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CARDIOPULMONARY ADAPTION TO ONE-LUNG VENTILATION AND NEOADJUVANT THERAPY DURING TREATMENT OF ESOPHAGEAL CANCER

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CARDIOPULMONARY ADAPTION TO ONE-LUNG VENTILATION AND NEOADJUVANT THERAPY DURING TREATMENT OF ESOPHAGEAL CANCER
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family.

ABSTRACT

Introduction: Esophagectomy is the main form of curative treatment for esophageal cancer and has a high morbidity rate. Neoadjuvant chemo- (CT) or chemoradiotherapy (CRT) is employed to increase long-term survival. To improve perioperative care the effects of measures taken to improve surgical exposure i.e. one-lung ventilation (OLV) and the effects of adding radio- to chemotherapy needs to be understood.

Aims

- **I.** Compare inflammatory response and factors regulation pulmonary vascular tone after one- and two-lung ventilation during esophageal resection (paper I-II).
- **II.** Compare the effects of chemotherapy and chemoradiotherapy on cardiac function, perioperative hemodynamics and inflammatory response (paper III-V).

Methods: Study one (papers I-II) was a randomised controlled trial comparing one- (n=16) vs. two-lung ventilation (n=14) during esophagectomy. Cytokines, complement activation markers, nitrite and endothelin were measured in plasma during and after surgery. Lung biopsies acquired before and after the thoracic part of the operation were accesses for levels of iNOS and vascular congestion. Study two (papers III-V) was a single centre cohort from within a multi-centre randomised controlled trial comparing CT vs. CRT regarding complete histological response. In paper III cardiac function following neoadjuvant treatment was evaluated in 40 patients as intention to treat (CT n=23, CRT n=17) using echocardiography with strain and tissue doppler analysis as well as plasma NT-proBNP. In paper IV the perioperative hemodynamic profile was investigated in 31 patients as per protocol (CT n=17, CRT n=14) using LiDCOplus. Measurements were performed before, during and after surgery. In addition plasma NT-proBNP and Troponin T were measured repeatedly. In paper V, concerning the same group of patients as in paper IV cytokines and complement activation markers in plasma were measured before, during and after surgery. In perioperative lung biopsies cytokine mRNA was measured and the number of CD45 positive cells counted.
Results: In study one, inflammatory markers, factors regulating pulmonary vascular tone, iNOS levels and pulmonary congestion were similar between the groups apart from the terminal complement complex, C5b-9, which was increased in the OLV group on the 3rd and 10th postoperative day. In study two, a decrease of septal systolic function and global diastolic function was seen in the CRT group as well as a lower preoperative cardiac index. However, when challenged by surgery a similar hemodynamic profile was seen in both groups. IL-β mRNA was higher in lung biopsies from patients that had received CRT, but there were no differences regarding systemic inflammation. Respiratory complications and other types of morbidity were similar in the CRT and CT groups.
Conclusions: One-lung ventilation during esophagectomy increases activation of the complement system compared to two-lung ventilation but does not appear to induce factors related to pulmonary vascular tone. CRT induces a slight impairment of cardiac function compared to CT, an effect that did not persist when the cardiovascular system was challenged by surgery. Thus CRT as administered in this study appears safe from a cardiovascular perspective. However, CRT increases local inflammation in the lung, which might affect postoperative morbidity.

LIST OF SCIENTIFIC PAPERS

I. **One-Lung Ventilation During Thoracoabdominal Esophagectomy Elicits Complement Activation**
J. A. Tsai, **M. Lund**, L. Lundell, K. Nilsson-Ekdahl.
Journal of Surgical Research, 2009 ,152, 331-7

II. **Nitric oxide and endothelin-1 release after one-lung ventilation during thoracoabdominal oesophagectomy**
M. Lund, L. Ny, R. E. Malmström, J. O. Lundberg, Å. Öst, M. Björnstedt, L. Lundell, J. A. Tsai
Diseases of the Esophagus 2013, 26(8):853-8.

III. **Effects on heart function of neoadjuvant chemotherapy and chemoradiotherapy in patients with cancer in the oesophagus or gastroesophageal junction - a prospective cohort pilot study within a randomized clinical trial.**
M. Lund, G. Alexandersson von Döbeln, M. Nilsson, R. Winter, L. Lundell, J. A. Tsai, S. Kalman.
Radiation Oncology, 2015, 10, 1-9

IV. **Effects of neoadjuvant chemo- or chemoradiotherapy for oesophageal cancer on perioperative hemodynamics– a prospective cohort study within a randomized clinical trial**
M. Lund, J. A. Tsai, M. Nilsson, R. Winter, L. Lundell, S. Kalman.
Submitted

V. **Postoperative inflammatory response after neoadjuvant chemoradiotherapy or chemoradiotherapy for cancer of the oesophagus or gastroesophageal junction – a substudy within a randomized trial**
M. Lund, H. Kozarcanin, T-E. Mollnes, K. Nilsson-Ekdahl, M. Rydén, M. Nilsson, L. Lundell, S. Kalman, J. A. Tsai
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
CI	Cardiac index
CO	Cardiac output
CRT	Chemoradiotherapy
CT	Chemotherapy
DO_2I	Oxygen delivery index
EA	Esophageal adenocarcinoma
EDV	End diastolic volume
EF	Ejection fraction
ESCC	Esophageal squamous cell carcinoma
GE-junction	Gastor esophageal junction
GS	Global strain
HR	Heart rate
IL	Interleukin
iNOS	Inducible nitric oxide synthases
MAP	Mean arterial pressure
NO	Nitric oxide
NT-proBNP	N-Terminal Pro-B-Type Natriuretic Peptide
OLV	One-lung ventilation
PEEP	Positive end expiratory pressure
POD	Postoperative day
SV	Stroke volume
SVI	Stroke volume index
SVR	Systemic vascular resistance
TAT	Thrombin antithrombin III complex
TLV	Two-lung ventilation

1 INTRODUCTION

1.1.1 Etiology of esophageal cancer

Already in the 1930s Craver and Watson reported excessive alcohol intake, smoking, low socioeconomic status, poor oral health and intake of hot drinks as risk factors for esophageal cancer[1]. Esophageal cancer is classified as into two subtypes based on histology, and their aetiologies differ. Esophageal squamous cell carcinoma (ESCC) has been and still is the dominant cancer type worldwide. However, since the 1970s there has been a steady increase in the incidence of esophageal adenocarcinoma (EA) in the distal third of the esophagus and in particular in the gastro-esophageal junction (GE-junction). High alcohol consumption, smoking, low socioeconomic status and low intake of fresh fruit and vegetables are still considered as risk factors for ESCC[2]. With the exception of smoking, these risk factors seem to be less important for the development of EA. Gastroesophageal reflux and obesity, which also may be related to each other, have been identified as the predominant risk factors for EA in the Western world[2]. The decreased incidence of Helicobacter pylori in the Western populations may be of importance for the pathogenesis although this theory is controversial[3].

1.1.2 Incidence and prevalence

Esophageal cancer is the 8th most common cancer in the world and is, because of its poor prognosis, ranked 6th in mortality[4]. The incidence of ESCC is stable or even declining slightly whereas the incidence of EA is increasing rapidly in the western world, where EA is now becoming the predominant form of esophageal cancer. The worldwide incidence of EA in 2012 was approximately 52 000 cases and the corresponding rate for ESCC was 398 000 cases[5]. In Sweden the age standardised incidence rates for have been reported to be 2.1 per 100 000 population for men and 0.4 per 100 000 for women regarding EA. These values are comparable to many European countries with the exception of national hot spots such as the United Kingdom (7.2) and The Netherlands (7.1). Corresponding values for ESCC in Sweden are 1.5 and 0.7[5]. The average incidence increase for EA was calculated to be 4.9% annually in men and 3.9 % in women between 1970 and 2004[6]. Esophageal cancer occurs mainly in males with a risk of ESCC being 3-4 times higher and the risk of EA 7-10 times higher in men compared to women [7]. Moreover these tumours develop predominantly in older persons (>65 years).

1.1.3 Prognosis

The long term survival for patients suffering from esophageal cancer continues to be poor. Early symptom may be vague and at the time of symptom driven medical consultations up to 70 % of the patients are found to be incurable due to advanced or metastatic disease [8]. Many patients are also considered inoperable because of concomitant disease. In Sweden 28 % of patients with esophageal cancer underwent curative resection 2006-2013[9]. Overall 5-year survival irrespective of treatment is 16.9 %, with only minor differences between genders and ethnic backgrounds[10]. Survival after treatment with curative intent have improved over the

past decades possibly because of better staging with exclusion of patients with metastatic disease, but also due to advances in surgical technique, postoperative care and the introduction of neoadjuvant therapy. Five-year survival after curative treatment is now reported to be as high as 44.4 %[11] and survival rates tend to be even higher in clinical trials compared to real life population-based studies. In Sweden, five-year survival after esophageal resection with curative intent was 38 % during 2011-2014[9]. Most patients die from recurrent local or metastatic disease mainly in the liver, lungs or bone despite curative treatment. Tumour stage is an important factor for long term survival[9].

1.2 TREATMENT MODALITIES FOR ESOPHAGEAL CANCER

1.2.1 Diagnosis and staging

Confirmation of disease is done by endoscopy with biopsy for histopathological analysis and this also helps to define the tumour level; upper-, middle- or lower-esophagus, or GE junction. The determination of the tumour level at the GE-junction is important for deciding treatment modality and may be challenging if a hiatal hernia is present or because the actual GE-junction may be obscured by the tumour. Further staging is performed with computerized tomography, preferably with the addition of positron emission tomography to increase the sensitivity for detecting metastatic disease.

1.2.2 Assessment for surgery and patient comorbidities

Patients with esophageal cancer are often burden by comorbidities and due to the invasiveness of esophagectomy, preoperative risk assessment is of the highest significance. Several risk scoring systems have been proposed to facilitate selection of patients for surgery, but their usefulness has often been found to be limited due to overestimating mortality and failing to identify patients at risk for severe morbidity precisely enough[12, 13]. Few data exist regarding patients >80 years, and the effect of age *per se* has not convincingly been demonstrated[14, 15]. Historically, esophageal cancer patients have been treated as one group but with the increasing incidence of EA this needs to be diversified. Patients with EA are often over weight to obese. Accordingly, diabetes, hypertension and cardiovascular diseases are frequently seen as comorbidities. In addition to these risk profiles, patient with squamous cell carcinoma often present with a history of alcohol abuse. Hypertension and cardiovascular disease are also common in this group and in both groups smoking and chronic obstructive pulmonary disease are common. All of these comorbidities are known to have an adverse impact on the postoperative course and should be optimally treated before surgery. Patients with esophageal cancer may also suffer from malnutrition, which adds another risk factor for postoperative morbidity[16].

Evaluation of respiratory function by spirometry is a valuable tool and a forced vital capacity <80 % or a forced expiratory volume in one second <70 % have been linked to both pulmonary and non-pulmonary postoperative complications[17]. Medication to relieve obstructive complaints should be optimised and preoperative smoking cessation is imperative. Preoperative respiratory muscle training (as supervised by a physiotherapist)

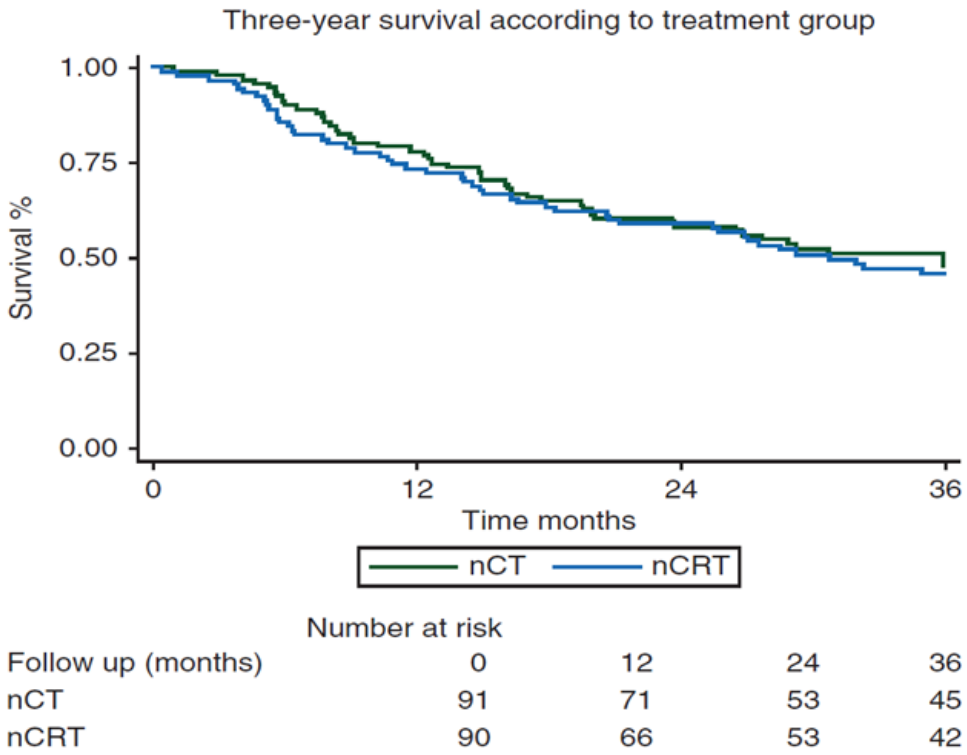
might also be of value[18]. A cardiac exercise test or cardiopulmonary exercise test where maximum work capacity, anaerobic threshold and maximum oxygen uptake are measured, is one of the best evaluated tests in the preoperative testing of each individual patient to objectify general physical capacity and thereafter the postoperative risk. However further research is needed to increase the discriminatory ability of these tests[19-21].

1.2.3 Oncological treatment

During the last decade studies have accumulated to show that neoadjuvant oncological treatment for esophageal cancer improves survival and neoadjuvant treatment is therefore regarded as standard of care in many countries today. Meta-analyses have shown that neoadjuvant chemotherapy (CT) as well as chemoradiotherapy (CRT) increase overall survival[22] even though there is substantial heterogeneity within the studies regarding tumour types, radiation doses, chemotherapy regimens, methods of preoperative staging and treatment delivery, as well as sample sizes in the respective studies. Chemotherapy is most often administered as a combination of drugs given in two to three cycles. Typically platinum-based drugs are combined with 5-Fluorouracil. Recently a combination of carboplatin and paclitaxel showed good results with good treatment tolerance [23]. However no real consensus exists regarding the optimal treatment plan. For adenocarcinoma perioperative chemotherapy is also an option and have shown improved survival rates compared to surgery alone[24]. Studies comparing neoadjuvant and perioperative chemotherapy are currently ongoing.

In all modern protocols radiotherapy is given concurrently with chemotherapy. Different regimes have been used in published studies with doses ranging from 1.75-3.7 Gy/fraction and total dose ranging from 35-50.4 Gy[22]. Doses above 2 Gy/fraction are avoided nowadays. It is of particular importance to reduce radiation toxicity to adjacent organs, primarily the lungs. In a study by Lee et al it was reported that postoperative pulmonary complications increased significantly when more than 40% of the total lung received radiation doses higher than 10 Gy (V10>40 %) [25]. Further studies by Wang indicated that the percentage of lung tissue spared from doses >5 Gy might be an even better indicator[26].

