocytes, the CD66b/CD8 immunostain index, had excellent prognostic discriminatory power. This measurement seems highly suited for routine application, robust to heterogeneity and automatically obtainable after quick and simple outlining of an easily definable ROI. The index is obtainable by a trained technician and, potentially, in a single, double-immunostained section. Still, DIA protocols may be sensitive to variations in for instance tissue processing and IHC staining protocols [1,5,6], and the inter-laboratory reproducibility of measurements should therefore be investigated further.

In early stage cervix cancer additional prognostic factors are especially needed to identify high-risk patients, who may benefit the most from adjuvant treatment [33,34], and those at very low risk of recurrence, who may be cured by minor surgery [35]. The results of the CD66b/CD8 immunostain index obtained by DIA should be addressed prospectively in these settings in future studies.

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AUTHOR CONTRIBUTIONS

BSN collected patient data and provided data on lymphocyte counts. AC planned the study with FD and ML. AC, FD, and ML made an equal contribution in data analysis. AC wrote the main parts of the manuscript with support from the other authors. PSN setup the automated digital analysis protocols and aided in the data analysis of the output. TSN provided the technical basis for digitalisation and automated digital analysis and aided in the interpretation of data. All authors revised the manuscript critically and approved the final manuscript.

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Table 1
Univariate comparison of prognostic impact with respect to recurrence-free survival (RFS) of cervical carcinoma, for digital image analysis (DIA) assessments in the global tumour area and for previously obtained observer-assisted stereological (OAS) assessments in the peritumoural compartment. $CD66b^+$ neutrophils, $CD163^+$ macrophages, and $CD8^+$ lymphocytes, stratified at median.

Risk factor	Median cutoff	DIA (HR)	OAS (HR)
$CD66b^+$	> vs. ≤	2.9 (1.3–6.3; P=0.007)	2.1 (1.0–4.5; P=0.04)
$CD8^+$	≤ vs. >	2.4 (1.1–5.1; P=0.02)	3.4 (1.5–7.7; P=0.003)
$CD163^+$	> vs. ≤	2.0 (0.97–4.2; P=0.06)	2.1 (1.0–4.4; P=0.05)

Hazard ratio (HR) for RFS (95% CI; P-value); vs. = versus;

FIGURE LEGENDS

Figure 1. (A) The digital imaging analysis (DIA) allows for discrimination between tissue (blue area) and irrelevant space within the region of interest (ROI; yellow line) (resolution x20). **(B)** DIA determines the density of immunostain (orange) relative to tissue (resolution x200) **(C)** Double staining with CD34/CD66b allows for distinction between intravascular $CD66b^+$ neutrophils and in the tumour nests (resolution x200).

Figure 2. Scatterplots of log+1-transformed values for immunostain density-assessments performed by automated digital image analysis (DIA) (y-axis) versus corresponding variables obtained by observer-assisted stereology (OAS) (x-axis). **(A)** $CD66b^+$ immunostain density vs. numerical density of $CD66b^+$ neutrophils. **(B)** $CD163^+$ macrophage immunostain densities obtained by DIA vs. OAS. **(C)** $CD8^+$ immunostain density vs. numerical density of $CD8^+$ lymphocytes.

Figure 3. Kaplan-Meier plots of recurrence-free survival of 101 patients with localized cervical squamous cell carcinoma according to the CD66b/CD8 immunostain index divided at quartiles. P-value obtained from log-rank test.

