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**The Morphometry of Lymph Node Metastases  
after Acetone Compression**

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5-FU	Fluorouracil	TME	Total Mesorectal Excision
AC	Acetone Compression	TRG	Tumor Regression Grading
APC	Adenomatosis Polyposis Coli	VEGF	Vascular Endothelial Growth
AP	Alkaline Phosphatase		Factor
CI	Chromosomal Instability	WME	Whole Mesorectal Embedding
CRC	Colorectal Carcinoma		
CRM	Circumferential Resection Margin		
DAB	3,3'- Diaminobenzidine		
DFS	Disease-free Survival		
Dpi	Dots Per Inch		
EGFR	Epidermal Growth Factor		
fDCS	Follicular Dendritic Cells		
H&E	Hematoxylin and Eosin		
HNPPC	Hereditary Nonpolyposis Colorectal Cancer		
HRP	Horse Raddish Peroxidase		
IHC	Immunohistochemistry		
ITC	Isolated Tumor Cells		
KDE	Kernel Density Estimation		
MD	Manual Dissectioning		
MHC II	Major Histocompatibility Complex II		
mi	Micrometastases		
MMR	Mismatch Repair		
MRI	Magnetic Resonance Imaging		
MSI	Microsatellite Instability		
Px	Pixels		
RCT	Radiochemotherapy		
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction		
SD	Standard Deviation		

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## **1. Introduction**

### **1.1 Colorectal Cancer**

Colorectal carcinoma (CRC) are malignant neoplasms arising from the caudal midgut and the hindgut. They exhibit local invasive growth, lymphogeneous metastatic spread to regional lymph nodes and hematogeneous spread to distant organs, most notably liver and lungs. The majority of CRC are caused by acquired DNA-mutations and epigenetic alterations (McCoy and Weinberg 1983, The Cancer Genome Atlas Network 2012), while a fraction of colorectal cancers are caused by hereditary susceptibilities (Dietmaier et al. 2000, Umar et al. 2004). As adenocarcinoma arising from the epithelial lining of the gut is the most common tissue of origin, CRC is often regarded synonymic for adenocarcinoma. However, any tissue belonging to the large intestine may undergo malignant transformation. Given their differences in anatomy and clinical treatment, colon and rectal cancer must clearly be defined. In spite of their distinct properties, their underlying biology seems comparable, as it is indicated by the emerging comprehensive molecular landscape of colorectal cancer (The Cancer Genome Atlas Network 2012). The histopathology of biopsies and surgical resections of colorectal cancer, including a detailed investigation of the lymph nodes for metastasis, is essential for diagnosis and the staging of CRC, as well as clinical decision-making and high-quality medical care (Virchow 1898, Nathan et al. 2011).

#### **1.1.1 The Epidemiology of Colorectal Cancer**

In 2008, 1.235 million new cases of CRC occurred worldwide, making it the third most common type of cancer in the world after lung cancer and breast cancer (data: IARC, Globocan Fast Stats 2008). It is also the second most frequent cause of cancer-related death in Europe (Ferlay et al. 2007), including Germany. In Germany, 35,360 men and 30,040 women suffered from CRC in 2008 and forecasts suggested these numbers would rise to 38,300 men and 31,100 women in 2012. Like most solid tumors, CRC predominantly affects the elderly: In Germany, over 50% of all patients diagnosed with CRC are 70 years or older and only 10% are diagnosed before the age of 55. The mortality rate, on the other hand, has declined by more than 20% since 1999 for both men and women, and was 0.24 for men and 0.14 for women in 2008 (data: "Krebs in Deutschland", chapter "Darm C18-21"). In the U.S., CRC is the third most common type of cancer after lung cancer and prostate cancer (Siegel et al. 2013). Incidence rates increase with age, the median age at diagnosis for CRC being 69 (data: SEER Cancer Statistics Review 1975-2009). Mortality rates in the U.S. have been decreasing since 1950 in women and since 1980 in men. In contrast to Germany, incidence rates have shown slight decreases in the U.S. since 1998, 3% per year for men and 2.3% per year for women (Kohler et al. 2011). Decreasing incidence and mortality rates in the U.S.



are accompanied by an increase in the 5-year relative survival rate for CRC, which increased for men and women from 51.7% in 1981-1983 to 68.1% in 2002-2008 (data: SEER Cancer Statistics Review 1975-2009).