Whether neoadjuvant CT or CRT is the best approach to increase long term survival is debated. Only two randomised head to head comparative studies have explored this important question[27, 28] but none of them was large enough to offer robust conclusions regarding e.g. long term survival. The NeoRes study, from which a cohort of patients was further studied in this thesis, was initiated to investigate the rate of complete histological response after neoadjuvant CT vs. CRT. Complete histological response has been proposed as a marker of increased long term survival[29, 30]. There are also concerns that neoadjuvant CRT may increase postoperative morbidity and mortality but the results are conflicting[23, 31-33]. Little is known about the underlying physiological mechanisms for this possible increase in morbidity and mortality from neoadjuvant CRT. Neoadjuvant CT has on the other hand never been shown to increase postoperative complications compared to surgery alone[22].



nCT, neoadjuvant chemotherapy; nCRT, naoadjuvant chemoradiotherapy

Figure 1. Kaplan–Meier plot of overall 3-year survival according to treatment group in the NeoRes study. Reproduced with permission from Annals of Oncology (Klevebro, Nilsson 2016).

Recently, during the preparation of this thesis, the main results from the NeoRes study were published. Complete histological response, which was the primary outcome was achieved in 28 % in the CRT group vs. 9 % in the CT group, p=0.002. CRT also yielded a higher R0 resection rate (87 % vs. 74 %, p=0.04), and a lower frequency of lymph-node metastases (35 % vs. 62%, p=0.001). However this did not translate into an increased 3-year survival rate (CRT 47 % vs. CT 49 %, p=0.77, Figure 1)[34]. There was also an increased mortality in the CRT group during the first year following randomisation from causes other than tumour recurrence (CRT: 11 out of 24 deaths, CT: 3 out of 20 deaths, p = 0.036).

Patients with ESCC can also be treated with definitive CRT, which includes a higher radiation dose compared to neoadjuvant treatment. Studies comparing definitive CRT with curative intent for ESCC have shown results comparable to neoadjuvant treatment and surgery[35-38], but these studies have been hampered by a substantial degree of protocol deviation. In patients who respond poorly to definitive CRT or have a local recurrence, rescue esophagectomy that carries a high risk of postoperative mortality can be performed in selected cases, provided that there is no metastatic disease[39]. Whether neoadjuvant therapy followed by resection or definitive CRT followed by rescue esophagectomy in selected cases is the best treatment for ESCC has yet to be determined in future studies.

1.2.4 Surgical technique

Historically surgery has been the curative treatment for esophageal cancer. The first transthoracic esophagectomy was performed by Franz Torek in 1913[40]. Today several techniques are available depending on tumour localisation and the need for lymphadenectomy. For T1a disease (no invasion of submucosa) organ sparing endoscopic resection and if present, eradication of residual Barret's esophagus mostly with radiofrequency ablation has >95 % local control rate[41]. The widely used two field open Ivor Lewis approach starts with a laparotomy to mobilise the stomach and resect the gastro-omental, gastric and celiac trunk lymph nodes. The stomach is usually formed into a narrow gastric tube. Thereafter the patient is turned into the left lateral position for a right posterior thoracotomy which allows access for resection of the esophagus and mediastinal lymph nodes. The gastric conduit is then anastomosed to the remnant esophagus in the thorax to restore intestinal continuity. Ivor-Lewis esophagectomy is performed for lesions of the GE-junction and lower or middle esophagus. For more proximal tumours, the McKewn procedure, which is performed in the reversed order and finished with a cervical incision for the anastomosis is applied. Trans-hiatal esophagectomy which excludes thoracotomy and therefore necessitates blind dissection of the upper thoracic oesophagus can be applied for distal tumours and an extended total gastrectomy including the distal esophagus can be performed for tumours in the GE-junction. These procedures have the advantage of being less invasive than a thoracoabdominal procedure, but do not allow for a complete mediastinal lymphadenectomy. In later years minimally invasive procedures involving laparoscopy and thoracoscopy either together or combined with open surgery have been developed and introduced to an increasing extent. So far results have mainly been obtained from non-randomised studies. These have shown benefits in favour of the minimally invasive approach including decreased major morbidity, pulmonary complications, anastomotic leakage, mortality, length of hospital stay, operating time and blood loss [42]. One multicentre randomised trial comparing total minimally invasive and open approach was in favour of the minimally invasive approach regarding postoperative complications and quality of life and a another study comparing laparoscopically-assisted with open esophagectomy showed similar results[43].

1.2.5 Anaesthetic technique

Anaesthesia for esophageal resection is challenging. Although ventilation of both lungs is feasible during the thoracic part of the procedure, commonly the right lung is deflated during open thoracotomy to facilitate the surgical access and exposure. A thoracic epidural is the main form of pain control and has been shown to decrease pulmonary complications[44, 45] and endorse early extubation as well as allowing early mobilization of the patient[46]. Early extubation is nowadays used routinely in clinical practice. Fluid administration is a major concern as excessive fluids both during and after surgery may be linked to increased morbidity[47] although this was not confirmed in a recent meta-analysis[48]. The concept of goal-directed therapy can be used to guide fluid administration. However, the application of goal-

directed therapies varies and these can also be questioned regarding their effectiveness although a decreased morbidity has been claimed[49]. It also appears that the group to benefit most from such therapy is the high risk surgical group, i.e. as represented by those undergoing an esophageal resection[50]. Hypovolaemia also needs to be avoided as impaired tissue perfusion will decrease organ function and might negatively affect the healing processes of the anastomosis. On the other hand the use of vasopressors to increase mean arterial pressure (MAP) did not affect the anastomotic micro circulation when studied in an animal experimental model[51]. However, the answer to the most important question: How different fluid regimes and vasoactive therapies affect the microcirculation in the anastomosis remains elusive, as an adequate methodology to measure anastomotic circulation over time in vivo is currently lacking. Recently, enhanced recovery protocols including early mobilization of the patients have reported decreased length of stay as well as decreased cardio- respiratory morbidity compared to conventional postoperative care[52, 53], albeit that robust scientific evidence behind the true relevance of the various components contained within these enhancement recovery programs is lacking. The type of anaesthetic used during surgery can affect the inflammatory response but results are conflicting and no clear advantage of any particular agent is currently known[54].

1.2.5.1 One Lung Ventilation

One-lung ventilation (OLV) with deflation of the right lung has traditionally been used during the open, thoracic part of the operation to facilitate surgical access and exposure. OLV is associated with a number of potential drawbacks such as the risk of oxygen toxicity by free radical formation after re-oxygenation. This risk is generated by high FiO_2 delivered to the ventilated lung and also from increased production of oxygen radicals at the re-ventilation of the occluded lung. Other risks related to OLV are barotrauma, atelectasis and possibly ischaemia-reperfusion injury[55], which adds to the already high risk of postoperative pulmonary complications seen after esophagectomy. During OLV pulmonary shunting increases from 10 to 30% due to hypoxic vasoconstriction in the non-ventilated lung[56]. Surprisingly this does not increase net lung vascular resistance when pulmonary hypoxia is not global, but the underlying mechanisms are poorly understood[57, 58]. Protective strategies consist of reducing plateau pressures to below 25 cm H_2O, low tidal volumes, pressure controlled ventilation, dependent lung positive end expiratory pressure (PEEP) and permissive hypercapnia[59]. These strategies have been shown to dampen some of the inflammatory responses caused by one-lung ventilation[60].The concept of protective ventilation is mainly carried over from the ARDSNet trial in which an improved outcome in patients with acute respiratory distress syndrome (ARDS) was demonstrated[61]. OLV may even trigger the inflammatory response to surgery, possibly originating mainly from the ventilated lung. This reaction may then be maintained locally over the following days as revealed by increased cytokine concentrations in pleural drains fluid compared to plasma[62].

1.3 COMPLICATIONS AFTER ESOPHAGECTOMY

The overall postoperative complication rate after esophageal surgery is high with a morbidity in the range of 30-50%[23, 63]and an in hospital mortality in the range of 2.9-5.3 %[64]. Respiratory complications are the most common and include acute lung injury, ARDS, pneumonia and respiratory insufficiency. Combined thoracoabdominal incision, micro- or macro aspiration from the gastric conduit, fluid overload and comorbidities (smoking and alcohol abstinence) contribute to the risk for respiratory complications. Early mobilization and the ability to cough are important preventive as well as treatment strategies. Atrial fibrillation is the most common cardiovascular complication affecting 20-25 % of the patients[65, 66]. This may also be the first symptom of a surgical complication and it is therefore important always to search for background-triggering factors[65, 67]. Besides surgical complications, fluid overload, local inflammation, partial vagal denervation and inability to continue regular beta-blockers may trigger atrial fibrillation. Anastomotic leakage is the most important surgical factor behind the high mortality after esophagectomy[68]. Other complications specific for this surgical procedure include chylothorax from thoracic duct leakage and recurrent laryngeal nerve palsy. To prevent severe complications early detection is fundamentally important. This requires familiarity with these patients amongst all hospital staff involved as well as established pathways for diagnosing and treating emerging complications. As a result, during the last decade esophageal cancer surgery have been centralised into high volume centres, where the mortality after esophagectomy is substantially lower compared to low volume centres[68, 69].

1.4 INFLAMMATORY RESPONSE AND RELATED REACTIONS

Inflammation, derived from the Latin word inflammatio (ignite, to set alight) is the process by which the body attempts to stop or remove harmful stimuli. Triggering factors are for example the presence of bacteria or bacterial endotoxins, tissue damage and cell death or ischaemia-reperfusion. A multitude of immune cells are attracted to the site of injury by endocrine and paracrine signalling from the damaged area as well as autocrine signalling among the cells from the innate immune system already present. Local blood flow and vascular permeability is increased to facilitate the recruitment of immune cells from the bloodstream to the site of injury. These cells then proceeds to eliminate pathogens and damaged cells through a plethora of mechanisms. During this process normal tissue function is impaired and further tissue damage can ultimately follow[70].

1.4.1 The immune system

The immune system is typically divided into the innate and adaptive systems. The innate system is mature at birth and is always active to provide an initial reaction to a stimulus. It consists of macrophages and neutrophils that ingest microbes, natural killer cells that kill infected cells and dendritic cells that together with macrophages present antigens to the adaptive immune system. Complement factors that either help to attract more immune cells (chemotaxis) or attack cells by disrupting the cellular membrane via an enzymatic cascade also belong to the innate system[70].

The adaptive or acquired immune system develops in response to an antigen. The response is slower than the innate response but the reaction to the antigen is stronger and once encountered the adaptive immune system will remember the antigen. The adaptive system is divided into humoral and cellular immunity. The humoral immunity consists of B-lymphocytes which secrete antibodies and also have an antigen-presenting capability. T cells are the cellular part of the adaptive system. There are several forms of T cells; the most abundant are T-helper cells and cytotoxic T-cells. T-helper cells are activated following major histocompatibility complex II presentation of an antigen by an antigen presenting cell. They then multiply rapidly and enhance and regulate the immune response through cytokine release. Cytotoxic T-cells kills host cells infected by intracellular microbes, viruses as well as tumour cells by inducing apoptosis [70]. Cytotoxic T-cells are activated by recognition of a foreign peptide presented by the host cell through major histocompatibility complex I which is present in all nucleated cells. All cells of the immune system produce cytokines and related factors that can regulate the function of other types of cells. CD45 or tyrosine phosphatase, receptor type C is a regulatory protein expressed on all leukocytes. It is routinely used to histologically identify leukocytes using immunohistochemistry.

1.4.2 Cytokines

Cytokines are proteins that have immune modulating effects by creating signalling pathways between different immune cells and also directed towards other cell types, which can both produce and react to cytokines. Cytokines are numerous and act in complex patterns to modulate the immune response as well as promote cell differentiation and growth. They are often divided into pro- and anti- inflammatory cytokines although many contribute to both effects to some extent. A large group of cytokines are called interleukins (IL) as they are produced by and act on leukocytes. Major pro-inflammatory cytokines are IL-1α and IL-1β, IL-2, IL-6, TNFα and IFN-y[71]. Major anti-inflammatory cytokines include IL-1ra, IL-4, and IL-10[72]. Chemokines (chemotactic cytokines) form a subclass of small cytokines with a molecular weight in the range of 20 kD produced primarily by endothelial cells and macrophages to facilitate leukocyte adhesion and migration through the vascular endothelium[73].Cytokine release is increased after esophagectomy and high levels of IL-6, TNFα, IL-1b, IL-10 have been linked to postoperative morbidity[74-78]. Perioperative corticosteroids have been shown to diminish the IL-6 and IL-8 response to esophagectomy, paralleled by a decreased postoperative morbidity. These data, although promising needs to be confirmed in future studies[79]. External radiation has been shown to increase the levels of IL-1b and IL-6, as well as lung specific TGF-beta1 and IL-13 when studied in a mouse model[80].

1.4.3 The complement system

The complement system is part of the innate immune system and consists of a family of proteins circulating in the general blood stream in the form of inactive zymogens. C3 is the most abundant of these and is hydrolysed to the active form C3b in plasma at a low rate. C3b is rapidly degraded if not bound to an activating pathogen. Once C3b is triggered it initiates

an enzyme cascade at the site of activation[70]. There are three different pathways to complement activation (Figure 2). In the classical pathway complement C1 binds to antibodies attached to an antigen and thereby promotes ingestion by phagocytes with complement receptors. This process is called opsonisation. In the alternative pathway C3b binds directly to the surface of a microbe or antigen thereby initiating the cascade. Finally in the lectin pathway, plasma mannose-binding lectin binds to an antigen. Plasma-mannose binding lectin is similar, in its structure, to a component C1 in the classical pathway and can activate C4. Once activated, all pathways lead to activation of C5 which is the initiating step in the formation of the terminal complement complex (sC5b-9 or TCC). sC5b-9 induces hydrolysis by forming a transmembrane pore in the cellular wall. sC5b-9 was formerly regarded as the most important effector mechanism for complement activation and remains so in special cases such as Neisseria meningitides infections. However, a modulatory effect of sC5b-9 on the humoral immune response such as opsonisation and chemo-attraction is today considered more important[81]. sC5b-9 has also been implicated to mediate increased hyperpermeability of the pulmonary endothelium in a rat model[82], and C3a and C5a are also important pro-inflammatory signals inducing chemotaxis of humoral immune cells. The complement system is complex and has numerous effects on the immune system and many of these are still poorly understood. Although initially thought to be activated only upon infection the complement system is now recognized to react to any form of tissue trauma such as, for example, surgery and ischemia reperfusion[83]. Dysregulation of the complement system is also involved in many auto-immune diseases as well as organ transplantation and targeted treatments of complement factors are today used for a few rare diseases[84, 85].