Figure 1

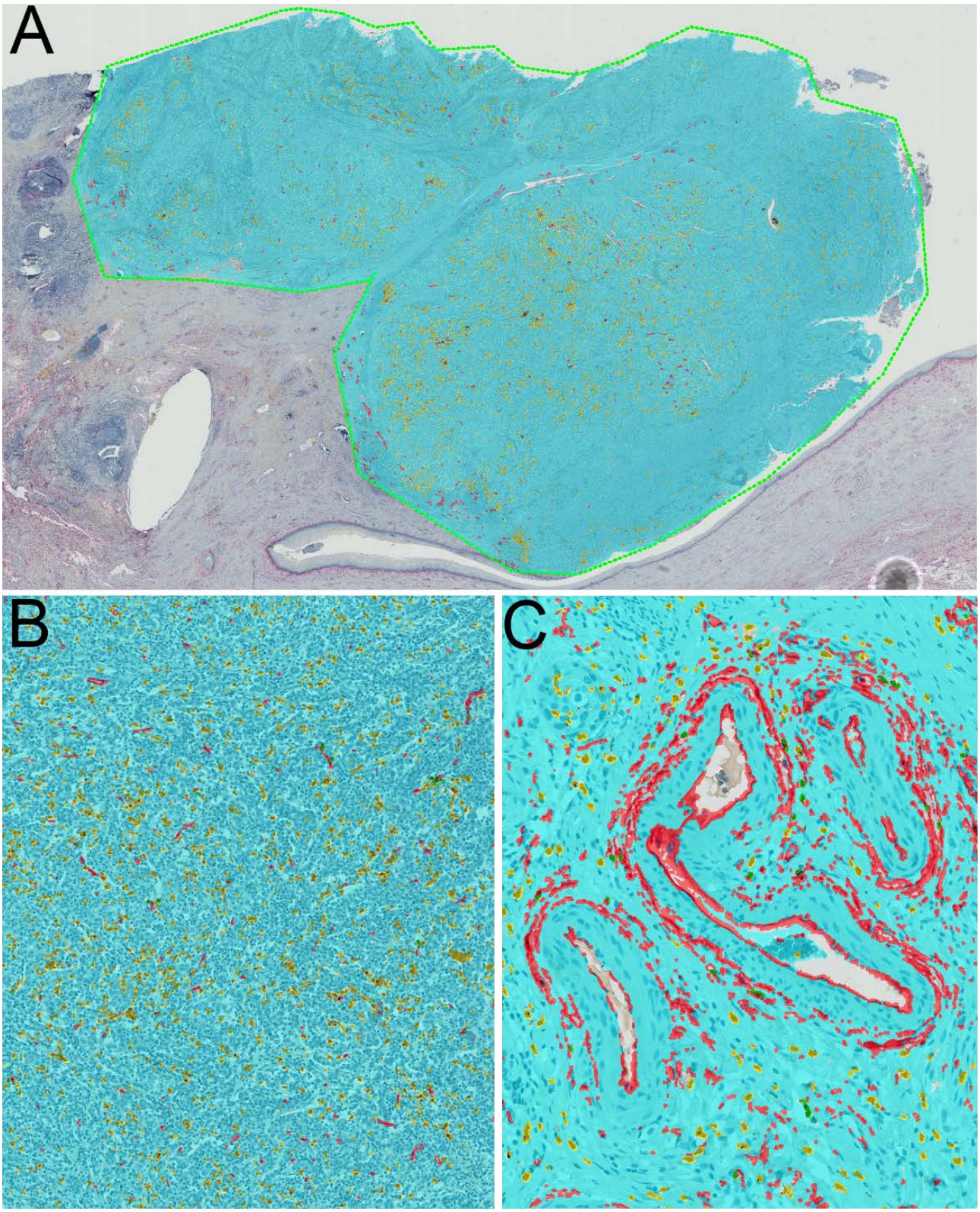


Figure 2