### **1.1.2 Risk Factors**

The known risk factors for CRC can be subdivided into modifiable and nonmodifiable risk factors. Nonmodifiable risk factors are sex, genetic susceptibility as represented by a personal or family history of adenomatous polyps or colorectal cancer and chronic inflammatory bowel disease. Incidence and mortality rates are about 35 to 40% higher for men than for women (American Cancer Society 2011). Patients who have first degree relatives with a colorectal carcinoma run a risk two to three times as high developing CRC themselves (Schmiegel et al. 2008). Patients with inflammatory bowel disease, most notably colitis ulcerosa, have an increased risk of cancer of the colon and the rectum, respective to the duration and extent of colitis. Patients with pancolitis, for example, have a relative risk of 14.8 of developing CRC compared to the general population (Ekbohm et al. 1990). The modifiable risk factors encompass life-style habits, including the so-called 'Western-type' diet with a high consumption of red or processed meat, highly caloric food and physical inactivity (Bosman et al. 2010). While incidence rates have stabilized or only slightly increased in economically developed countries such as Western Europe or Australia, *Center et al.* observed a continuing increase in CRC incidence rates in newly-industrialized countries such as Slovakia or Poland, reflecting the association of CRC to a recently-adopted western lifestyle (Center et al. 2009). The EPIC study investigated dietary habits of 478,040 men and women from 10 different European countries over a 6-year period and observed a linear increase in the hazard ratio by 1.49 for colon cancer and by 1.65 for rectal cancer per 100g consumed red and processed meat per day (Norat et al. 2005). Based on such findings, both the current German guidelines on CRC and the 2012 guidelines on Nutrition and Physical Activity for Cancer Prevention from the American Cancer Society recommend limiting the consumption of red and processed meat and alcohol, while regular consumption of high-fiber foods, fruits and vegetables is encouraged (Schmiegel et al. 2008, Kushi et al. 2012). Since male smokers have an estimated relative risk of 1.32 and female smokers an estimated relative risk of 1.42 of developing CRC (Chao et al. 2000), both guidelines also advise not smoking (Schmiegel et al. 2008, Kushi et al. 2012). Protective factors include a fiber-rich diet, physical activity and maintaining a healthy weight. A recent Danish study concluded that about 23% of CRC cases might be avoided by adhering to a healthy lifestyle (Kirkegaard et al. 2010).

### **1.1.3 Pathogenesis**

Colorectal cancer is a genetic disease, approximately 90% of all cases resulting from sporadic DNA mutations (Riede et al. 2004). Among all cancers, CRC is possibly best understood on the molecular level. The first known cellular proto-oncogene, k-ras, was initially isolated from colon cancers (McCoy and Weinberg 1983), and the concept of step-wise carcinogenesis was derived from observations in CRC (Fearon and Vogelstein 1990). Malignant neoplasms have defined properties that distinguish them from normal tissues. These 'hallmarks' (Hanahan and Weinberg 2000 and 2011) are mediated by changes in gene expression. Each hallmark may be caused by several different kinds of alterations affecting different genes. CRC might be considered the archetypical model for the step-wise acquisition of these hallmarks: a common first step is a protein-coding mutation in the Adenomatosis Polyposis Coli (APC) gene. The resulting loss of APC function causes stabilization of the protein beta-catenin, which acts as a transcription factor together with TCF/LEF proteins to activate a set of genes involved in cell growth and self-renewal, including cell-cycle protein Cyclin D1 and proto-oncogene c-Myc (Kumar et al. 2010). Subsequent mutations may include activation of proto-oncogene k-ras, which promotes PI3 kinase-related cell growth and possibly cellular plasticity (Ischenko et al. 2013), loss of tumor suppressor DCC and finally loss of function of the central tumor suppressor TP53. The acquisition of activating and silencing mutations is thought to underlie the adenoma-carcinoma-sequence, the clinically observable formation of CRC: initial events such as loss of APC cause formation of adenomatous polyps by mediating self-sustained growth. Within years, these benign precursor lesions may eventually undergo malignant transformation and become invasive carcinomas, e.g. by TP53-loss. On the molecular level, two major phenotypes of CRC adenocarcinomas can be distinguished: tumors with chromosomal instability (CI) and tumors with DNA-mismatch-repair defects, indicated by the presence of microsatellite instability (MSI). The 'classical' adenoma-carcinoma sequence is usually accompanied by CI causing alterations in the copy number and structure of chromosomes (Markowitz and Bertagnolli 2009). MSI results from inherited or acquired mutations in DNA mismatch-repair genes and underlies about 15% of all CRC (Umar et al. 2004), and about 90% of the cases involving patients with hereditary nonpolyposis colorectal cancer (Bocker et al. 1997), the most common type of hereditary CRC-disposition.