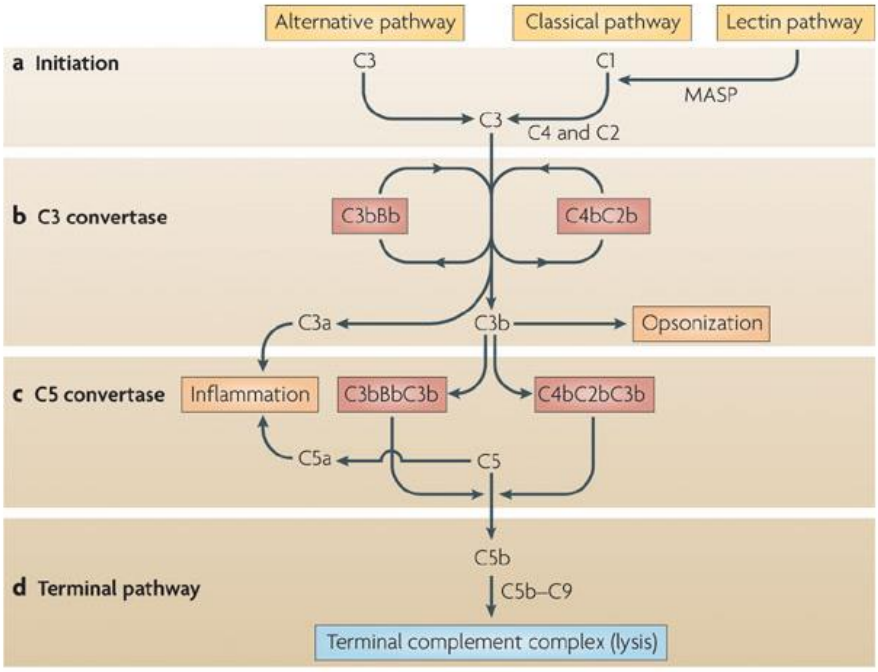


Figure 2. Schematic view of the complement system illustrating the three activation pathways as well as the main effector mechanisms of opsonization, inflammation and lysis. Reproduced with permission from Nature Reviews Immunology (Zipfel, Skerka 2009).

1.4.4 Nitric oxide

Nitric oxide (NO) is a free radical consisting of one nitrogen atom with a covalently bound oxygen atom with an unpaired electron. It is an odourless and colourless gas in its free form. In the late 1980s an unknown endothelial relaxant was discovered which was later on demonstrated to be NO[86]. Since then many different functions of NO have been discovered. In 1998 Furchgott, Ignarro and Murad were awarded the Nobel Prize "for their discoveries concerning NO as a signalling molecule in the cardiovascular system".

NO is a potent vasodilator by relaxing smooth muscle and is the active substance in nitroglycerine, long used to treat angina pectoris. However, NO also contains anti- as well as pro-inflammatory effects leading to impaired platelet adhesion, regulation of cytokine production in the endothelium and among the immune cells, and is involved in the inflammation seen after ischemia-reperfusion[87]. In vivo NO is synthesized from L-arginine by three isoforms of nitric oxide synthases (NOS), further named from the tissue in which they were found: neuronal NOS, endothelial NOS and inducible NOS (iNOS) which is induced in several cell types including macrophages and neutrophils in response to inflammation.

NO has a short half-life of only milliseconds depending on the micro environmental conditions under which it is released. Local NO concentration, its ability to diffuseand the concentration of other bio reactants are major factors that regulate NO stability[88]. Its short half-life makes NO difficult to measure. Therefore the terminal end products of NO breakdown, nitrite and nitrate, are often used as indirect estimates of NO production. Nitrate is the major end product when a sufficient amount of O_2 is present[88].

NO has been shown to reduce pulmonary vasoconstriction in healthy volunteers during hypoxemia[89] and to reduce pulmonary arterial pressure rise during cardiac surgery[90]. OLV, on the other hand, does not increase pulmonary artery pressure[57, 58] and NO administered during OLV, did not affect mean pulmonary artery pressure during OLV[91].

1.4.5 Endothelin

Endothelins are small polypeptides, which exist in three isoforms (1-3), are produced by many cell types throughout the body and secreted in an auto or paracrine fashion[92]. Endothelin-1 is the main contributor to vascular tone. It is formed predominantly in endothelial cells and is a powerful vasoconstrictor considered to act as a natural counterpart to NO. There are two major receptors for endothelin called ET_A and ET_B. ET_A is selective for endothelin 1 and 2 and thought to be the main pathway for the vasoconstrictive effect while ET_B is non-selective and believed to mediate a broader range of effects such as endothelin-1 clearance, endothelial cell survival, and induction of NO through regulation of endothelial NOS. There are several feed-back loops between NO and endothelin. Moreover NO inhibits the release of endothelin and endothelin elicits release of NO[93, 94]. ET_A receptors are present both in the pulmonary vascular endothelium and in the airway smooth muscle cells mitigating both vascular as well as bronchial constriction[92]. Increased levels are present both in

symptomatic asthma and in pulmonary hypertension. Endothelin levels has been found to increase following ischemia reperfusion injury in animal studies and blockage of endothelin by receptor antagonists have been shown to attenuate lung injury following ischemia reperfusion[95, 96]. This indicates that endothelin also plays a role in ischemia reperfusion injury.

1.4.6 Ischemia-reperfusion injury

Ischemia-reperfusion injury is a complex pathophysiological event characterized by intracellular injury, dysregulation of circulation, inflammation and increased permeability of capillaries and arterioles[97, 98]. Ischaemia induces an anoxic cell injury with loss of electrolyte and imbalanced water homeostasis leading to necrosis (cell death) or apoptosis (programmed cell death without release of harmful substances). When reperfusion occurs, reactive oxygen species are formed augmenting oxidative stress and recruiting cytokine synthesizing neutrophils. The NO-Endothelin balance is shifted leading to a decrease of NO and increase in endothelin. The net effect of that is vasoconstriction and even arrested microcirculatory flow which will further increase tissue damage. Apart from vasodilation, NO appears to have both beneficial and harmful effects in this pathophysiological setting. During oxidative stress endothelial NOS may produce the highly reactive peroxynitrite which can induce cell death although it might also act as a free radical scavenger[99]. Beneficial effects include decreased neutrophil infiltration through down regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines as well as inhibition of apoptosis[100].

Ischaemia reperfusion injury is important in clinical settings such as myocardial infarction, stroke, cardiopulmonary bypass and organ transplantation[101-103]. One-lung ventilation during esophagectomy has been suggested to induce an ischaemia-reperfusion injury when the collapsed lung is re-ventilated. In a porcine model the NO metabolite nitrite was decreased following OLV and there were signs of vascular congestion in the lung[104].

1.5 CENTRAL HEMODYNAMICS

Tissue oxygen delivery is the end product of the circulatory system. With the exception of the small amount of free dissolved oxygen in the blood it is directly proportional to haemoglobin levels, haemoglobin saturation and cardiac output (CO). Cardiac output is controlled by heart rate (HR) and stroke volume (SV). Stroke volume is determined by preload, afterload and cardiac contractility. Preload is the grade of stretch in the sarcomeres of cardiac myocytes at the end of diastole. It is commonly assessed by end diastolic volume (EDV) and/or end diastolic pressure. Afterload is the pressure the heart must overcome in order to eject blood out of the heart i.e. the systemic (SVR) or pulmonary resistance. SVR is calculated as SVR= (MAP-CVP)/CO where MAP is mean arterial pressure and CVP is the central venous pressure measured at the right atrium. Contractility is the force generated in the sarcomeres of the myocytes by actin myosin cross-bridges during systole. Contractility is dependent on a number of factors including stretching of the myocytes. At low levels of stretch (preload) fewer actin-myosin cross-bridges are formed and less force is generated.

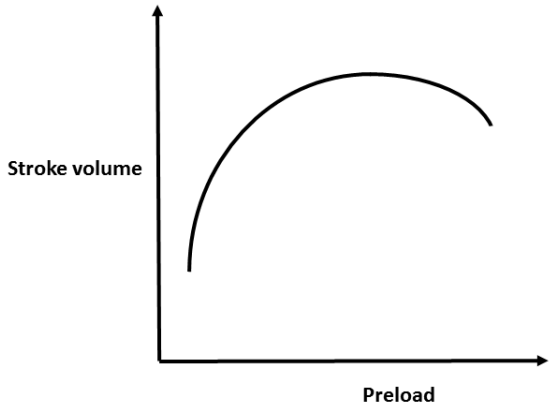


Figure 3. Illustration of the Frank-Starling relationship. Stroke volume will increase rapidly with increasing preload when preload is low. As the myocytes become overstretched further increase in preload will lead to a decreased stroke volume.

With increasing preload more cross-bridges are formed and more force is generated. However, if preload increases too much, actin and myosin become too separate to form cross-bridges and contractile force will decrease. This is called the Frank-Starling relationship (Figure 3) and means that given a static afterload and HR, SV will increase with increasing preload until the myocytes become overstretched, at which point SV will start to decrease.

Measuring contractility is difficult and in vivo involves the direct and simultaneous measurement of pressure volume curves during sudden changes of preload[105, 106]. Today this can be accomplished by heart catheterization using a conductance catheter and intermittent vena cava occlusion but the method is still complex and invasive. Other indirect methods such as echocardiography and SV measurement exist. This is discussed further below.

1.6 THE HEART CYCLE

In the early part of systole, ventricular pressure rises and the pulmonary and aortic valves are closed. This is called isovolumetric contraction. Thereafter the aortic and pulmonary valves open and blood is ejected from the heart. Flow is initially fast as ventricular pressure exceeds aortic pressure and then slows down as the pressure gradient drops. Systole ends with closure of the aortic and pulmonary valves when vascular pressure exceeds the ventricular pressure. At that point the remaining blood volume in the left ventricle, referred to as the residual volume, is used to calculate the ejection fraction (EF) as EF= (EDV-residual volume)/EDV. Early diastole is called isovolumetric relaxation where there is a substantial drop of pressure inside the ventricle as the myocytes relax. When the ventricle pressure drops below the atrial pressure, the mitral and tricuspid valves open and the rapid filling phase begins. During the rapid filling phase blood is sucked passively into the ventricles, and this phase contributes the largest portion of the EDV. A small volume is added by blood returning through the atria from the periphery or lungs during the following phase named diastasis. Finally the atria contract thus adding the final portion of EDV. Contribution to EDV from atrial contraction increases with increasing heart rate as the time for diastasis is shortened. However if HR is so high that the rapid filling phase is impaired, EDV will decrease[107].

1.7 HEART FAILURE

Heart failure is defined as the physiological state in which CO is insufficient to meet the oxygen needs of the body. In systolic heart failure contractility is decreased. EDV is preserved or enlarged due to dilation but SV is decreased leading to a lower EF. In diastolic heart failure the rapid filling phase is impaired i.e. the relaxation of the heart. As a result, EDV is decreased leading to a smaller SV but with preserved contractility and EF. Both systolic and diastolic heart failure produce similar symptoms of fatigue and stress intolerance, although patients with diastolic failure are more sensitive to tachycardia. Left ventricular failure generally produces respiratory symptoms such as dyspnoea, orthopnoea caused by pulmonary congestion while right ventricular failure tends to produce peripheral oedema from congestion in systemic capillaries.

1.8 NT-PROBNP

N-Terminal Pro-B-Type Natriuretic peptide (NT-proBNP) is a peptide hormone with vasodilatory and natriuretic effects. It is released from cardiac myocytes in response to myocardial stretch from volume overloading as well as from underlying structural abnormalities in patients with heart failure[108]. It is used to diagnose and exclude heart failure and also gives prognostic information[109, 110]. During the last decade preoperative and postoperative levels of NT-proBNP have been shown to predict postoperative cardiac morbidity and also mortality[111-115]. Radiotherapy for oesophageal cancer has also been shown to increase NT-proBNP levels[116], but the prognostic significance of this finding is unknown.

1.9 ECHOCARDIOGRAPHY

Based on the principle of reflected ultrasonic waves, echocardiography has become the most common way of assessing cardiac function. There is a multitude of parameters for evaluating different functions and anatomical structures in the heart. Guidelines on how to acquire images and interpret them have been published[117]. The most widely used parameter, which is also easiest for the non-specialist to interpret, is EF. However both the acquisition and the interpretation of images are user-dependent, which reduces reproducibility. Many echocardiographic parameters are also affected by preload and afterload making assessment of contractile function more difficult. In recent years strain has emerged as a parameter to assess contractile longitudinal or circumferential function of the heart[118-120]. Strain is the relative shortening of the distance between naturally occurring acoustic markers (speckles) within the endocardium. The length of shortening as well as the velocity of the speckles can be measured using a method called speckle tracking. Global strain (GS) is the average longitudinal strain of the different cardiac segments obtained from one projection (Figure 4). It is considered more sensitive than EF and less user dependent[121-123], the latter largely due to its angle independence (having the transducer tilted slightly out of plane does not affect the values measured). Strain has been shown to have an excellent agreement in accuracy with methods considered to be have the highest accuracy; R^2=0.9 vs sonomicrometry in dogs and R^2=0.89 vs. magnetic resonance imaging[118, 124].

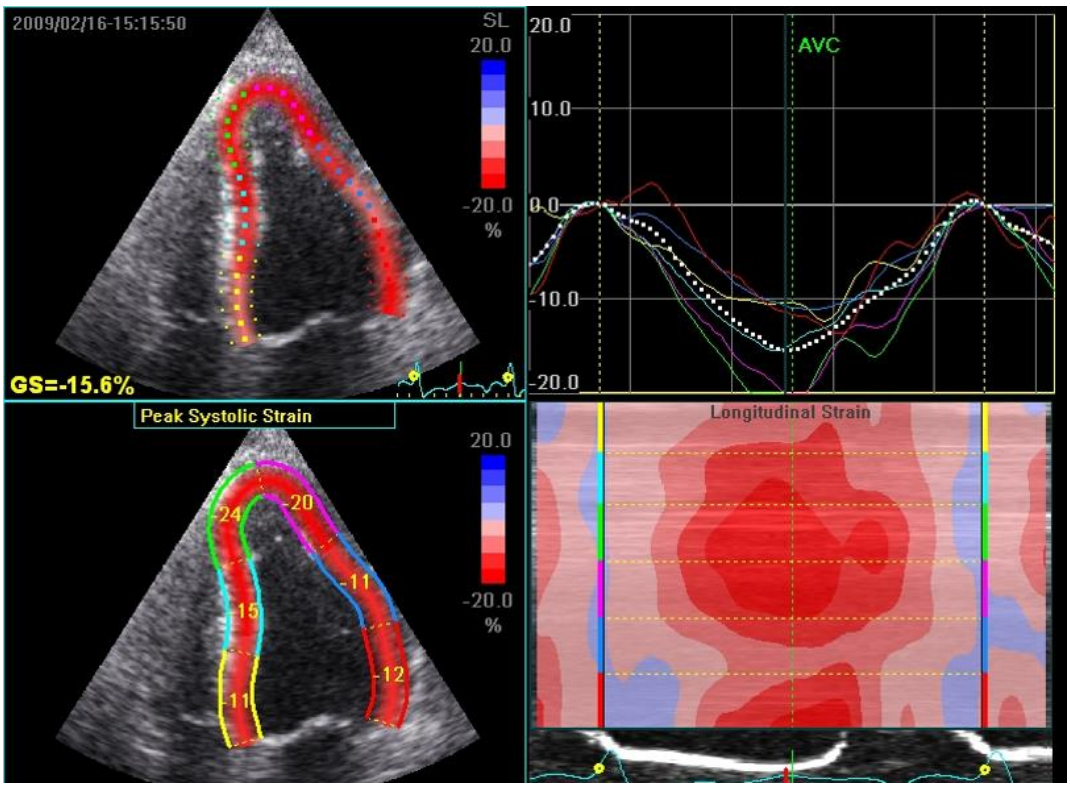


Figure 4. Analysis of global longitudinal strain (GS) using EchoPac in a four chamber apical view. The strain of the six segments analyzed are shown in the bottom left window and the resulting GS in shown in the top left window.