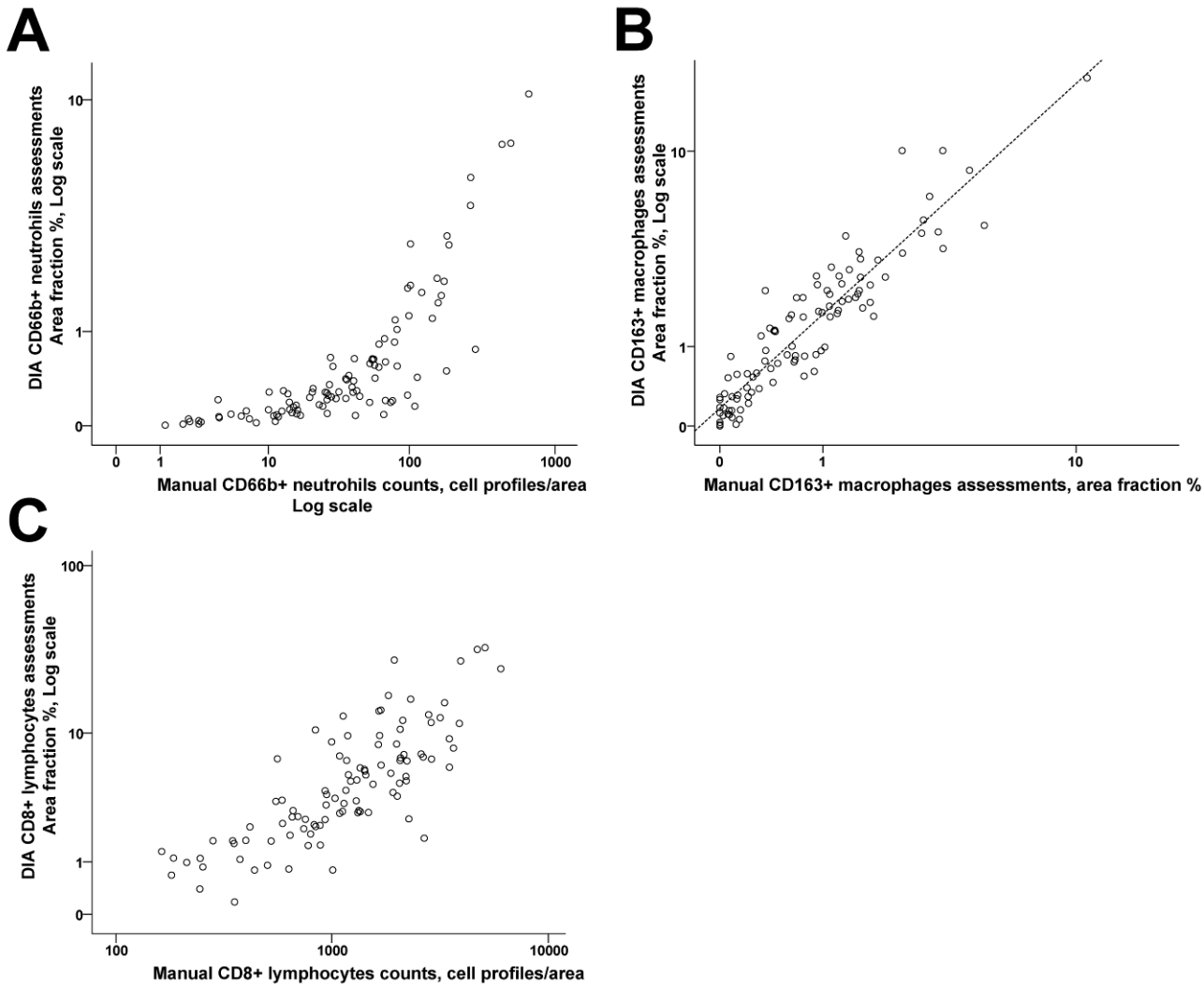
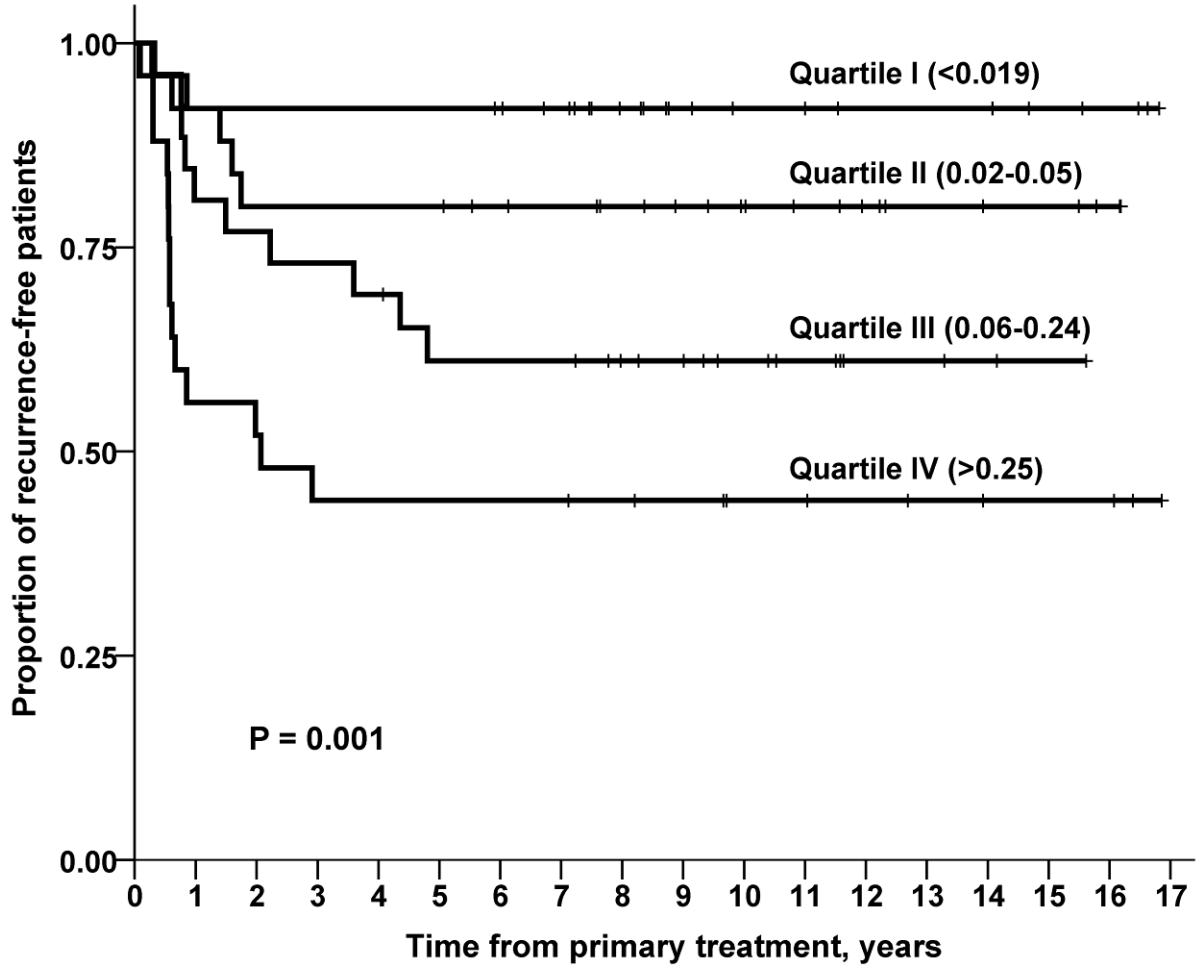