### **1.1.4 Genetic Factors**

In about 5% of all cases, CRC is caused by a genetic syndrome. These syndromes may manifest themselves as colonic polyposis as in Familial Adenomatous Polyposis caused by hereditary APC mutation and in Peutz- Jeghers- Syndrome. Other syndromes, such as the hereditary nonpolyposis colorectal cancer syndrome (HNPCC), do not feature increased numbers of polyps. HNPCC, or Lynch syndrome, is the most common genetic syndrome

responsible for CRC, causing 2 to 4% of all CRC cases (Jasperson et al. 2010) and is characterized by a deficiency in DNA-mismatch-repair, autosomal dominant inheritance and neoplastic lesions that occur in young adults. Identification of HNPCC patients is facilitated by reference listings of diagnostic criteria, most notably the Amsterdam criteria and the revised Bethesda criteria (Herold 2011). Patients with HNPCC have a risk of up to 80% of developing CRC throughout their lifetime and have increased rates of kidney, ovary and skin cancer. Thus, in such cases, an annual colonoscopy is recommended as early as the age of 25 (Schmiegel et al. 2008). On the genomic level, the mismatch-repair-deficiency is indicated by length-changes of small repetitive DNA-sequences, so-called microsatellites. Microsatellite instability is defined as an alteration of the length of these microsatellites and occurs in about 90% of patients with HNPCC and in about 15 to 30% of all patients with sporadic CRC (Bocker et al. 1997). A panel of five defined microsatellite sequences in the tumor should be tested for mutations in patients suspected of having HNPCC. Depending on whether 2, 1 or none of the five microsatellite sequences of the tumor DNA have been mutated, MSI can be further classified as MSI-high, MSI-low or MSI-stable (Umar et al. 2004). A practical approach to screening for MSI is the detection of the most commonly involved mismatch-repair enzymes by immunohistochemistry: MSH2, MLH1, MSH6 and PMS2 on tissue sections of a biopsy of a surgical specimen. Loss of either of these proteins is highly predictable for HNPCC or acquired MSI (Jasperson et al. 2010).

### **1.1.5 The Classification and Metastatic Spread of CRC**

Colorectal carcinoma show both lymphatic and hematogenous metastatic dissemination. The first targets of lymphatic metastases are regional lymph nodes of the section of the bowel the tumor is located in (Riede et al. 2004). Organotypic hematogenous metastatic spread depends on the location of the tumor. Blood from the rectum is drained by the hemorrhoidal plexus, which forms an anastomosis between the portal system and the systemic circulation. The lower part of the rectum and the anal canal are drained by the middle rectal vein, which connects with the inferior vena cava via the internal iliac vein and by the lower rectal vein, which connects to the inferior vena cava via the internal pudendal vein and internal iliac vein (Aumüller et al. 2007). Thus, tumors of the upper and middle rectum cause liver metastases in over 50% of patients, and tumors of the lower rectum may cause early lung metastases (Herold 2011). The systemic venous drainage of the lower rectum is pharmacologically employed by suppositories, as hepatic first-pass metabolism is circumvented. Because of this anastomosis between hemorrhoidal plexus and portal system, portal hypertension especially in liver cirrhosis, may cause rectal varices (Aumüller et al. 2007).