1.10 MINIMALLY INVASIVE HEMODYNAMIC MONITORING AND GOAL DIRECTED THERAPY

The pulmonary artery catheter was the first method used to measure CO in routine practice. Today it has largely been replaced with less invasive methods even though it is still useful in some settings. A number of methods have been developed such as esophageal Doppler ultrasound, pulse contour analysis and pulse power analysis which is used in this thesis. The application of these techniques for preload optimization has been developed based on predicted changes of SV from dynamic flow parameters (stroke volume variation, pulse pressure variation, aortic blood flow velocity waveform)[125]. Optimization can also be performed by challenging the Frank-Starling curve of the heart either by rapidly infused fluid boluses or a passive leg rise test which increases venous return to the heart and therefore also preload[125, 126]. A patient is generally deemed a responder (on the ascending part of the pressure volume curve) if a SV increase >10 % is achieved by the intervention, i.e. an increase in preload.

2 AIMS

The overall objective of this thesis was to explore a clinically challenging field closely connected to the surgical management of oesophageal cancer. In particular the thesis focused on physiological effects of single lung ventilation and neoadjuvant treatment and on the potential impact of these therapeutic concepts on the perioperative courses.

Specifically we aimed to:

- Compare the systemic inflammatory response and factors regulating pulmonary vascular tone, as elicited by the alleged ischaemia-reperfusion injury following one- or two-lung ventilation during the thoracic part of esophageal resection for cancer.
- Compare acute effects on the preoperative cardiac function following neoadjuvant chemo- or chemoradiotherapy for cancer of the esophagus or gastro-esophageal junction.
- Compare the perioperative hemodynamic profile, inflammatory response and respiratory function following neoadjuvant chemo- or chemoradiotherapy in patients undergoing thoracoabdominal esophageal resection for cancer.

3 MATERIALS AND METHODS

The general outlay and outcome measures in papers I-V are shown in Table 1. Both studies were performed at the Karolinska University hospital in Huddinge and included only patients with oesophageal or GE junction cancer.

Table 1. Study design and outcome measures

	Paper	Design	Intervention	Setting	Outcome	N
Study one	Paper I & II	Single centre prospective randomised controlled trial	OLV or TLV	Perioperative	Cytokines, complement activation markers, NO/endothelin, clinical outcome	30
Study two	Paper III	Single centre prospective randomised cohort	CT or CRT	Post neoadjuvant treatment	Systolic and diastolic echocardiographic parameters. NT-proBNP	40
	Paper IV & V	Single centre prospective randomised cohort	CT or CRT	Perioperative	Hemodynamic parameters, echocardiographic parameters, NT-proBNP, cytokines, complement activation markers, clinical outcome	31

3.1 ETHICAL CONSIDERATIONS

In study one patients were randomised to either standard treatment with OLV or the experimental treatment of two lung ventilation (TLV) during the thoracic part of the operation. OLV is standard during esophagectomy, and although TLV has been used to a lesser extent there has been no indication that TLV has any specific safety issues. Blood sampling of pulmonary veins and arteries adds a small risk but any bleeding during surgery is easily detectable and treated during the operation. Lung biopsies also add a small risk for air leakage and pneumothorax, but this is not expected to have any clinical consequences since

all patients that underwent esophagectomy at this unit received bilateral thoracic drainages as standard care. No extra vascular accesses were used for blood sampling. However, the acquisition of lung biopsies represents an ethical dilemma. Given the high incidence of postoperative pulmonary complications following esophagectomy it is imperative to study the underlying factors in order to improve treatment. Given this, we considered the ethical dilemma as minor. In study two, patients with esophageal cancer were randomised to either CRT or CT, both of which are standard neoadjuvant treatments. Therefore this study does not have any particular ethical dilemmas from that perspective. The extended protocol at Karolinska Huddinge could be considered as cumbersome for the patient in terms of more hospital visits for different examinations but did not with the exception of lung biopsies entail any increased risks.

The protocols for papers I-V were approved by the Stockholm regional ethics committee. All patients received written and oral information and were included after signing an informed consent form. For the lung biopsies in paper V, an additional consent was obtained.

3.2 PATIENT CHARACTERISTICS

A brief description of patient characteristics in each study is given in Table 2 below. A detailed description is provided in each paper.

Table 2. Patient characteristics

	Group n	Age Median (range)	BMI Median(range)	Cardio-vascular disease n (%)	Smoking ongoing/ previous n (%)	ASA class Median (range)
Paper I-II	OLV n=16	62.5 (33-74)	24.1 (17.3–32.8)	-	10	2 (2–3)
	TLV n=14	66.5 (43–83)	22.0 (18.0–35.3)	-	11	2 (2–3)
Paper III	CRT n=17	66 (56–75)	26 (21–35)	10 (59)	4 (24)	2 (2–3)
	CT n=23	62 (46–71)	23 (18–33)	7 (30)	10 (43)	2 (2–3)
Paper IV-V	CRT n=14	66 (56-75)	26 (21-34)	7 (50)	8 (47)	2 (2–3)
	CT n=17	60 (51-71)	23 (18-33)	6 (35)	2 (14)	2 (2–3)

3.3 SURGERY

Thoracoabdominal surgery (papers I, II, IV, V) was performed as described under 1.2.4. Reconstruction was performed using a narrow gastric tube in all but four cases (Study 1: OLV group 2, TLV group 1; Study 2: CT group 1) All patients received bilateral thoracic drains with active suction at the end of surgery as well as a jejunal catheter for postoperative nutrition.

3.4 ANAESTHESIA

Anaesthesia (papers I, II, IV, V) was induced with Propofol 2-3 mg/kg and Fentanyl 2 µg/kg after placement of an epidural in the 6-8th epidural space. A bolus dose of Fentanyl 50 µg was administered in the epidural after which an infusion with Bupivacain 2 µg/ml, Adrenaline 2 µg/mL and Fentanyl 50 µg/ml was started at 10-15 ml/h. Anaesthesia was maintained using Sevoflurane at a concentration of 0.7-0.9 % minimal alveolar concentration together with the epidural and muscle relaxants. A Norepinephrine 40 µg/ml infusion was used as needed in order to maintain MAP at 60-70 mm Hg. Fluids were administered at a rate of 1 ml/kg/h of Glucose with electrolytes, Ringers acetate 2 ml/kg/h and 2 ml/kg/h of poly-hydroxyl-ethyl starch 130/0.4 (Voluven 60 mg/ml, Fresenius Kabi). Hypovolaemia was treated with poly-hydroxyl-ethyl starch or blood products as deemed appropriate by the anaesthesiologist. All patients received a double lumen central venous catheter in the right jugular vein and an arterial catheter in the radial artery. All patients were extubated at the end of surgery and transferred to the postoperative recovery ward.

3.4.1 Ventilation

A double lumen endobronchial tube was used in all patients and placement was verified by bronchoscopy at insertion and after repositioning of the patient. During laparotomy patients were normoventilated using volume controlled ventilation with a PEEP of 4 cmH_20 and peak pressures < 30 cmH_2O. FiO_2 adjusted to maintain PaO_2>10 kPa. For papers I and II one-lung ventilation was commenced in the OLV group after thoracotomy by deflation of the right lung. Tidal volume was reduced to maintain the same top pressures as during TLV and respiratory rate increased as necessary to maintain a similar minute volume. FiO_2 was increased to maintain PaO_2 >10 kPa. A PEEP of 5 cm H_20 was applied to the right lung using a Bains system with 100 % FiO_2. In the TLV group top pressures and minute ventilation were maintained and respiratory rate and tidal volume was changed as needed.
For papers IV and V the ventilation protocol was the same as for papers I and II during the abdominal part of the procedure. During the thoracic part, OLV was employed in all patients with the same goals regarding pressure levels and arterial PaO_2. However PEEP was only applied to the right lung if PaO_2 >10 kPa could not be maintained using FiO_2 100 %.

3.5 NEOADJUVANT TREATMENT

In Study two (papers III-V) neoadjuvant therapy was employed as follows (Figure 5): Chemotherapy was given in three cycles of 21 days. Cisplatin 100 mg/m^2 was given on day 1 and 5-fluorouracil 750 mg/m^2/24h was given on days 1–5. Cisplatin was switched to Carboplatin or Oxaliplatin in case of hearing impairment or renal dysfunction. Dose reduction was allowed in case of severe side effects. In the CRT group, concomitant radiotherapy was administered during cycles two and three in 2 Gy/day fractions 5 days per week for a total of 40 Gy. Radiotherapy was planned using a computer tomography-based three-dimensional treatment planning system and dosing to adjacent organs was minimized using a multiple field technique [127]. Surgery was performed 4-6 weeks after neoadjuvant treatment.

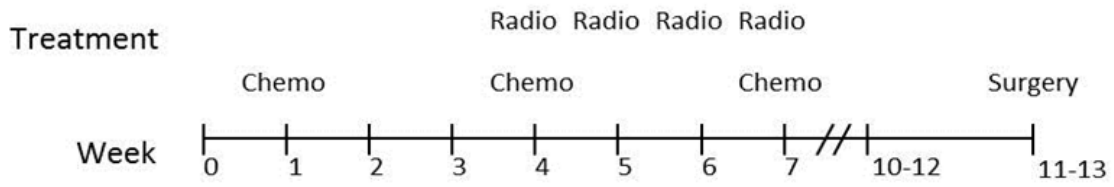


Figure 5. Neoadjuvant treatment. Cisplatin and 5-fluorouracil was given in three cycles. Radiotherapy 2 Gy/day was given concomitantly for a total of 40 Gy during cycles two and three.

3.6 STUDY ONE; PAPERS I & II

Study one was a prospective interventional single centre study including consecutive patients undergoing thoracoabdominal esophageal resection for esophageal or GE-junction cancer. None of the patients had received neoadjuvant therapy. Patients were randomised to receive ventilation of one or two lungs during the thoracic part of the procedure. Randomisation was performed during the operation, before thoracotomy by use of sealed, opaque envelopes. Analysers of samples were blinded to study group allocation. The same study population was used in papers I and II.

3.6.1 Data acquisition paper I

The first blood samples were collected from the central venous catheter after induction of anaesthesia. After thoracotomy, but before adjusting ventilator settings and instituting OLV, a second set of samples was drawn from the central venous catheter as well as the right inferior pulmonary vein. This was repeated before closure of the thorax (10 min after re-ventilation in the OLV group). Further central venous samples were collected in the mornings of POD 1-3 and 10. Samples were centrifuged at 2500 g/4°C for 10 min before storing the supernatant at -80°C in aliquots. Data regarding in hospital morbidity was obtained continuously from patients clinical records.

3.6.2 Data acquisition paper II

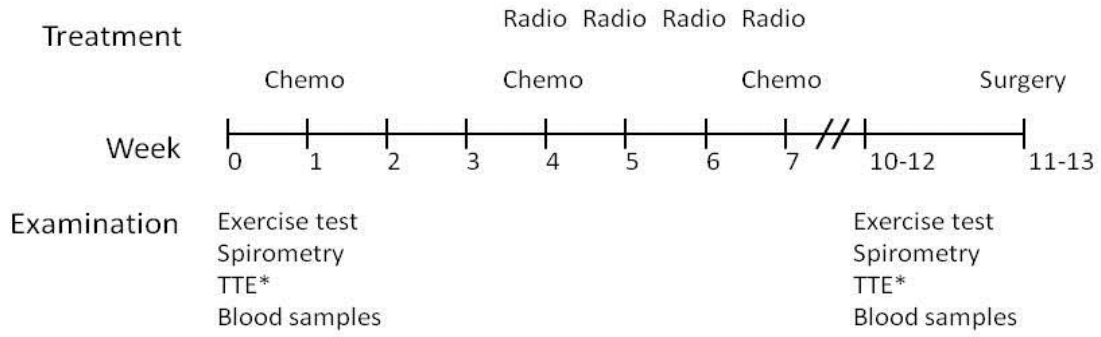
The first blood samples were collected from the central venous- and arterial catheters immediately after thoracotomy (before adjusting ventilator settings/instituting OLV). This was repeated before closure of the thorax (10 min after re-ventilation in the OLV group). Further central venous samples were collected in the mornings of POD 1-3 and 10. Samples were centrifuged at 2500 g; 4 °C for 10 min before storing the supernatant at -80 °C. Lung biopsies were taken from the lower lobe of the right lung before start of dissection of the thorax. Before closure of the thorax (10 min after re-ventilation OLV group) a second biopsy was taken from the same site. Biopsies were divided and fixed in a formaldehyde solution or immediately frozen in liquid nitrogen and stored at -80°C. Data regarding in hospital morbidity was obtained continuously from patients clinical records.

3.7 STUDY TWO; PAPERS III-V

Study two, the NeoRes study, was a prospective, none blinded, interventional multicentre study in Sweden and Norway regarding histological response following neoadjuvant chemo- or chemoradiotherapy (CT and CRT) for esophageal or GE-junction cancer. Patients eligible for inclusion were adults <75 years deemed accessible for surgery with curative intent and with no major illness making neoadjuvant therapy unsuitable. Randomisation was performed by a computer based program at the Regional Oncological Centre in Stockholm and patients were stratified by histological type prior to randomization. A detailed description of the inclusion and randomisation process has been published by Klevebro et.al. [127].
Patients scheduled to be operated on at Karolinska University Hospital were from January 2009 also included in the prospective single centre observational cohort studies presented in this thesis. Added inclusion criteria were planned thoracoabdominal resection. Of the 41 patients fulfilling these criteria 40 were analysed in paper III as intention to treat. In papers IV and V 31 of the 41 patients were analysed as per protocol (completion of resection).
Analysers of blood samples and the performer of echocardiography were blinded to study group allocation. Study group allocation was not blinded during acquisition of hemodynamic measurements.

3.7.1 Data acquisition paper III

Before start of neoadjuvant therapy and 4-6 weeks after its completion an exercise test, echocardiography and measurements of NT-proBNP were performed in an outpatient setting (Figure 6). For details regarding the methodology of the respective test please see the respective sub heading below.



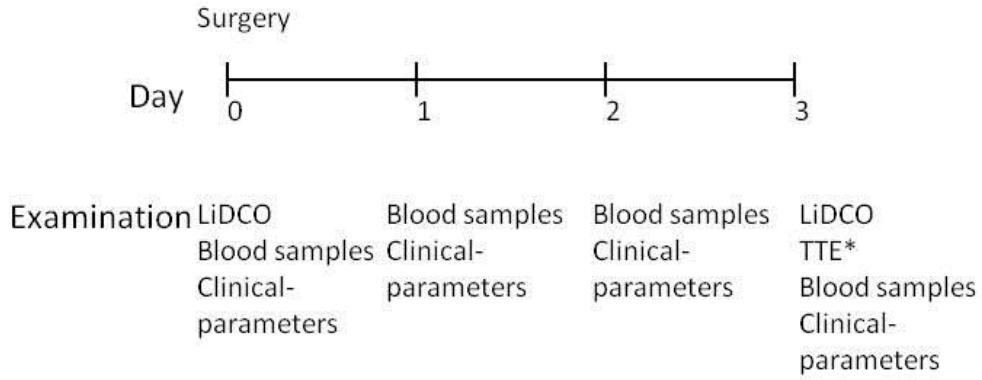
*TTE, trans thoracic echocardiography

.**Figure 6.** Time line for data acquisition in paper III in relation to neoadjuvant treatment.

3.7.2 Data acquisition paper IV

Echocardiography after neoadjuvant treatment was performed in an outpatient setting (the same examinations as in paper III) and repeated in the morning of the third postoperative day (POD).
Baseline NT-proBNP was measured upon admittance for surgery. NT-proBNP and Troponin T were also measured after arrival to the postoperative ward POD 0 and in the mornings of POD 0-3.