A
DIA CD66b+ neutrohils assessments
Area fraction %, Log scale
10
1
0
0
1
10
100
1000
Manual CD66b+ neutrohils counts, cell profiles/area
Log scale
B
DIA CD163+ macrophages assessments
Area fraction %, Log scale
10
1
0
0
1
10
Manual CD163+ macrophages assessments, area fraction %
C
DIA CD8+ lymphocytes assessments
Area fraction %, Log scale
100
10
1
0
100
1000
10000
Manual CD8+ lymphocytes counts, cell profiles/area

Figure 3


1.00
0.75
0.50
0.25
0.00
Proportion of recurrence-free patients
Quartile I (<0.019)
Quartile II (0.02-0.05)
Quartile III (0.06-0.24)
Quartile IV (>0.25)
P = 0.001
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time from primary treatment, years

5. Discussion and conclusions

5.1 Discussion

Detailed discussions of the different aspects of the thesis are given in the separate manuscripts. Here selected topics and methodological considerations will be further discussed.
The main focus of the present thesis was the prognostic impact of neutrophils and macrophages on patient outcome with the hypothesis that neutrophils and macrophages correlated with harmful effects. This was explored in selected solid tumor types. For assessments in the tumor compartment we used robust, reproducible unbiased stereological methods.

Our main novel result is that CD66b$^+$ neutrophils independently predict recurrence in cervical cancer, both when assessed with stereology and automated digital image analysis (DIA) (Paper II and IV). Cervical cancer is foremost a viral-induced cancer that activates the adaptive immune system and thus previously the main focus of research has been on the favourable role of lymphocytes. However, as in other cancers, adverse effect of elevated WBC, neutrophils, neutrophil/lymphocyte ratio, and monocyte count in the blood has been documented (Table 1). Likewise, a few studies have explored the adverse impact of macrophages in the tumor microenvironment with the relatively unspecific CD68 marker. To our knowledge the impact of neutrophils and CD163$^+$ M2 macrophages in cervical cancer has not been investigated before. Macrophages have been implicated with angiogenesis and lymphangiogenesis in cervical cancer (Table 1) that may facilitate spread to lymph nodes or distant metastasing. Neutrophils likewise harbour characteristics that facilitate angiogenesis and lymphangiogenesis[70]. Generally neutrophils and macrophages share various tumor-promoting features, and are in some cases mutually redundant[38]. CD8 is a glycoprotein expressed on cytotoxic T cells that have the capability of killing tumour cells. The cytotoxic T cells are very important in cancer defence but are dependent on the presence of other cells[71]. The favourable impact of lymphocytes is likely related to either tumor elimination or tumor equilibrium where cancer cells are eliminated or kept at bay[72]. Macrophages on the other hand have been implicated in tumor escape[73]. The fact that neutrophils can inhibit cytotoxic lymphocytes may likewise facilitate tumor-escape[74].
For NSCLC patients we observed no significant correlation with tumor-associated neutrophils or macrophages directly with RFS or OS; however assessments correlated with other poor prognostic factors; CD66b$^+$ assessments correlated with elevated blood CRP and WBC, histologic necrosis, T-stage, and squamous cell histology; CD163$^+$ macrophages correlated with elevated blood CRP, histologic necrosis, N-stage, and squamous cell histology. (Paper I). One previous study has reported the impact of tumor-associated neutrophils[42]. An association with RFS was observed, however, they found no significant effect on OS. Our findings support the latter. For tumor-associated macrophages, studies in NSCLC have used different IHC markers that are unspecific (i.e. CD68), markers of both M1 macrophages (i.e. HLA-DR) and M2 macrophages (i.e. CD163, CD204) (Table 1). Furthermore, researchers have used assessments in whole tissue sections or TMA and different tumor compartments have been explored. Thus some authors report favourable impact of TAMs on patient outcome (M1 macrophages), and others report detrimental impact (M2 macrophages), or no impact (Table 1). In paper I we found no significant impact of CD163$^+$ (M2) macrophages on RFS or OS. In our experience NSCLC sections are often difficult to assess by immunohistochemistry. Many tumors contain significant necrosis and necrotic areas were often heavily stained for CD66b (Appendix, figure A and B). Often aggregation of viable CD66b$^+$ neutrophils in areas adjacent to necrotic areas was observed (Appendix, Figure C and D). As necrosis has been associated with unfavourable prognosis in NSCLC[75] this may in part explain diverse results in different studies. Inflammatory infiltration in the

lungs may also be the result of pneumonia, abscesses, pneumonitis, bronchitis, and chronic obstructive pulmonary disease (COPD) that complicates assessment, and is not directly associated with tumour growth. Moreover, non-small cell lung cancers arise after number of years with exogenous irritants such as smoking, pollutants, asbestos, radon etc. Thus, when cancers become symptomatic they are usually advanced and contain a number of mutations. Hence, a large number of chromosomal abnormalities can be detected, reflecting very complex karyotypes[76]. This further adds to the heterogeneity of NSCLC which suggests that a single prognostic marker may be difficult to find in NSCLC. In addition, the role of manipulation of the immune system has yet to show true promise in NSCLC[77].

An important novel finding in the present thesis was the prognostic impact of combined baseline and nadir neutrophils in both NSCLC and ovarian cancer (Paper III). For NSCLC and ovarian cancer we observed absence of chemotherapy-induced neutropenia as a poor prognostic factor in multivariate analysis. This was independent of relative dose intensity of chemotherapy. Neutrocytosis may be a secondary phenomenon to smoking or use of prednisone, pneumonia or other infections, comorbidity such as coronary disease, diabetes, or adipositas, and constitutional symptoms such as poor performance, weight loss. It may also a marker for increased tumor burden or a more aggressive tumor. Most importantly we identified a poor prognostic group of patients with baseline elevated neutrophils who did not achieve treatment-induced neutropenia. These patients were unlikely to benefit from chemotherapy treatment. However, we also observed an intermediate patient group with baseline elevated neutrophils who achieved chemotherapy-induced neutropenia and had improved OS compared to the poor prognostic group. This may suggest that, for some patients, it is possible to intervene on the adverse effect of neutrocytosis. These results may have clinical impact and should be assessed further prospectively.

We chose to evaluate whole-tissue sections for the surgery cohorts in paper I and II based on previously published methods from our institution[78-80]. Especially for NSCLC, tumors are generally heterogeneous. Thus, concern can be raised whether studies in tissue-micro arrays would yield information that is generalizable to the whole tumor. Some information will be lost as demonstrated by Jensen et al[81]. Another concern as stated by Fridlender et al is "whether using TMA sections is a good idea because you are typically looking at core tumor when the vast majority of tumor associated inflammation tends to be on the edges" [82]. On the other hand, TMA techniques allow for consistent staining conditions and low cost, and stainings can be performed in a short time frame.

Whether our findings in whole tissue sections of selected tumor blocks can be generalized to the whole tumor is uncertain. Especially for NSCLC tumor we were dependent on which tumor blocks were sampled from the lobectomy or pneumonectomy specimens by routine pathology procedures. Furthermore, we selected tumor blocks with as little necrosis as possible (although many still contained considerable necrosis) that could possible skew results. Finally, we did not adjust for tissue shrinkage or expansion that can impact relative parameters (i.e. neutrophil count per tumor area).

Stereological quantification of tumor-associated cells allows for reliable, unbiased, and reproducible estimation. These methods allow for assigning specific observations to different tumor compartments (tumor nests, peritumoral area, and stromal area) in a tumor section. Different studies have demonstrated the microlocalization of tumor-associated neutrophils and macrophages is of importance[83].