The classification of colorectal cancer is based on the UICC TNM classification 7<sup>th</sup> edition, 2010:

Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Every T	N1, N2	M0
Stage IIIA	T1, T2	N1a	M0
	T1	N2a	M0
Stage IIIB	T3, T4a	N1	M0
	T2, T3	N2a	M0
	T1, T2	N2b	M0
Stage IIIC	T4a	N2a	M0
	T3, T4b	N2b	M0
	T4b	N1, N2	M0
Stage IVA	Every T	Every N	M1a
Stage IVB	Every T	Every N	M1b

**Table 1.1**

### **Tumor Stage according to the UICC:**

#### **T-Tumor**

- T1-** Tumor invades submucosa
- T2-** Tumor invades muscularis propria
- T3-** Tumor invades the muscularis propria in subserosa or nonperitonealized pericolic or perirectal tissue
- T4-** Tumor invades adjacent organs or other structures and/or perforates visceral peritoneum
  - T4a-** Tumor perforates visceral peritoneum
  - T4b-** Tumor invades other organs or structures

#### **N-Regional Lymph Nodes**

- N1-** Metastasis in 1 to 3 regional lymph nodes
  - N1a-** Metastasis in 1 regional lymph node
  - N1b-** Metastasis in 2 to 3 regional lymph nodes
  - N1c-** Tumor deposits or satellites in the fatty tissue of the subserosa or the

nonperitonealized pericolic or perirectal fatty tissue without distant metastasis

- N2-** Metastasis to 4 or more regional lymph nodes
  - N2a-** Metastasis to 4 to 6 regional lymph nodes
  - N2b-** Metastasis to 7 or more regional lymph nodes

#### **M-Distant Metastasis**

- M0-** No distant metastasis
- M1-** Distant metastasis
  - M1a-** Metastasis confined to one organ or site (liver, lung, ovaries, non-regional lymph nodes)
  - M1b-** Metastases in more than 1 organ or the Peritoneum

(Wittekind and Meyer 2010, p.96-97)

### 1.1.6 The Staging of Isolated Tumor Cells and Micrometastases

Micrometastases are defined as infiltrated lymph nodes with a diameter of 0.2 to 2mm, while isolated tumor cells are defined as single cells or cells cluster with a diameter no larger than 0.2mm. The distinction between macrometastasis, micrometastasis and isolated tumor cells should be observed and a distinction in the classification of nodal status of micrometastasis and isolated tumor cells should be made as well:

Status	Classification
Both macro- and micrometastases are found	<b>pN1 or pN2</b>
Solely micrometastasis is found	The supplement 'mi' can be used. E.g.: pN1 ( <b>mi</b> ) or pN2 ( <b>mi</b> )
Isolated tumor cells are detected	The supplement 'i+' or 'mol+' can be used, depending on the method the isolated tumor cells were detected with:  -pN0 ( <b>i+</b> ) if the isolated tumor cells were detected using morphologic methods, such as H&E staining -pN0 ( <b>mol+</b> ) if the isolated tumor cells were detected using non-morphologic methods, such as PCR

**Table 1.2 The Staging of Isolated Tumor Cells and Micrometastases**

The usage of the abovementioned supplements, though optional, is nevertheless recommended (Wittekind and Meyer 2010).

### 1.1.7 Symptoms and Screening

Colorectal carcinoma is anatomically unevenly distributed. Two thirds of CRC are located in the colon, 30% can be found in the rectum, which forms 10% of the length of the large intestine (data: "Krebs in Deutschland", chapter "Darm C18-21"). Patients with colorectal cancer rarely show early symptoms, and the disease can remain undetected for a long time. Once tumor symptoms occur, patients may present with rectal bleeding due to the ulceration of the tumor, which can also cause anemia and patients feeling fatigued. Obstructive growth of the tumor may result in pain or changes in bowl habits, such as constipation or diarrhea. As 10% of rectal tumors can be palpated (Herold 2011), gold standard in screening and diagnosis is performing a digital rectal examination, followed by a colonoscopy and possibly polypectomy, which have proven to reduce incidence rates up to 90% (Winawer et al. 1993). According to current treatment guidelines, colonoscopy is recommended every ten years (Schmiegel et al. 2008) since studies have shown that it takes 10-15 years from initial formation of a polyp to invasive carcinoma (Kelloff et al. 2004). Alternately, an annual fecal blood test can be used as a screening method. The test relies on the detection of occult

blood and is less sensitive compared to colonoscopy but has also proven to significantly decrease the incidence of colorectal carcinoma (Mandel et al. 2000).