In the morning of the day of surgery haemodynamic measurements after fluid optimisation were performed using LiDCOplus. This was done in the preoperative ward with the patient awake and at rest. During surgery haemodynamic data were collected at predefined time points (after 2 hr of abdominal surgery, 1 hr after start of OLV and after closure of the thorax). The monitor was not available to clinical staff to guide intraoperative treatment. On POD 3 haemodynamic measurements were repeated after fluid optimisation (Figure 7). In-hospital morbidity and mortality were accessed daily by the research team.



*TTE, trans thoracic echocardiography.

Figure 7. Time line for data acquisition in paper IV-V.

3.7.3 Data acquisition paper V

Baseline blood samples for routine chemistry were measured upon admittance for surgery and baseline blood samples for cytokine and complement activation markers were taken in the morning of the day of surgery in the preoperative ward. Further samples were collected at POD 0 after arrival at the postoperative ward and in the morning of POD 1-3 (Figure 7). Samples for routine chemistry were analysed by the Karolinska University Hospital accredited laboratory for clinical chemistry. Blood samples for cytokine and complement activation marker were centrifuged at 2500 g; 4 ^{0}C for 10 min before storing the supernatant at -80 ^{0}C.

Arterial blood gases were drawn in the preoperative ward and at POD 0-3. Samples were analysed bedside using ABL 800 Flex (Radiometer Medical, Brønshøj, Denmark).

Lung biopsies from the medial part of the right inferior lobe (within the radiation field in patients from the CRT group) were taken before closure of the thorax with a linear staple device, divided and immediately frozen in liquid nitrogen or stored in formaldehyde solution.

In hospital morbidity and mortality were accessed daily by the research team.

3.8 ANALYTICAL METHODS

The methods used in paper I-V are outlined in Table 3 and further described in the following sections

Table 3. Analytical methods used

	Paper I	Paper II	Paper III	Paper IV	Paper V
ELISA	X				X
Radio immuno assay		X			
Chemiluminescence		X			
Histological assessment		X			X
Echocardiography			X	X	
Exercise test			X		
Routine laboratory analyses	X		X	X	X
LiDCOplus™				X	
Fluid optimisation*				X	
Blood gas analysis				X	X
Multiplex analyses					X
mRNA analysis					X

*Optimisation of stroke volume by fluid boluses and measured using LiDCOplus™.

3.8.1 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used for analysis of cytokines and markers of complement activation in plasma (paper I, V). ELISA is a common method for biochemical analysis based on attaching the substance studied to a surface coated with a substance specific antibody. A second specific antibody is then applied over the surface, binding to the substance. This antibody is linked to an enzyme and, in the final step a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, usually a colour reaction which can be quantified by a photo sensor. The method can be used to study a variety of substances and is considered highly quantitative and generally reproducible[128]. However only one cytokine per well can be analysed which increases costs and the sample amount used. Commercial kits are available for many analyses although results can vary according to antibody quality and kit manufacturer as well as operator skill. Analyses were performed by our research group and by the research group of Professor K. Nilsson-Ekdahl at Department of Immunology, Genetics and Pathology, Uppsala University. For details on specific kits and reagents used see papers I and V (page333 and page 9).

3.8.2 Radioimmunoassay

Radioimmunoassay was used for plasma Endothelin analysis (paper II). Briefly, this method is based on the competition of radioactively labelled antigen and non-labelled (cold) antigen in the study sample in binding to an antigen-specific antibody. When the cold antigen is added, marked antigen is displaced from the antibody binding sites into the solution. By measuring radioactivity in the solution the concentration of antigen in the sample can be calculated. Analyses were performed by the research group of Professor J.O. Lundberg at Department of Physiology and Pharmacology, Karolinska Institutet. The exact methodology used has been described in detail by Lundberg et al.[129].

3.8.3 Chemiluminescence

Chemiluminescence was used to analyse plasma nitrite (paper II). In brief, the method entails reductive cleavage of nitrate by iodine to form NO. NO then reacts with ozone to form N_2O. A portion of the N_2O formed arises in an electronically excited state and emits light upon decay which is measured by a photo sensor. Analyses were performed by the research group of Professor J.O. Lundberg at Department of Physiology and Pharmacology, Karolinska Institutet. The exact methodology used has been described in detail by Lundberg et al.[130].

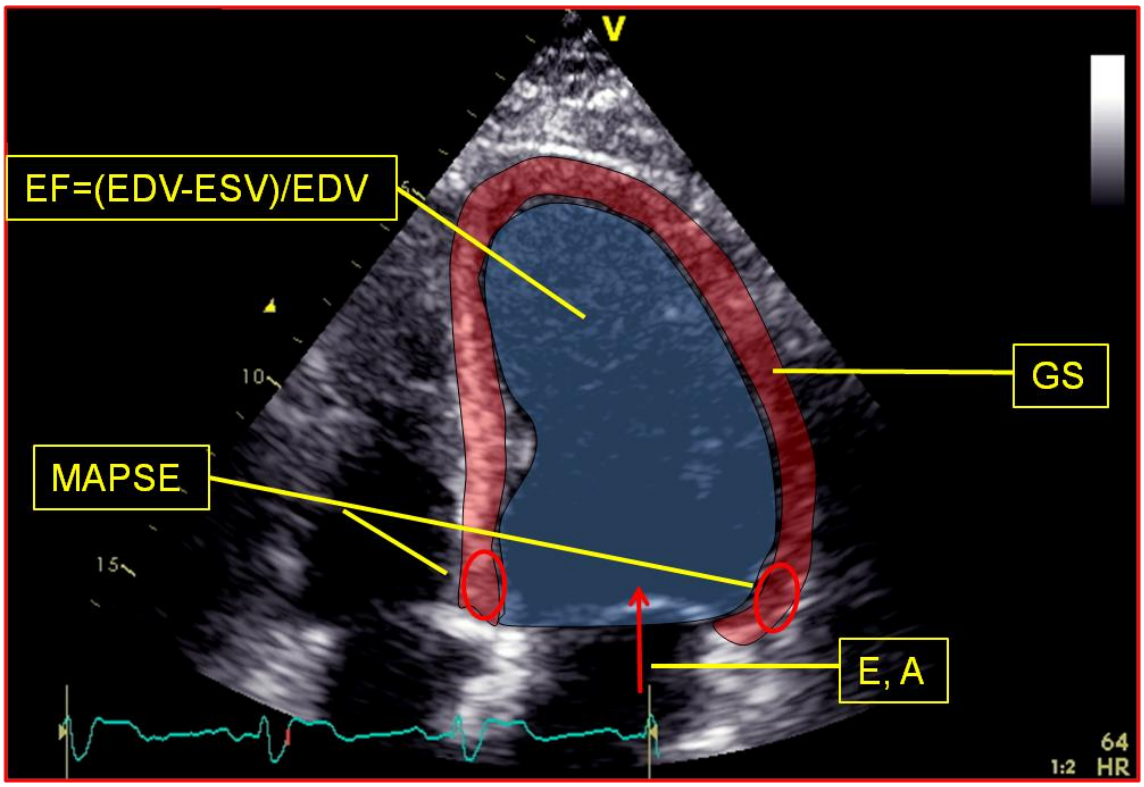
3.8.4 Histological assessment of lung biopsies

Lung biopsies were cut to 8-10 µm thickness and mounted onto slides. In paper II immunofluorescence was used to score levels of iNOS in lung biopsies at 60x magnification and hematoxylin-eosin staining was used for arbitrary scoring of vascular congestion. The immunofluorescence method utilizes a primary antibody against iNOS. A secondary antibody against the constant part of the first antibody and finally a tertiary antibody against the secondary antibody. The tertiary antibody also contains a fluorescent dye. Examination is then performed by fluorescence microscopy. Analyses were performed by Associate Professor L. Ny at Department of Oncology, Gothenburg University. For details on specific kits and reagents used see paper II (page 855). In paper V the numbers of CD45 positive cells in the interalveolar stroma were counted in three high power fields per biopsy at 40x magnification. Staining for CD45 was performed by the Karolinska University Hospital accredited laboratory for pathology using commercial immunohistochemistry kits (DAKO, Agilent Technologies, Inc.), counting was performed by Professor Å. Öst at the Department of Pathology, Karolinska Institutet.

3.8.5 Echocardiography

Transthoracic echocardiography was used in papers II & IV to assess global and regional systolic function as well as diastolic function of the left ventricle. As there is significant intra-observer variability in acquiring and interpreting ultrasound images, all examinations except three in papers III and four in paper IV were performed by the same laboratory technician using the same Vivid 7 ultrasound scanner and a standard 2.5 mHz transducer (GE Vingmed, Horten, Norway). Furthermore, all post-processing analysis was performed by me using Echo

PAC (GE Vingmed, Horten, Norway). A standard cardiac examination was performed in all patients. One or more loops of three heartbeats were recorded for each online view and stored for post-processing. As echocardiography results are greatly dependent on image quality, this was optimised as much as possible during the examination by use of probe and patient positioning, probe frequency, focus depth, sector width and gain (strength of incoming signals i.e. image brightness).The best cardiac cycle from each view was used in post processing. Further quality optimization was also performed during post-processing. Both performer and interpreter of the examinations were blinded to study group allocation at the time. In each examination the following measurements were made (Figure 8): Global systolic function was evaluated by EF (using the Simpson biplane method) and global strain (GS). Regional systolic function was evaluated by mitral annular plane systolic excursion (MAPSE) of the septal septum (sept) and the basal anterolateral wall (lat) using tissue doppler. Diastolic function was evaluated by measuring peak blood flow velocities across the mitral valve during diastole, E- and A-wave. The E-wave (E) is generated during the early filling phase when the ventricle relaxes and the A-wave (A) from the late filling phase when the atria contract. Details on measurement acquisition are presented in paper III (page 3).



E and A denotes blood flow velocities across the mitral valve. EF, Ejection fraction; EDV, end diastolic volume; ESV, end systolic volume; GS, global strain; MAPSE, mitral annular plane systolic excursion.

Figure 8. Illustration of measurement points from a four chamber apical view during echocardiography. Reproduced with permission from Radiation Oncology (Lund, Kalman 2014).

3.8.6 Exercise test

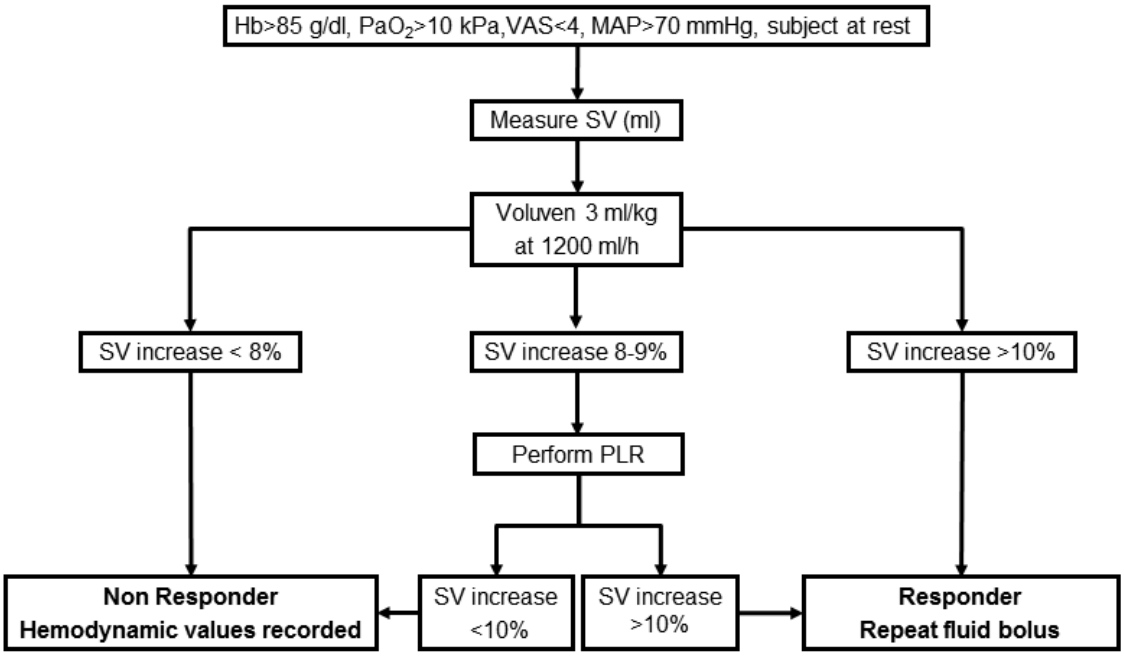
Standard exercise tests of maximum working capacity by use of a bicycle ergometer were performed before start of neoadjuvant treatment and repeated 4-6 weeks after completion of treatment (paper III). The purpose was not primarily to detect cardiac ischemia but rather to provide the maximum working capacity as an objective measurement of the patients' general fitness. All tests were performed at a hospital physiology department and supervised by a dedicated member of staff.

3.8.7 Routine laboratory analyses

C-reactive protein, leukocyte count, NT-proBNP and Troponin-T (papers I, III-V) were all analysed by the Karolinska University accredited laboratory for clinical chemistry (ISO 15189) by electrochemiluminescence immunoassay.

3.8.8 Haemodynamic measurements

LiDCOplus^{TM} (LiDCO Ltd, London, UK) (paper IV) is a device for continuous minimally invasive measurement of SV, utilising changes in the energy content of arterial resistance vessels (pulse power analysis). It is based on two steps. The first is calibration of the volume of the arterial resistance vessels (arterial waveform stroke volume curve) by use of lithium dilution. A small dose of lithium chloride is injected into a (central) vein and blood is drawn through a lithium sensitive sensor by use of a roller pump connected to an arterial line in the radial artery. The ensuing concentration curve is then integrated to achieve a direct measurement of CO much like thermodilution when using a pulmonary-artery catheter. This is then used to correct the algorithm used for continuous monitoring and in the second step data from the arterial line are copied from the regular monitor, analysed and displayed on the LiDCO monitor. During continuous monitoring, beat to beat measurement of SV can be performed and events added to track if changes in SV and CO occur following an intervention. As with all methods for measuring SV, it is sensitive to atrial fibrillation as that will cause a variation of SV. Other sources of measurement error include dampening and reflected arterial waves. However, using pulse power analysis, the impact of these sources can be minimised using autocorrelation. The method is well validated and has shown good correlation with the pulmonary-artery catheter over a wide range of stroke volumes[131-136]. This method was chosen for its ability to be used in an awake patent, during surgery and then again on the third postoperative day with an awake and mobilized patient. During measurement great care was taken in order to standardize the measurement situation. Pain was assessed using a visual analogue scale with a range of 0-10 and managed until the score was below 4. The patient was resting in bed with the researcher and monitor behind a screen during all measurements. All volume measurements were indexed for body surface area before statistical analysis.



Hb, haemoglobin; PaO_2, arterial partial pressure of oxygen; VAS, visual analogue scale; MAP, mean arterial pressure; SV, stroke volume; PLR, passive leg rise.

Figure 9. Flow chart of fluid optimization. A stroke volume increase > 10 % following a 3 ml/kg bolus of poly-hydroxy-ethyl-starch 130/0.4 indicated a responder. Boluses were repeated as long as SV increased >10 % per bolus. If SV increase was 8-9 % a passive leg rise was performed. After optimization hemodynamic values were recorded.