A systematic random sampling technique, using an unbiased counting frame, is efficient for obtaining average densities of cell profiles in tissue sections; such as densities of $CD66b^+$ neutrophils. Because of the counting principle of a positive immunohistochemical signal counted only when a visible nucleus was present, the influence of variation in section thickness is reduced. Validation of these two-dimensional variables as compared to true, three-dimensional estimates has been performed previously[69]. In contrast, $CD163^+$ macrophages are highly variable in shape and the cell borders difficult to delineate in paraffin-embedded specimens. Therefore, point-counting of immunostains was chosen as a substitute for cell numbers, which however, yields an area fraction, not a number. Consequently, $CD66b^+$ cell densities and $CD163^+$ area fractions cannot be compared directly.

In the fourth study of cervical cancer, we applied automated digital image analysis (DIA) and compared the results with the manual observer-assisted stereologic sampling (OAS) assessments obtained in study II. Assessment of CD163 immunostain in the OAS study was directly comparable with the area fraction obtained the automated DIA protocol. We observed a good correlation between OAS (all three tumor compartments combined) and DIA results (Study IV, Figure 2). This is indication of a high precision of subsampling with grid count with the manual OAS method and confirms the validity of results obtained with an automated DIA protocol. In the current setup compartmental information was not achievable by the DIA method. However, the results were sufficient for prognostic information and the global tumor density of neutrophil immunostain relative to that of cytotoxic lymphocytes, the $CD66b^+/CD8^+$ immunostain index, had excellent prognostic discriminatory power.

5.2 Conclusions

In summary, the main conclusions of the thesis were

- The densities of tumor-associated CD66b$^+$ neutrophils and CD163$^+$ macrophages in NSCLC were correlated with adverse prognostic factors and peripheral blood inflammation markers, but not directly correlated with RFS or OS.
- Tumour-associated neutrophil count is an independent prognostic factor for short recurrence-free survival in cervical cancer. Combining CD66b and CD8 immunostains may further improve prognostic stratification.
- Elevated baseline blood neutrophil count was an independent adverse prognostic factor in NSCLC. Failure to achieve chemotherapy-induced neutropenia was and independent risk factor in NSCLC and ovarian cancer. Combined baseline elevated blood neutrophils and failure to achieve neutropenia identified a high-risk group for NSCLC.
- Digital image analysis assessments of tumor-associated leukocytes are highly correlated with corresponding variables obtained by observer-assisted stereology and is of similar prognostic impact. The tumour-associated CD66b$^+$ neutrophil/CD8$^+$ lymphocyte immunostain index obtained by DIA is a strong and cost-efficient prognostic variable, which may prove useful for routine application.

6. Perspectives

The identification of tumor-associated CD66b$^+$ neutrophils as prognostic markers for recurrence in cervical cancer is novel. This may have the implications for identifying e.g. subgroups of patients who require closer follow-up or more aggressive therapy. The exact role of these neutrophils needs to be determined and the prognostic significance should be confirmed in larger, prospective studies.

The relationship between the favourable prognostic effect of CD8$^+$ lymphocytes and the negative influence of CD66b$^+$ accumulated in a prognostic index may be an easily achievable marker for patient outcome. Double staining of whole tissue sections would allow for rapid estimates of neutrophil cell profile to lymphocyte cell profile ratio. Cell to cell ratios are statistically robust to heterogeneity and easily obtainable. Computerized image analysis (such as TissuemorphDP, Visiopharm, Denmark) may allow for automatization of this process in a fairly homogeneous tissue such as cervical cancer. This is an objective, efficient, and very reproducible method; however, DIA assessments depending on cell morphology and compartment information may not be feasible. Therefore validation of DIA by comparisons with observer assisted methods and correlation with patient outcome is required.

Our findings of a poor and intermediate prognostic group in patients with NSCLC and ovarian cancer treated with chemotherapy require prospective validation. If validated these findings could have clinical implications. Future studies may focus on the poor prognosis group where chemotherapy seems to have negligible effect. Other therapies that target the tumor microenvironment either alone or in combination with chemotherapy may be necessary.

Chemotherapy affects neutrophils in a non-specific manner and whether pro-tumor neutrophils are specifically affected during chemotherapy treatment is unclear. Strategies to interfere with the pro-tumor function and enhance the antitumor function of neutrophils and macrophages may be an emerging research area.

7. English summary

The primary focus of the present thesis was the prognostic impact of neutrophils and macrophages in patients with selected solid malignant tumors. We also investigated the possible role of intervening elevated blood neutrophils with chemotherapy.

First, in a cohort of patients resected for stage I-IIIA NSCLC between 2003 and 2006 (N=335) we investigated the prognostic impact of peripheral white blood cell counts and $CD66b^+$ neutrophils and $CD163^+$ macrophages in the tumor microenvironment. We identified increased densities of $CD66b^+$ tumor-associated neutrophils (TANs) and $CD163^+$ macrophages (TAMs) as correlated with adverse prognostic factors, but not directly correlated with recurrence-free survival (RFS) or overall survival (OS). Elevated densities were correlated with lymph node positive disease which suggests that tumor-associated macrophages can facilitate regional lymph node spread.

Second, in a cohort of patients with FIGO stage IB/IIA squamous cell cervical cancer treated from 1990 to 2000 (N=101) we explored the prognostic impact of $CD66b^+$ TANs and $CD163^+$ TAMs in the tumor microenvironment. We identified TANs as an independent poor prognostic factor for recurrence-free survival, whereas TAMs were not independently correlated with RFS. Observations performed in the peritumoral compartment were in general prognostically most informative. Furthermore, an index of intratumoral and peritumoral $CD66b^+$ neutrophils to $CD8^+$ lymphocytes had good discriminatory power for each quartile with 5-year RFS of 92%, 80%, 62%, and 44%.