## **1.2 The Therapy of CRC**

### **1.2.1 The Definition of CRC**

The colon is approximately 1.5 meters in length, subdivided into the ascending, the transverse, the descending and finally the sigmoid colon. Of these four parts, only the transverse and the sigmoid colon are intraperitoneal, while the ascending and descending colon are attached to the rear wall of the abdominal cavity and located secondarily retroperitoneal. The peritoneal covering of the sigmoid colon can expand to the front of the upper rectum, causing it to be secondarily retroperitoneal or even intraperitoneal, depending on the extent of the peritoneal covering. The rectal ampulla and anal canal are located in the small pelvis below the abdominal cavity (Martini et al. 2006). In Germany, the gold standard for distinguishing between colonic and rectal cancer is the distance from the anocutaneous line to the distal edge of the tumor measured by rigid rectoscopy. Tumors more than 16cm from the anocutaneous line are considered colonic whereas tumors 16cm or less are considered rectal tumors. Tumors of the rectum are further subdivided into tumors of the upper, middle or lower part of the rectum according to the distance of the tumors from the anocutaneous line (12 to 16cm, 6 to 12cm and less than 6cm respectively) (Schmiegel et al. 2008). These compartments differ in abundance of lymph nodes and of metastatic spread (Sprenger et al. 2010, Sprenger et al. 2013b). American guidelines, on the other hand, define rectal cancer as tumors located 12cm or closer to the anocutaneous line. The American definition is based on the local recurrence rates of tumors located 12cm or more from the anal verge, which are similar to the rates of colon tumors (Nelson et al. 2001).

### **1.2.2 The Therapy of Rectal Cancer**

In the treatment of rectal cancer, all three modalities of oncological treatment are employed: surgery, radiation and chemotherapy. Decisions regarding therapy are based on rectoscopy for tumor localization and biopsy, on endosonography to determine the depth of tumor penetration and possibly on MRI to establish the distance of the tumor from the mesorectum (Schmiegel et al. 2008). Surgical treatment is essential for the patient's prognosis and depending on the size and the location of the tumor within the rectum, different surgical procedures may be used. Each surgical approach aims at complete tumor removal (R0) by removing of the tumor using the no-touch-technique and taking into consideration recommended safety margins for surgical resection. To ensure an optimal outcome and low local recurrence rates, surgical treatment includes resection of the mesorectum and the removal of the mesorectal fatty tissue as well as adjacent lymphatic vessels and lymph nodes. Mesorectal excision can be performed as Partial or Total Mesorectal Excision (PME,