3.8.9 Fluid optimisation

Fluid optimisation using LiDCOplus was performed in the preoperative ward before surgery and repeated on postoperative day 3. Boluses doses of poly-hydroxy-ethyl-starch 130/0.4 (Voluven 60 mg/ml, Fresenius Kabi) 3 ml/kg (2.5 ml/kg if BMI> 30) were infused at a rate of 1200 ml/hr. A SV increase of > 10 % following a bolus was considered to indicate a responder. Boluses were repeated as long as SV increased >10 % per bolus. When SV increase was borderline (8-9 %) a passive leg rise (PLR) was performed[137] (Figure 9). The haemoglobin value used in calculations of DO_2I was adjusted down by 5 % per bolus to account for hemodilution.

3.8.10 Blood gas analysis

Arterial blood gases were drawn during adequate pain relief (visual analogue scale below 4) and with the patient breathing room air for a minimum of 10 min for measurement of PaO_2/FiO_2-ratios (paper V). If saturation was less than 85 % on ambient air a Hudson RCI® oxygen mask (Teleflex, Morrisville, USA) with an oxygen flow of 5 L min^{-1} was used for a FiO_2 of 0.4 according to the manufacturer. For intubated patients FiO_2 was recorded from the ventilator. For the haemodynamic measurements preoperatively and on POD 3 FiO_2 was adjusted to achieve PaO_2 > 10 kPA (paper IV). Samples were analysed bedside using ABL 800 Flex (Radiometer Medical, Brønshøj, Denmark).

3.8.11 Multiplex analysis

Cytokines in plasma (paper V) were analysed using a flow-cytometry based multiplex assay (Luminex, Biorad systems, California, USA). This is a form of immune assay allowing simultaneous and rapid quantification of multiple cytokines from the same sample utilising a smaller sample volume than traditional ELISA. In brief, antibodies against the target cytokine are attached to different coloured beads. Then fluorescence or streptavidin labelled detection antibodies bind to the specific cytokine-capture antibody complex on the bead set. The resulting changes in light absorbance can be measured using flow cytometry. Compared to ELISA this method is cheaper, more time efficient and requires less volumes of sample[128]. Analyses were performed by the research group of Professor Mollnes at Centre of Molecular Inflammation Research, Norwegian University of Science and Technology. For details on specific kits and reagents used see paper V (page 8).

3.8.12 mRNA analyses

In paper V mRNA from lung biopsies acquired during operation were analysed using commercial kits. Total RNA was extracted and reverse transcribed to cDNA after concentration and quality measurements. Assessment of mRNA expression was performed with 18S rRNA as a reference gene and relative levels were calculated using a comparative Ct-method (i.e., $2^{\Delta Ct\text{-target gene}}/2^{\Delta Ct\text{-reference gene}}$). Analysis was performed by the research group of Professor M. Rydén at the Department of Medicine, Karolinska Institutet. The exact methodology used has been described in detail by Gao et al[138].

3.9 STATISTICS

Table 4 below presents an outline of the statistical methods used for papers I-V in this thesis

Table 4. Statistical methods used

	Paper I	Paper II	Paper III	Paper IV	Paper V
ANOVA	X				
Mann-Whitney U-test	X	X	X	X	X
Fischer´s exact test	X	X	X	X	X
Bonferroni post-hoc test	X		X		
ANOVA on ranks		X			
Linear mixed models			X	X	X
Holm-Šidàk correction				X	

3.9.1 Paper I

Analyses of repeated measurements were performed on relative changes from baseline with analysis of variance (ANOVA) and time as the repeating variable. Data are presented as mean ± standard error of the mean (SEM) of relative changes from baseline, except for CRP,

which is presented as median and range. The Bonferroni correction was used to account for multiple testing.

3.9.2 Paper II

Analyses of repeated measurements were performed with ANOVA on ranks with time as the repeating variable. Data are presented as median and interquartile range.

3.9.3 Paper III

A linear mixed model was used to test repeated measurements for changes within the groups over time (within group changes) as well as changes between the groups over time (interaction effect). Several mixed models were used for each variable and to each model one of a set of covariables was added to test for any impact on the results. Covariables were chosen from patient characteristic and comorbidities with possible effects on the variables tested, for example age, hypertension, and ischaemic heart disease. Non parametric data were normalised using log transformation. The covariance structure was unstructed (assuming no relationship between the repeated measurements). The Bonferroni correction was used to account for multiple testing.

3.9.4 Paper IV

A linear mixed model was used to test for within group change and the interaction effect over time. Several mixed models were used for each variable and to each model one of a set of covariables was added as described above to test for any impact on the results. Non parametric data were normalized using log transformation. The covariance structure was autoregressive meaning less correlation was assumed between repeated measurements the further apart they were in time. In post hoc testing the Holm-Šidàk correction was used. The Holm-Šidàk correction was chosen as it is slightly less restrictive than the Bonferroni correction. Thus it might be seen as more appropriate in hypothesis generating post hoc testing.

3.9.5 Paper V

A linear mixed model was used to test for within group change and the interaction effect over time. Several mixed models were used for each variable and to each model one of a set of covariables was added as described above to test for any impact on the results. Non parametric data was normalised using log transformation. The covariance structure was autoregressive.

4 RESULTS AND DISCUSSION

The present thesis is based on two parts, mutually interconnected, addressing various aspects of the perioperative courses connected with esophagectomy for cancer. In total this work comprises five individual papers designated as I through V. Details regarding methods and results can be found in the respective papers. The key results are discussed here in the context of pathophysiology and implications for the management of esophageal or GE-junction cancer

4.1 STUDY ONE (PAPERS I-II)

This was a randomised trial comparing OLV (n=16) and TLV (n=14) during esophagectomy. The two groups were well balanced regarding patient characteristics. Operative characteristics in terms of bleeding, operative time and time of modified ventilation were also similar in the two groups, which suggest that there were no major technical difficulty associated with TLV during the thoracic part of esophagectomy. No differences in postoperative morbidity were observed.

The main finding in paper I was that the terminal complement complex (sC5b-9) increased more in the OLV group on POD 3 and 10 compared to the TLV group (Figure 10) while the increase of C3a was similar in both groups throughout the study period. Thrombin Antithrombin III complex (TAT) peaked in plasma at the time of reventilation while IL-6 peaked on POD 2 and CRP on POD 3. Both groups expressed a similar pattern and there were no differences between the groups regarding these compounds.

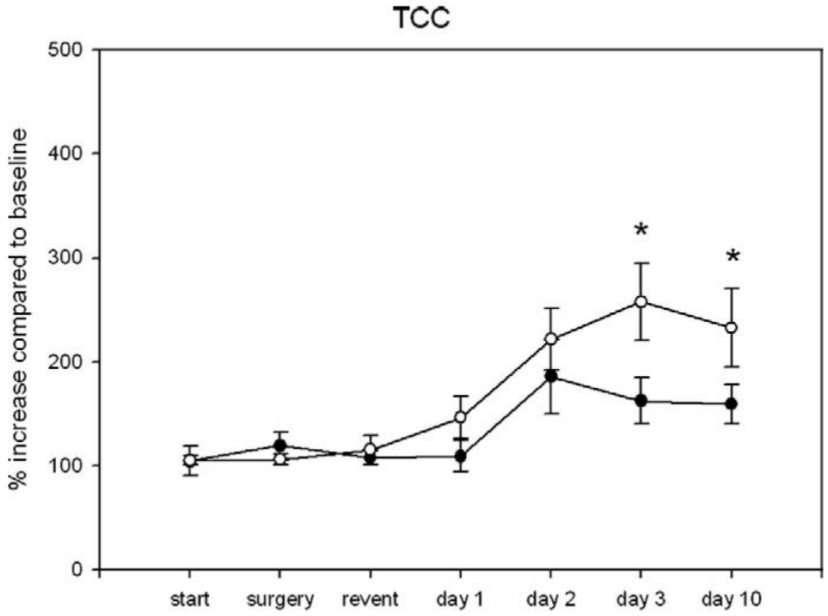
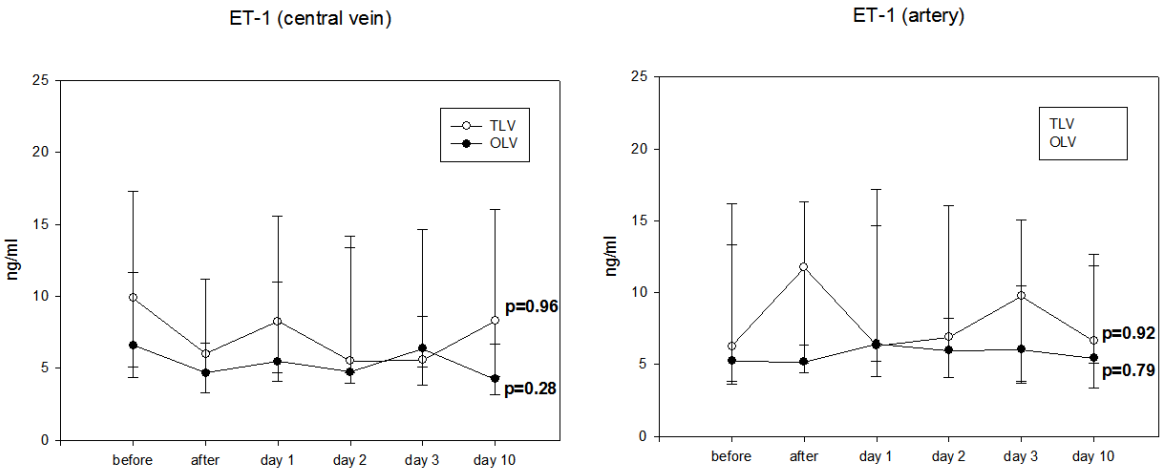


Figure 10. Plasma levels of C5b-9 (TCC) expressed as % increase from baseline. Analyzed with ANOVA and presented as mean ±SEM. *$p<0.05$. Reproduced with permission from Journal of Surgical research (Tsai, Ekdahl 2009).

In paper II plasma levels of nitrite and endothelin did not display any differences between the groups. In fact nitrite levels measured from the superior vena cava (central venous catheter) and radial artery did not display any significant within group change (directly before vs directly after the thoracic part of the procedure) in either group. Nor did endothelin levels change significantly during the study period in either central vein or radial artery (Figure 11). The levels of iNOS and vascular congestion, assessed in the 14 cases where lung biopsies were available, did not indicate any difference between the groups.



(○) OLV= one lung ventilation group, (●) TLV=two lung ventilation group

Figure 11. Endothelin (ET-1) in central vein and radial artery before and after altered ventilation as well as on POD 1-3 and 10. There were no significant changes in either group during the study period. Analyzed with ANOVA on ranks and presented as median and interquartile range. Reproduced with permission from Diseases of the Esophagus (Lund, Tsai 2012).

4.1.1.1 Aspects on inflammation and complement activation markers

The postoperatively increased levels of sC5b-9 in the OLV group vs. the TLV groups is an indirect sign of an increased systemic inflammation after OLV since sC5b-9 is coupled to a parallel increase of C5a, which has pro inflammatory properties such as chemotactic effects on immune cells. C5a was not measured directly. sC5b-9 has also been linked to hyperpermeability of the pulmonary endothelial barrier[82]. C3a as well as sC5b-9 started to increase on POD 2 in both groups but there were no differences between the groups regarding C3a. POD 2 is the approximate time point where respiratory complications typically start to manifest after esophagectomy[139], and the increase of sC5b-9 in the OLV group might indicate a relationship to respiratory postoperative complications. There was also an increase of TAT that may be linked to the increase of sC5b-9. TAT is a marker of thrombin generation, which is connected to the complement system through its ability to activate C5[140]. However this pathway of activation of C5 did probably not occur herein as there were no differences in TAT levels between the groups and because the increase of sC5b-9 was delayed compared to TAT. Kvarnström et al have also reported a similar temporal pattern of sC5b-9, C3a and IL-6 in a study on major abdominal surgery during 24 h after surgery[141].

C5b-9 and C3a, IL-6 and TAT were also analysed in samples from the right inferior pulmonary vein after re-ventilation and found to be increased to a similar extent in both treatment groups compared to before start of thoracotomy. However, except for TAT, the levels were low compared to those measured in the general circulation indicating that the lungs might not be the primary source of these factors at this time point. The levels of TAT and complement factors in the pulmonary vein later on during the postoperative course are unknown as postoperative sampling from the pulmonary veins was not possible to perform. Complement activation has been shown to correlate with ARDS and inhibition of complement reduces lung injury both in the experimental[142]and in the clinical setting[143, 144].The delayed increase of sC5b-9 that was shown could be speculated to represent a "second hit" in the lungs following OLV. Furthermore any possible effects on local inflammation were probably not mediated by sC5b-9 induced lysis, but rather from its potential ability to increase pulmonary endothelial permeability or from a pro inflammatory effect induced by C5a, although this was not measured.

Our data seem to indicate a temporal correlation between sC5b-9 and the emerging of postoperative complications, although the mechanism of action for sC5b-9 and its origin is unknown. This small study could not show any correlation between the levels of sC5b-9 and postoperative complication, although it should be noted that the four patients with the highest levels of sC5b-9 all developed complications requiring an extended time in the intensive care unit. It is also interesting to note that we found no differences regarding plasma levels of CRP or IL-6, which have been associated with postoperative complications in several studies[74, 78, 145] suggesting that OLV does not induce more systemic inflammation compared to TLV.

Based on these observations it can be concluded that OLV induces a delayed increase of sC5b-9 that in our study seems to coincide with the development of postoperative respiratory complications. However the pathway of activation of sC5b-9, its precise effect and clinical relevance is unknown.

4.1.1.2 Aspects on factors regulating pulmonary vascular tone

Acute lung injury from OLV may occur both in the ventilated and non-ventilated lung but for different reasons. The dependent lung is mainly damaged from hyperperfusion and over distension while the non-ventilated lung is damaged from surgical trauma, hypoperfusion and possibly also ischaemia-reperfusion injury. However the end result after any of these events will always be acute lung injury with an increase of inflammatory cells, cytokines and vascular oedema in the lung[146]. Ischaemia reperfusion injury in the lung triggers an inflammatory reaction including a large number of mediators including NO and endothelin[87]. Ischaemia reperfusion occurs in transplantation, cardiopulmonary bypass and arguably from all forms of OLV[146], but this has not been studied in detail during esophagectomy. From studies in rodents it has been shown that both NO and endothelin are increased by OLV[95, 147]. However the balance might be shifted by a down regulation of NO as reported in a porcine study[104]. On the contrary, arterial endothelin levels were reported to be increased only in patients undergoing pneumonectomy compared to lobectomy in a human study[148]. NO has

been used as treatment for hypoxaemia and increased pulmonary vascular pressure during OLV in several studies and although results are not completely consistent, it appears that NO treatment is only beneficial in the presence of pulmonary hypertension[149].

In paper II we were unable to detect any effect from OLV compared to TLV on major factors influencing pulmonary vascular tone (NO, iNOS and endothelin) and there were no signs of increased vascular congestion in the lungs in the analysis of lung biopsies. By use of these respective methods we could thus not find any signs of ischaemia-reperfusion injury after OLV in contrary to what is often proposed. Although not statistically significant, the largest change recorded regarding endothelin-1 was the increase of arterial levels immediately at the end of surgery in the TLV group. This could be due to manipulation and compression of the right deflated lung which might lead to endothelin release.

Based on these observations we suggest that OLV does not seem to affect pulmonary vascular tone or induce an ischemia reperfusion injury and thus maintains its place as standard care during open thoracoabdominal esophagectomy.