Third, we studied patients with stage III-IV NSCLC (N=111) and stage I-IV (N=118) ovarian cancer treated according to a chemotherapy-toxicity adjusted protocol. We identified elevated baseline blood neutrophils as an independent adverse factor for overall survival in NSCLC. Chemotherapy-induced neutropenia was correlated with a favourable outcome in NSCLC as well as ovarian cancer. Patients with elevated blood neutrophils and failure to obtain neutropenia during chemotherapy had the poorest prognosis, but patients that obtained neutropenia during chemotherapy had a better prognosis despite elevated baseline neutrophils. It may therefore be hypothesized that chemotherapy can intervene the negative impact of elevated blood neutrophils.

Finally, we applied an automated digital analysis protocol to the cohort of 101 cervical cancer patients. This protocol did not give compartmental information but produced highly comparable assessments as the manual stereological protocols. The tumour-associated $CD66b^+$ neutrophil/$CD8^+$ lymphocyte index obtained by DIA was a strong and cost-efficient prognostic variable, which should be investigated further in larger, prospective studies.

In conclusion, we confirmed that increased blood neutrophils are a poor prognostic marker. Tumor-associated neutrophils were correlated with poor prognosis in cervical cancer, but not in NSCLC. Increased densities of tumor-associated macrophages are correlated with lymph node metastases in both NSCLC and cervix cancer. We furthermore hypothesize that the seemingly detrimental effect of elevated blood neutrophils might be intervened with chemotherapy exemplified for ovarian cancer and NSCLC patients.

8. Danish summary

Fokus for den aktuelle Ph.d. afhandling var at undersøge den prognostiske betydning af tumor-associerede neutrofilocytter (TANs) og makrofager (TAMs) hos patienter med solide tumorer. Endvidere undersøgte vi om en toksicitets-justeret kemoterapi protokol kunne modvirke den ugunstige effekt af forhøjede niveauer af blod neutrofilocytter.

I det første studie af patienter med stadium I-IIIA NSCLC (N=335) behandlet mellem 2003 og 2006 undersøgte vi den prognostiske betydning af leukocyttallet i blodet samt betydningen af CD66b$^+$ TAN og CD163$^+$ TAM. Vi fandt at øgede densiteter af CD66b$^+$ TAN og CD163$^+$ TAM var associerede med andre ufavorable prognostiske faktorer, men der var ingen direkte associering med recidiv-fri overlevelse (RFS) eller generel overlevelse (OS). Øgede densiteter af TAM var associerede med lymfeknudemetastasering, hvilket antyder at TAMs kan medvirke til lymfogen spredning.

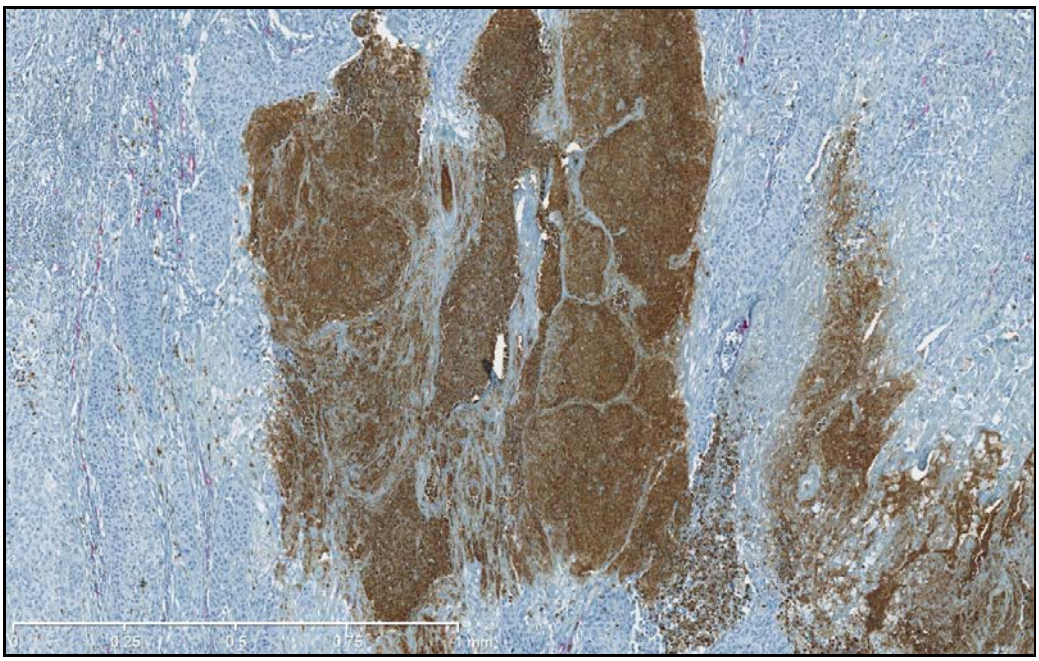
I det andet studie af patienter med FIGO stadium IB og IIA cervix cancer (N=101) behandlet mellem 1990 og 2000 undersøgte vi den prognostiske betydning af CD66b$^+$ TANs og CD163$^+$ TAMs i tumor mikromiljøet. Vi fandt at TANs var en multivariat uafhængige faktor for RFS, hvorimod TAMs ikke havde nogen selvstændig betydning. Især TANs peritumoralt havde prognostisk betydning. Et index mellem intratumorale og peritumorale CD66b$^+$ TANs divideret med intratumorale og peritumorale CD8$^+$ lymfocytter kunne separere patienter i forhold til 5 års RFS i hvert kvartil på hhv. 92%, 80%, 62%, and 44%.

I det tredje studie undersøgte vi patienter med stadium III-IV NSCLC (N=111) og stadium I-IV æggestokkekræft (N=114) som blev behandlet i forhold til en toksicitets-justeret kemoterapi protokol. For NSCLC var et forhøjet neutrofilocyt tal i blodet en uafhængig prognostisk faktor for OS. kemoterapi-indiceret neutropeni var korreleret med bedre OS for patienter med både NSCLC og æggestokkekræft. Vi identificerede en gruppe af patienter med specielt dårlig prognose med forhøjede baseline neutrofilocyt tal i blodet der ikke opnåede neutropeni under kemoterapi behandlingen. Patienter der opnåede neutropeni havde en bedre prognose trods forhøjet baseline neutrofilocyt tal. Vi opstiller derfor en hypotese at kemoterapi kan modvirke den ufavorable effekt af forhøjet neutrofil tal i blodet.

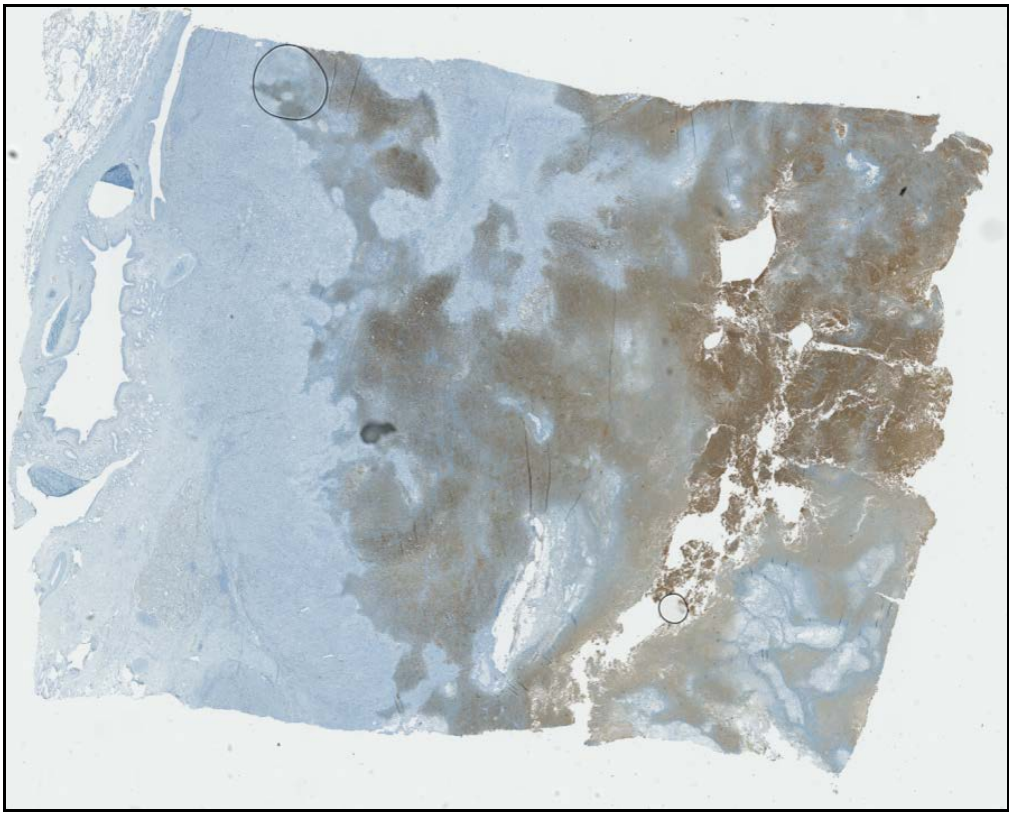
I det fjerde studie undersøgte vi betydningen af automatisk computerbaseret digital billedanalyse (DIA) af kohorten med 101 livmoderhalskræftpatienter. Dette gav sammenlignelige resultater som manuel stereologisk evaluering, men kunne kun vurdere densiteter i hele tælleområdet og ikke i de forskellige tumor sektioner. Et index mellem CD66b$^+$ neutrofile og CD8$^+$ lymfocytter via DIA var en omkostningseffektiv, stærk prognostisk faktor.

Konklusivt identificerede vi forhøjet baseline neutrofilocyt tal som en ufavorabel prognostisk markør. Tumor-associerede neutrofile var korreleret med dårlig prognose ved patienter med livmoderhalskræft, men ikke ved patienter med NSCLC. Øgede densiteter af TAMs var korrelereret med lymfeknudemetastaser ved både NSCLC og livmoderhalskræft. Endvidere opstillede vi hypotesen at kemoterapi kan modvirke den ufavorable effekt af forhøjet neutrofil tal i blodet som vist ved æggestokkekræft og NSCLC.