4.1.2 Limitations to be considered for papers I-II

There are several important limitations that needs to be considered. The primary endpoint (frequency of re-intubation) was not analysed due to early closure of the study after the start of a new multicentre study (the NeoRes study, paper III-IV), which resulted in a limited study population (n=30). Results are further limited by missing data from lung biopsies (paper II). Furthermore, we did not analyse C5a in paper I, which would have been interesting to do since it represents earlier steps in the complement cascade, possibly offering the option to elucidate which pathway that was increased. Also, the addition of a PEEP of 5 cmH_20 with a FiO_2 of 100 %, to the non-ventilated lung during OLV, may have attenuated the hypoxic vascular response sufficiently to prevent an ischemia-reperfusion injury becoming manifest upon reventilation (paper II).

The statistical analyses in paper I were performed with ANOVA and in paper II with ANOVA on ranks for repetitive measurements. Although acceptable in parametric testing and possibly in non-parametric testing after normalisation of data by rank scale or log transformation, these approaches may be suboptimal. Corresponding studies are always exposed to the risk of type II error (false positive) even although the Bonferroni correction was applied to adjust for multiple testing.

4.2 STUDY TWO (PAPERS III-IV)

All patient were consecutively included from the NeoRes trial but the demographics were slightly different in paper III compared to papers IV and V (Table 2), as the patients in paper III were included in the analysis at an intention to treat base while patients in papers IV and V were analysed as per protocol (i.e. those who completed an esophagectomy). This difference in inclusion for analysis allowed us to analyse all patients that underwent echocardiographic assessment in paper III. Data regarding radiation doses to the heart and lungs and rate of incomplete neoadjuvant treatment according to protocol are given in Table 5.

Table 5. Heart and lung radiation and rate of incomplete neoadjuvant treatment

	Paper III		Paper IV-V	
V10 heart,% median (range)	74.9 (50.0-92.2)		72.0 (50.0-92.2)	
V30 heart, % median (range)	29.0 (0-80.1)		40.0 (0-80.0)	
V20 lung, % median (range)			8.7 (1.0-14.6)	
Avrlung, Gy median (range)			6.9 (3.4-10.4)	
Incomplete chemotherapy n (%)	CT 3 (13)	CRT 2 (12)	CT 4 (24)	CRT 2 (14)
Incomplete radiotherapy n (%)		1 (6)		1 (7)

CT, chemotherapy; CRT, chemotradiotherapy; Avrlung, average dose to total lung volume.

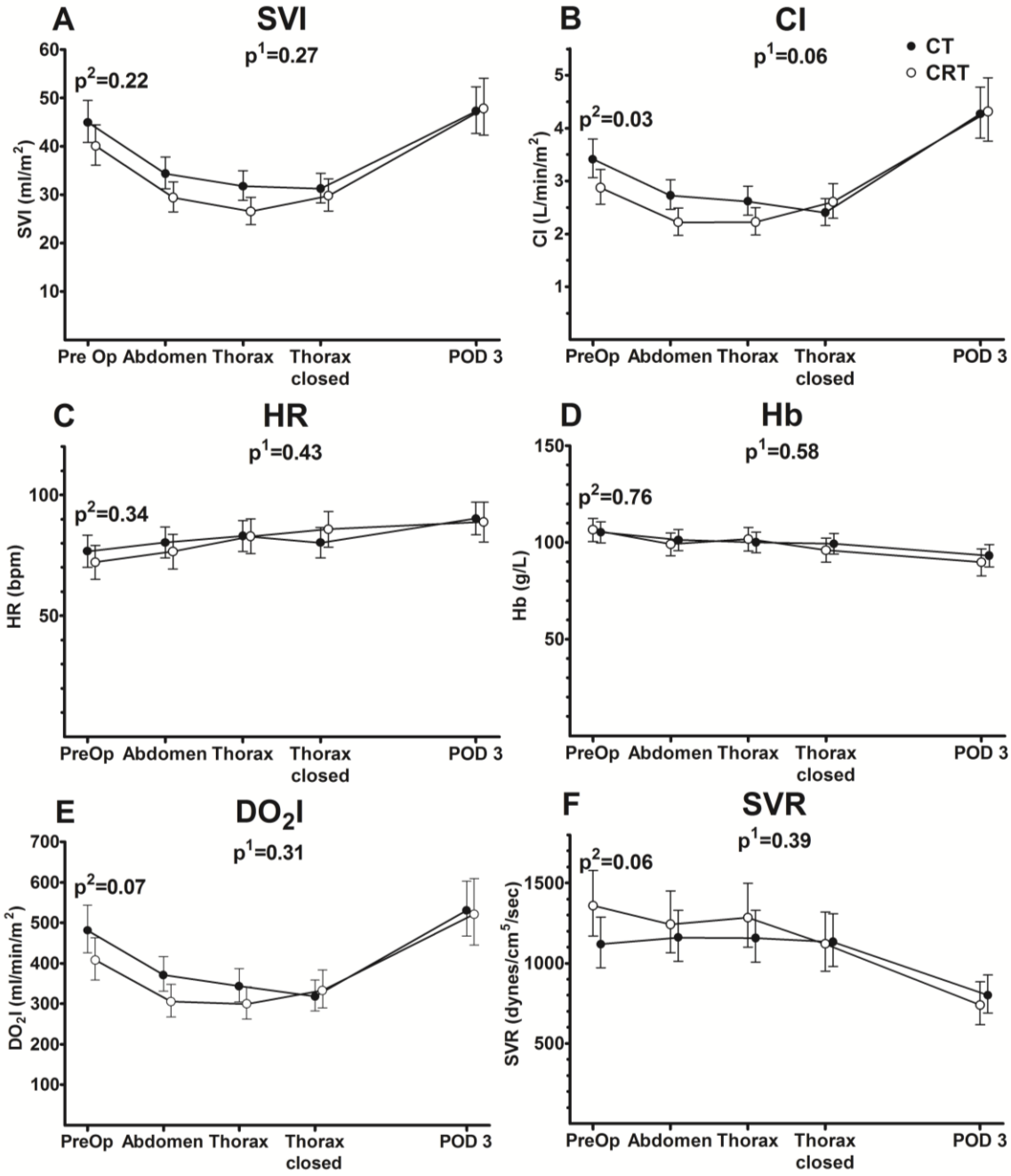
Summary of radiation doses to the heart and lungs and incomplete neoadjuvannt treatment in papers III-V. V10, V20 and V30 denote the % of organ volume having received more than 10, 20 and 30 Gy respectively.

The main findings in paper III (CRT, n=17; CT, n=23) were a decrease in regional systolic function (MAPSE sept) and diastolic function (E and E/A ratio) and a concomitant increase of NT-proBNP associated with CRT (within group effect, Table 6). However the corresponding interaction effects did not reach significance (p=0.09, 0.09, 0.39 and 0.07 respectively).No changes were seen following CT. Maximum work capacity also decreased in both groups following neoadjuvant treatment but the interaction effect did not reach significance (p=0.10).

Table 6 Results of Echocardiography, NT-proBNP and exercise tests in paper III

	Chemotherapy			Chemoradiotherapy			
Variable	Pre neoadjuvant	Post neoadjuvant	P[a]	Pre neoadjuvant	Post neoadjuvant	p[a]	p[b]
EF (%)	59 (56–62)	57 (53–60)	>0.99	60 (57–64)	59 (55–63)	>0.99	0.80
GS (%)	–17.6 (–16, –19)	–15.7 (–14, –17)	0.26	–17.3 (–16, –19)	–16.1 (–14, –18)	>0.99	0.59
MAPSE sept (cm/s)	12.5 (11.5–13.5)	12.1 (11.2–13.1)	>0.99	12.6 (11.4–13.8)	11.1 (10.1–12.2)	0.02	0.09
MAPSE lat (cm/s)	11.5 (10.4–12.6)	11.2 (10.2–12.3)	>0.99	11.2 (10.0–12.4)	11.0 (9.8–12.1)	>0.99	0.96
E (cm/s)	72.0 (62.6–81.4)	68.1 (62.2–74.1)	>0.99	78.8 (68.4–89.3)	64.1 (57.2–70.9)	0.01	0.09
A (cm/s)	67.8 (58.2–77.5)	74.6 (63.9–85.3)	0.37	82.0 (71.1–92.7)	83.7 (71.6–95.9)	0.98	0.41
E/A	1.08 (0.93–1.25)	0.95 (0.81–1.10)	0.43	0.97 (0.82–1.14)	0.77 (0.65–0.92)	0.03	0.39
NT–ProBNP (ηg/l)	93 (58–149)	108 (70–167)	>0.99	65 (32–130)	154 (92–260)	0.05	0.07
Exercise test (W)	150 (135–165)	133 (115–151)	0.03	151 (133–151)	118 (96–140)	0.001	0.10

Data are presented as mean (95% confidence interval). [a]Mixed models test of within-group changes; [b]Mixed models test of interaction effect; EF, ejection fraction; GS, global strain; MAPSE, mitral annular plane systolic excursion; NT-proBNP, N-terminal pro-brain natriuretic peptide.Reproduced with permission from Radiation Oncology (Lund, Kalman 2014).



(○) CRT=chemoradiotherapy, (●) CT=chemotherapy. SVI, Stroke volume index; CI, cardiac index; HR, heart rate; Hb, Haemoglobin level; DO_2I, Oxygen delivery index; SVR, Systemic vascular resistance.

Figure 12 A-F. Haemodynamic profiles during the study period. There were no significant interaction effects (p^1). B; Preoperative CI was lower in the CRT group (p^2). Linear mixed models, presented as mean and 95 % confidence interval.

In paper IV (CRT, n=14; CT, n=17) we observed a decreased CI at baseline (as measured in the pre-operative ward before surgery) in the CRT group compared to the CT group. This did not translate into a significant interaction effect for lower CI in the CRT group during the study period (p=0.06). Haemodynamic values changed in both groups during the study period but there were no significant interaction effects between the groups (Figure 12). On POD 3, both groups displayed an almost identical hyperdynamic state compared to baseline.

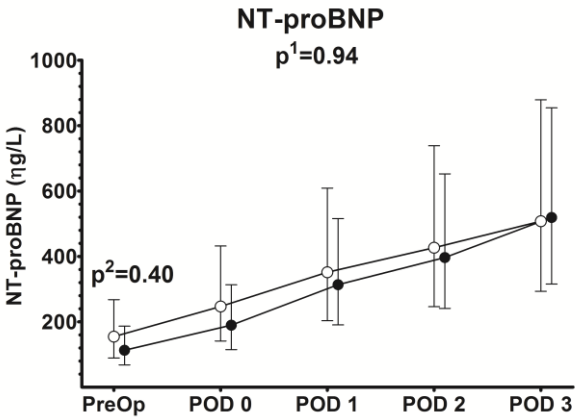


Figure 13. NT-proBNP levels increased similarly in both groups during the study period. Linear mixed models. p^1 interaction effect, p^2 preoperative differences, presented as mean and 95 % confidence interval.group.

The biochemical marker NT-proBNP increased similarly throughout the study period in both groups (Figure 13) while there were no differences in the levels of Troponin T in either group. In a post hoc statistical analysis of NT-proBNP levels on POD 3 we found higher levels in the patients that required time in the intensive care unit (p=0.03) but not in those that developed cardiovascular complications (p=0.06). Operative characteristics such as operating time, bleeding, transfusion and the volume of intravenous fluids administered during the study period were similar between the groups. There were no differences in cardiovascular complications between the groups. A downside of the per protocol analysis was that we detected a difference in patient characteristic in that the CRT group were older (66 vs. 60 years, p=0.03) and had a higher body mass index (26 vs. 23, p=0.04).

In paper V (CRT, n=14; CT, n=17) we detected that mRNA levels of IL-1β in lung biopsies from the medial part of the right inferior lobe were significantly higher in the CRT group compared to patients receiving CT alone 2.0 vs. 4.4 AU (p=0.007, Table 7). mRNA levels of other cytokines (IL-6, IL-8, IL-10, MCP-1) analysed were generally higher in the CRT group but these differences never reached statistical significance. Plasma levels of the same cytokines with the exception of IL-1β peaked immediately after surgery and there were no interaction effects between the groups. Plasma levels of IL-1β did not change at all (within group effect) during the study period and there were no interaction effects. The plasma levels of markers of complement activation all displayed within group changes of a similar pattern during the study period with no interaction effect. The number of leukocytes in the interalveolar stroma did not differ between the groups (p=0.53). PaO_2/FiO_2-ratios declined similarly in both groups and showed no tendency to improve at POD 3. Pulmonary complications were frequent in both groups (CRT 93 %, CT 65 %) but the difference in incidence between the groups did not reach statistical difference (p=0.09, Table 8).

Table 7. mRNA levels in lung biopsies

	Chemo-therapy (n=15)	Chemoradio-therapy (n=11)	p
IL-1β	2.00 (1.37-2.73)	4.41 (2.65-6.43)	0.007
IL-6	6.52 (4.16-8.85)	8.94 (3.27-20.53)	0.16
IL-8	9.53 (2.28-14.41)	17.45 (2.36-29.24)	0.39
IL-10	3.71 (1.19-4.76)	4.24 (2.66-6.84)	0.28
MCP-1	7.10 (2.35-11.16)	9.65 (5.01-21.19)	0.16

The level of IL-1β mRNA in lung biopsises was significantly higher in the CRT group. Mann-Whitney U-test, presented as median and interquartile range of arbitrary units.

Table 8. Postoperative complications in study two (paper IV-V)

Complication n(%)	Chemo-therapy (n=17)	Chemoradio-therapy (n=14)	P
In hospital mortality	0 (0)	1 (7)	0.45
Surgical complication[a]	9 (53)	5 (36)	0.47
MACE[b]	5 (29)	5 (36)	0.72
Atrial fibrillation[b]	4 (24)	4 (29)	1.0
Myocardial infarction[b]	1 (6)	0 (0)	1.0
Pulmonary complications[b]	11 (65)	13 (93)	0.09
Pulmonary infection[b]	3 (18)	4 (29)	0.67
Respiratory failure[b]	11 (65)	11 (79)	0.46
Sepsis[c]	3 (18)	3 (21)	1.0

Respiratory failure was the most common complication. There were no significant differences in postoperative morbidity or mortality between the CT and CRT groups. Fischer´s exact test. [a]Defined as any complication directly linked to the surgical procedure i.e. anastomotic leakage, chylothorax or similar. [b]Defined according to ESA-ESICM guidelines (Jammer et al 2014). [c] Defined according to 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. MACE: major adverse cardiovascular event.

4.2.1.1 Aspects on cardiac function following neoadjuvant treatment

CRT has been suggested to increase postoperative morbidity and possibly also mortality, but the results from randomised studies of CRT are inconsistent[27, 28, 32, 33]. As radiotherapy for esophageal cancer will inevitably also affect adjacent thoracic organs, such as the heart and lung, impaired cardiac function might well be an important pathophysiological factor behind the alleged increase in morbidity and mortality. It is well known that delivery of external radiation to the thoracic cavity causes long term negative cardiac effects such as coronary artery disease, valvular disease, heart failure and pericardial disease[150]. However, many of the studies are old and used techniques for radiation therapy that are considered to be outdated today. On the other hand, with increasingly longer survival rates for many cancers such as breast cancer, these potential adverse effects have emerged as an expanding problem. Since these effects represent very late complications occurring 5-10 years or more after treatment, they may well be severely underreported[151]. There are few data concerning long term cardiac effects from radiotherapy for esophageal cancer, as long term survival historically has been very poor. In a study by Morota et al severe cardiotoxic events were found in patients receiving definitive CRT for esophageal cancer during a median follow up time of 26 months[152].