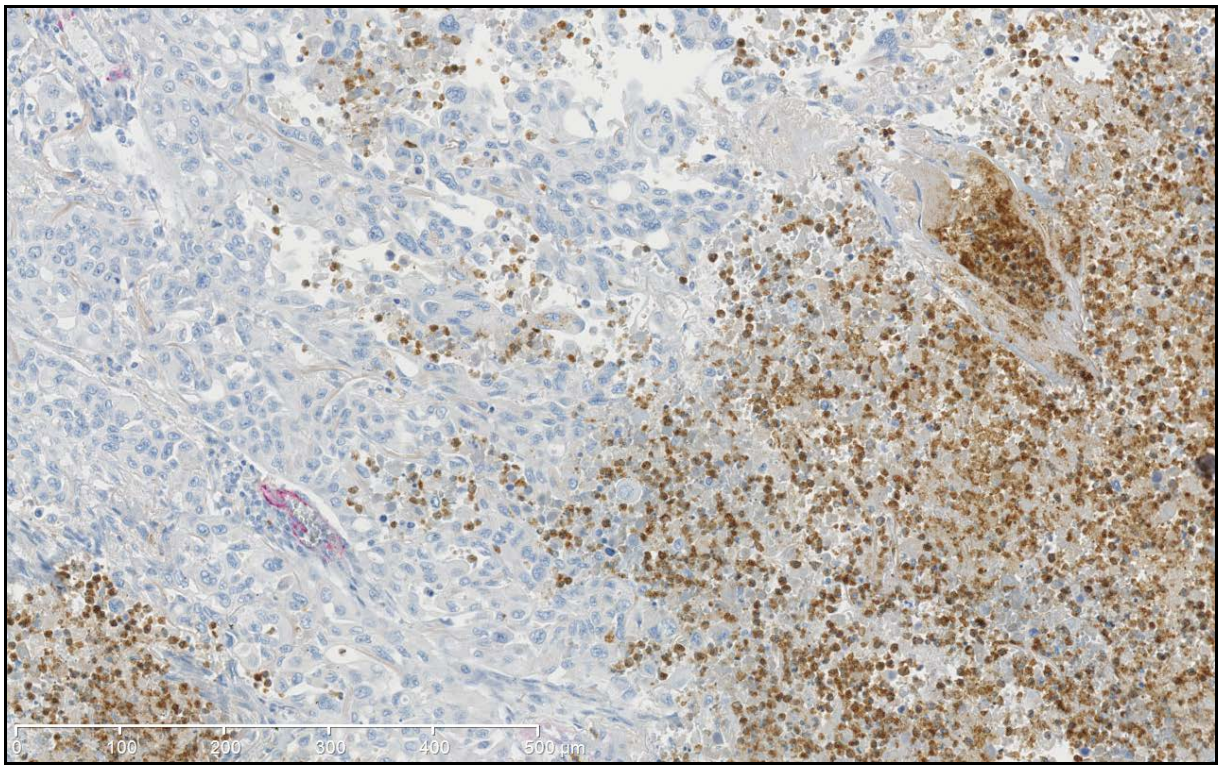
9. Appendix



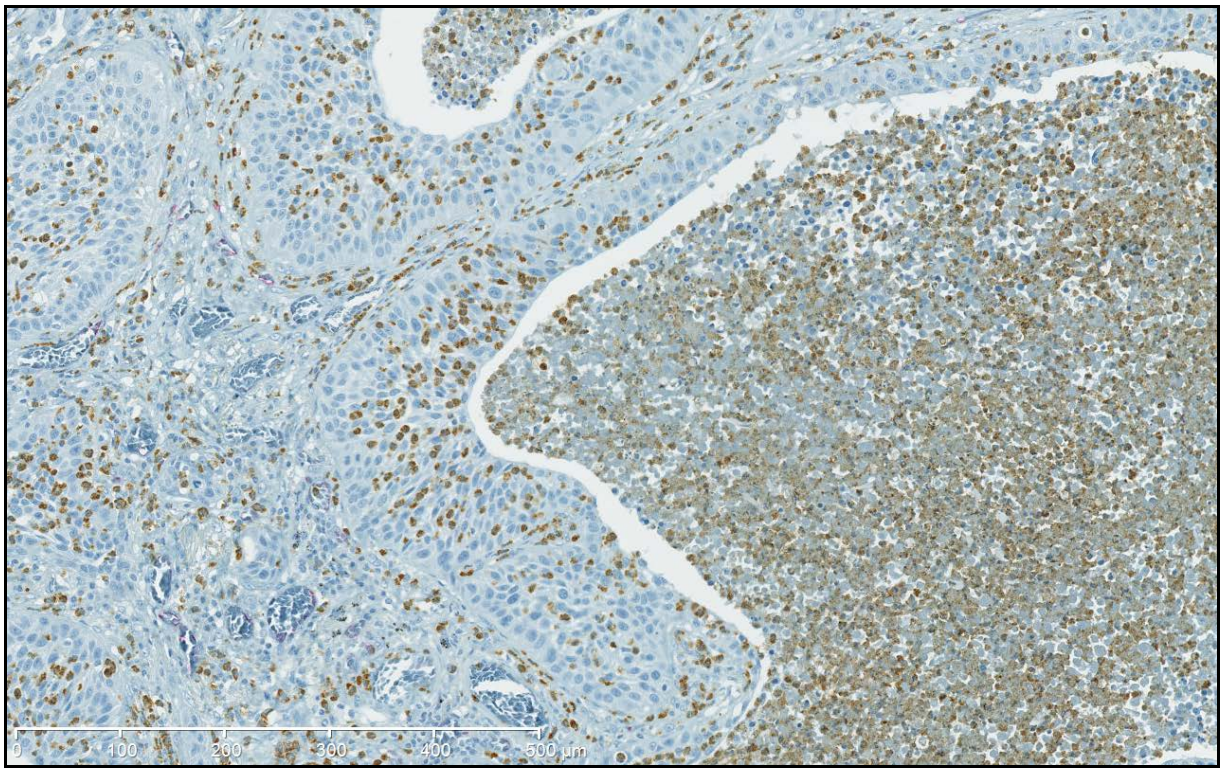
Appendix, figure A. Necrotic areas often stain heavily postive for CD66b.



Appendix, figure B. Some tumors are heavilynecrotic and necrotic areas are positive for CD66b staining.



Appendix, Figure C. Necrotic areas are sometimes with viable CD66b⁺ neutrophils in the adjacent tumor area



Appendix, Figure D. Tumor nests adjacent to necrotic areas are sometimes heavily infiltrated by CD66b⁺ neutrophils.

10. References

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10.2 References for table 1A and 1B

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