There is some knowledge about acute radiation damage to the heart that originates mainly from old studies on the treatment of lymphoma[153]. During the last decade additional studies employing modern diagnostic techniques have indicated that acute cardiac impairment may follow thoracic radiotherapy for cancer. Mostly these studies have concerned radiotherapy for breast cancer[154-156]. Some studies have been performed in esophageal cancer patients although most of those studies are retrospective and limited by small sample sizes[157-159]. In a prospective study by Hatakenaka et al[157] the level of cardiac impairment was even larger than presently observed and also included a global impairment of systolic function. The most likely explanation to these conflicting results could be that the radiotherapy treatment administered in that study (mainly palliative) contained larger doses and that other chemotherapeutic agents were administered. Otherwise our results concur with the those reported by Hatakenaka, in that the greatest systolic impairment of wall motion was found in the mid anteroseptal, mid inferoseptal and mid inferior segments, which is in accordance with our findings of decreased mitral annular plane systolic excursion of the septum (MAPSE sept). We also observed that CRT decreased the blood flow velocities over the mitral valve during the fast, passive filling phase of the left ventricle (E), coupled with an unchanged blood flow during atrial contraction (A) and accordingly a decreased E/A. These data suggest an impaired diastolic function as a consequence of impaired relaxation of the left ventricle. This impairment of diastolic function could well occur as a result of acute inflammation affecting the myocardium and/or pericardium with oedema and decreased compliance caused by radiotherapy. It should be emphasized that we have no confirmatory observation on this as our study was not designed to investigate underlying mechanisms.

Diastolic dysfunction is normally divided into five separate categories based on severity: Normal, impaired relaxation, pseudo normal, restrictive with reversibility and restrictive

without reversibility. A number of echocardiographic characteristics are used to differentiate between the groups. We abstained from applying such a classification since this would have severely diluted the power of our data, given the small sample size. Moreover the primary outcome variable in this paper was to evaluate systolic function by GS why only a limited number of variables for evaluating diastolic function were measured. We did not find a dose –response relationship between V30 and the echocardiographic or NT-proBNP changes. This could again be related to the small sample size or to the fact that V30 reflects the radiation dose to the whole heart volume rather than different segments. Due to the small sample size a segmental analysis was not performed.

The increase of NT-proBNP, which we found in the CRT group, was small and not indicative of heart failure. However, NT-proBNP has also been launched as a predictor for the risk of perioperative cardiac complications, with cut off-levels between 201-791 ng/l[112, 113]. Pre and perioperative levels of NT-proBNP may accordingly act as predictors of postoperative atrial fibrillation even if cut-off levels have to be better defined[115]. It cannot be ruled out that the increase observed in the CRT group might be related to increased inflammation elicited by radiotherapy rather than cardiac impairment[160]. It should also be noted that we found no indication of cardiac impairment by echocardiography or NT-proBNP in the chemotherapy group.

Both groups displayed a decrease in maximum work capacity assessed by exercise testing before and after neoadjuvant treatment and there was a trend towards a greater change in the CRT group (p=0.10). A decrease in exercise capacity following neoadjuvant treatment has previously been documented and has been suggested to be a predictor of postoperative morbidity[20, 161]. However when exercise tests from the entire NeoRes study population were analysed, the decrease in work capacity was completely abolished when adjusting results for haemoglobin levels[162]. This suggests that the decrease in work capacity might be mainly due to bone marrow depression from neoadjuvant treatment rather than from an effect on cardio respiratory function.

Our data thus indicate that by adding external radiation therapy to neoadjuvant CT, a small acute impairment of regional systolic and diastolic function occurs and there is a slight increase in NT-proBNP levels. The clinical implications of this remain unclear both regarding long term effects, but also regarding the perioperative period which was further investigated in paper IV.

4.2.1.2 Aspects on perioperative haemodynamics following neoadjuvant therapy

Preoperative haemodynamic variables were generally lower in the CRT group although these differences never reached statistical significance except for CI. This resulted from a slight decrease of mean SVI coupled with a somewhat lower mean heart rate in the CRT group. This is in accordance with our findings in paper III which indicated a regional systolic as well as a slight diastolic impairment associated with CRT. These differences in preoperative testing are unlikely to be the cause of hypovolaemia, as measurements were performed in

both groups after fluid optimisation. With the exception of low haemoglobin levels, the preoperatively assessed mean haemodynamic values were all within normal limits in both groups.

During surgery both groups behaved similarly with a decrease in all haemodynamic parameters. The lowest values were generally attained during the thoracic part of the operation. This pattern, as well as the absolute vales recorded, corresponded well to what has been reported previously during esophagectomy[163]. On POD 3, both groups displayed an almost identical hyperdynamic circulation with increased SVI, CI, DO_2I and HR compared to baseline, which was coupled with a decreased SVR. This indicates that when exposed to the per- and postoperative stress, both groups were able to increase their cardiac performance to the same extent irrespective of which type of neoadjuvant therapy had been given. We found no significant interaction effect between the groups during the study period.

NT-proBNP levels increased over time for the duration of the study period, reaching levels that might be predictive of an increased risk for postoperative cardiac events and mortality, as suggested by Rodseth et al[164]. This could potentially arise from the postoperative inflammatory response and fluid administration given to maintain intravascular volume. No correlation was found, however, between CRP (as a marker of inflammation) and NT-proBNP (data not shown). Regarding fluid administration the largest fluid volume was administered during the day of surgery (about 8000 ml in each group) and the mean volume of fluids (enteral and parenteral) remained around 4000 ml/day in both groups. This corresponded to a mean weight gain of about 2.5 kg from baseline to POD 1 and a further weight gain of about 0.6 kg up until POD 3 (data not shown) which is commonly seen following major surgery.
We did not analyse echocardiographic data as images acquired on POD 3 were of very low quality due to the mediastinal dissection and presence of drains and wound dressings. This is indicative of the difficulty in performing functional tests postoperatively in this group of patients, as previously reported by us concerning postoperative spirometry (paper I).

The results from paper IV seem to confirm a slight but clinically questionable impairment of cardiovascular function as detectable in the resting state after CRT. More importantly these data suggest that a deficient adaptation of the cardiovascular system, when challenged by surgery, was not prevailing. Thus CRT as administered in this study does not appear to add an increased cardiovascular risk compared to CT in the perioperative setting.

4.2.1.3 Aspects on perioperative inflammation following neoadjuvant treatment

The enhanced local levels of IL-1 β mRNA in the lung parenchyma harbours an obvious potential to play a pathophysiological role in lung injury. Two recent studies, which investigated the respective effect of protective ventilation and continuous positive airway pressure (CPAP) during esophagectomy, found that both protective ventilation and CPAP was followed by lower levels of IL-1β in bronchial lavage fluid compared to the control groups[165, 166]. In addition, a relationship between systemic inflammatory responses and

postoperative complications after esophagectomy has been documented in several studies[74, 77, 167]. It needs to be pointed out that the pulmonary IL.-1β gene expression can be up regulated following radiation without a concomitant increase in the circulation unless a second stimulus such as an infection or a trauma like surgery is added[168]. Besides having pro inflammatory properties, including induction of other cytokines and attraction of inflammatory cells, IL-1β has also been associated with increased wall thickness of the major airways, vascular congestion and fibrosis in rodent models[169, 170]. One of the key cytokines triggered by IL-1β is IL-6[168] which is considered to be of importance for the postoperative inflammatory responses[171]. IL-6 production was found to increase in pulmonary epithelial cell cultures after esophagectomy in a study by Abe et al[76]. On the other hand, we were unable to detect significant differences in IL-6 between our study groups, both in plasma and in mRNA from lung biopsies. Again, clinical research addressing similar subtle pathophysiological mechanisms always face the issues of ample sample sizes and the correct timing of sample collection.

Data from the complete NeoRes trial showed that although the final number of postoperative complications was not increased by CRT, the severity of the respective complications was greater[127]. The trial also found that the mortality rate from serious adverse events and postoperative complications was higher in the CRT group during the first year following randomisation[34]. However the underlying cause of the increased one year mortality is unknown. In the present cohort derived from the entire group of patients enrolled, we were able to study the respiratory complications in more details, including measurements of arterial blood gases, respiratory rates and detailed definition of respiratory complications. We report a pulmonary complication rate of 93 % in CRT vs. 65 % in CT group (p=0.09). Our data thus suggest that there might be a relationship between the enhanced expression of IL-1β mRNA expression in lung tissue and postoperative occurrence of pulmonary complications. These observations, taken together, describe a consistent pattern that suggests an enhanced risk for severe respiratory complications after esophagectomy when adding the current type of radiotherapy to chemotherapy. In a non-randomised cohort study that compared neoadjuvant CRT with surgery only[172], a significant impairment of respiratory parameters both before and after surgery was shown in the CRT group. Postoperative heart rates and the duration of the systemic inflammatory response syndrome were also increased. We were unable to demonstrate corresponding clear differences between the CRT and CT groups.

4.2.2 Limitations to be considered for papers III-V

It is important to consider the impact of the small sample size (40 patients in paper III and 31 patients in paper IV-V) on results. As the design of these studies included a cohort of patients enrolled in a larger study, no formal power calculations were found relevant for the endpoints addressed in papers III-V.

Results are also limited by missing data. In paper III we were able to perform echocardiography examinations in only 67 % of patients, while in paper IV we were able to perform postoperative haemodynamic measurements in only 77 % of the patients.

These figures illustrate the complexity and many hurdles that affect the completion of complex perioperative physiological studies associated with demanding surgical procedures.

In papers IV and V we observed an imbalance in age and body mass index between the two treatment groups. We handled this by adjusting the statistical model used for a set of co-variables. This decreased the tendency for changes in CI as previously detected in paper IV, but otherwise no significant effects of these potential confounders were found.

Possible limitations arising from our methodology were several. In paper III we used echocardiography which is known to be user dependent. Consequently, we used the same laboratory technician for performing the examinations and all post processing was performed by me without knowledge of treatment group allocation. Also, most echocardiographic parameters are influenced by preload (volume status). This could have affected our results as patients might be differently affected by catabolic/hypovolaemic issues depending on group allocation to neoadjuvant treatment.
In paper IV LiDCOplus was used for haemodynamic measurements. While this is a validated method approved for clinical use it has limitations such as limited accuracy, especially during arrhythmias and in cases of aortic insufficiency. To increase accuracy we used three instead of the recommended two standard calibration points[132]. In the presence of arrhythmias, the average values acquired during 30 sec were used. Haemodynamic measurements are also sensitive to the level of physical activity, pain or emotional stress. This makes it important to standardise patient activity and overall situation during measurements especially following surgery. In order to mitigate this, great care was taken to control pain before measurement and to have the patient resting awake in bed. To avoid any influence from clinical staff or the researcher, no other activities took place during measurements and the researcher and monitor were hidden behind a screen. Values acquired during movement or coughing of the patient were disregarded. We also employed fluid optimisation during haemodynamic measurements. Although fluid optimisation is widely employed, often stroke volume variation or pulse pressure variation are used to assess the fluid responsiveness. This was not applicable in an awake, spontaneously breathing patient and so a modified protocol derived from Pearse et al [126] was used. This protocol was based on absolute changes of SV following a fluid bolus to guide further fluid administration rather than predicting the response from stroke volume or pulse pressure variation. This introduced the possibility of giving unnecessary fluid and possibly even worsening a situation with heart failure (as fluids given could even decrease SV). However no patient displayed a decrease in SV following any bolus during the study.

In paper V, Multiplex analysis was used for measuring plasma levels of cytokines. Although the method has several advantages compared to traditional ELISA, some limitations have to be recognized. In Multiplex analysis it is critical that cross reactivity between any of the involved antibodies is avoided and that the antibodies are sufficiently specific. This is the case more so than for ELISA, as many cytokines are analysed in the same sample. The plasma preparation can also affect the levels of detectable cytokines as many of these are

bound to plasma proteins which may be affected by the preparation process. Moreover the underlying disease and concurrent state of inflammation have the potential to affect the levels of circulating proteins [128]. Thus the use of plasma in paper V for Multiplex analysis instead of serum can hypothetically have affected the levels of cytokines detectable.

5 CONCLUSIONS

- One-lung ventilation induces a delayed increase in C5b-9 compared to two-lung ventilation during the thoracic part of esophagectomy.
- One-lung ventilation during the thoracic part of esophagectomy seems not to affect factors regulating pulmonary vascular tone when compared to two-lung ventilation during esophagectomy.
- Neoadjuvant chemoradiotherapy has an acute negative effect on both systolic and diastolic function of the heart but these effects do not prevail when the cardiovascular system is challenged by the stress of surgery.
- The addition of radiotherapy to neoadjuvant chemotherapy increases the local inflammation in the right lung contained within the radiation field.

6 FUTURE PERSPECTIVES

With the development of minimally invasive techniques for esophagectomy the use of OLV has declined but surgical treatment will remain the mainstay for curative treatment of esophageal cancer for the foreseeable future. However, as CRT with curative intent is starting to emerge as a possible treatment option in ESCC, surgery might not be the future primary treatment in this group even though data is currently lacking regarding how to best select patients. CRT with curative intent will invariably contain larger radiation doses than are currently given for neoadjuvant treatment and as such damage to surrounding organs will also increase. In any definitive CRT treatment rescue esophagectomy will have to be an option in cases with treatment failure. As a consequence the problem of radiation induced tissue damage may be even more important in the future management of patients with ESCC, particularly in those submitted to the very high risk procedure of rescue esophagectomy. Research avenues that have to be explored are those focusing on the mechanisms behind lung and cardiac injuries with the objective to better target radiotherapy and minimise the tissue damage, as well as targeting the negative effects in the perioperative setting.

Furthermore, it is an open question as to how long a time should elapse between the cessation of neoadjuvant treatment and the resection of the esophagus. Accordingly, longitudinal studies on the natural course of changes in organ function, as presently observed, have to be performed in order to define the optimal time point for surgical intervention. The on-going NeoRes2 study investigates the effects of a longer time interval between neoadjuvant treatment and surgery and also includes serial NT-proBNP measurements.

The introduction of minimally invasive esophagectomy techniques and enhanced recovery programmes for the postoperative rehabilitation of the patients, have and will have an important influence on the perioperative outcome. Still, the challenges of performing surgery consecutively in the two major body compartments persists and the pathophysiological mechanisms involved have to be better understood. Also the impact of age needs to be further explored. The age profile of future patients will change and more octogenarians will in the future be offered surgery for esophageal cancer. Therefore the specific impact of age on many of those parameters which have currently been explored in regard to neoadjuvant treatment has to be studied in more detail in order to adapt to the future demands of clinical medicine.

With the predominance of adenocarcinoma in the current and most probably future population with malignancy of esophageus, it is most likely that a variety of aspects on the further development of neoadjuvant CT have to be taken into account. Novel drugs, and combinations of these, will be developed and tested in clinical situations. Many of those (together with those currently in use) will exert adverse effects on central organ functions such as the heart and respiratory tree. Given all of these preconditions, the respiratory complication rates will most probably remain significant and cannot be ignored. As a consequence of these changes, additional and complementary observational studies, based on the current methodology have to be designed and hypotheses need to be explored.

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