TME) and the GAST-05 study is investigating possible benefits of applying PME instead of TME in tumors located in the upper part of the rectum (Hofheinz et al 2012b). For resection of tumors located in the sigmoid-rectal junction or the proximal rectum, treatment guidelines recommend anterior resection, which includes resection of the upper part of the rectum carrying the tumor as well as PME. This procedure allows preservation of continence by establishing an anastomosis between the descending colon and the remaining lower part of the rectum. To further improve the patient's continence, prior to establishing the anastomosis, a J-shaped pouch of the distal part of the descending colon can be formed. If the tumor is located in the central part of the rectum, the entire rectum is removed, resulting in a so-called low anterior resection including TME. Resection of tumors located in the lower part of the rectum either requires low anterior resection and TME or abdominoperineal resection and TME. Abdominoperineal resection includes removal of the entire rectum, closing the perineum and forming a sigmoidostomy or descendostomy (Schmiegel et al. 2008, Siewert et Stein 2012). Besides location and size of the tumor, one of the most important factors affecting treatment decisions is the patient's lymph node status, determined after surgical resection, pathological grossing and histopathological examination. The presence of lymph node metastases strongly influences tumor stage (table 1.1). Whereas perioperative therapy is not recommended for stage I tumors, the standard therapeutic approach for colorectal cancer stage II and stage III is preoperative neoadjuvant radiochemotherapy (RCT) followed by surgical resection and postoperative chemotherapy (Hofheinz et al. 2012b). This procedure was established by the CAO/ARO/AIO-94 study: Administering RCT neoadjuvantly instead of postoperatively caused a significant reduction in local recurrence rates (Sauer et al. 2004). The effect was recently confirmed by 11-year follow-up data (Sauer et al. 2012). Both neoadjuvant and adjuvant chemotherapy rely on fluorouracil (5-FU) and folic acid (Schmiegel et al. 2008). Ongoing studies focus on adding additional chemotherapeutic agents: the CAO/ARO/AIO-04-trial investigates a regimen supplemented by Oxaliplatin. Early data indicate an increased pathological complete response of 17% compared to 13% by treatment with 5-FU, folic acid and radiation (Rödel et al. 2012). *Hofheinz et al.* compared the usage of intravenous 5-FU with orally available prodrug Capecitabine in the neoadjuvant and adjuvant treatment of locally advanced rectal cancer, and observed an increased 5-year overall survival (76% vs 67%) and significantly fewer distant metastases (19% vs 28%) (Hofheinz et al. 2012a). Radiotherapy is most frequently applied in 25 to 28 fractions with a dose of 45 to 50.4 Gy, followed by surgery after 4 to 6 weeks (Schmiegel et al. 2008). The currently planned CAO/ARO/AIO-12 study will investigate if a longer interval after irradiation might increase pathological complete response (data: German Rectal Cancer Study Group, 9<sup>th</sup> newsletter, February 2013). Despite the

progress in local therapy, however, distant metastasis occurs with an unchanged rate in about 30% of CRC patients 10 years after resection (Sauer et al. 2012).

### **1.2.3 The Therapy of Colon Cancer**

The treatment of colon cancer is based on surgery and chemotherapy as radiotherapy is not applicable. The extent of the surgical removal of the bowel depends on the lymph drainage area the tumor is located in and the adjacent blood vessels. The tumor is removed using the so-called no-touch technique (Müller 2011), and adjuvant chemotherapy may be supplemented. The application of adjuvant chemotherapy depends on the patient's risk for local and distant recurrent disease and is usually only indicated if lymph node metastases are present (Schmiegel et al. 2008). For stage II patients the benefit of adjuvant chemotherapy is still questionable and several studies advise against it (Benson et al. 2004). Stage III colon cancer patients, on the other hand, have proven to benefit from adjuvant chemotherapy (Gill et al. 2004). Commonly used treatment regimens for adjuvant chemotherapy are based on combinations of folinic acid, 5-FU and oxaliplatin (FOLFOX), or folinic acid, 5-FU and irinotecan (FOLFIRI) (Schmiegel et al. 2008).

### **1.2.4 The Therapy of Patients with MSI**

According to *Umar et al.*, MSI is found in about 15% of all colorectal cancers (Umar et al. 2004), both in patients with HNPCC and sporadic CRC. However, the decision on therapy is currently solely based on tumor stage, while MSI status is not taken into consideration. *Jover et al.* point out that patients with MMR-proficient stage II or III CRC show an improvement in overall and disease-free survival of about 20% after being treated with adjuvant chemotherapy, whereas this increase in survival could not be found in patients with MMR-deficient tumors (Jover et al. 2009). Patients with MMR-deficient tumors stages II and III, on the other hand, were found to have a better clinical outcome than patients with MMR-proficient tumors, which has led *Lanza et al.* to recommend the immunohistochemical analysis of MLH1/MSH2 expression as a faster and more economic prognostic marker (Lanza et al. 2010). Treatment of MMR-deficient colon tumor cells with nonsteroidal anti-inflammatory drugs such as aspirin has led to a reduction in the MSI-phenotype (Rüschoff et al. 1998), suggesting that aspirin might be a possible chemopreventive for patients with MMR-deficient tumors (Mc Ilhatten et al. 2007). However, current treatment guidelines on CRC do not recommend regular use of aspirin as primary prophylaxis (Schmiegel et al. 2008).

### **1.2.5 Possible Biomarkers for the Treatment and Prognosis of CRC**

Since neoadjuvant RCT in combination with standardized surgical tumor removal has led to a significant reduction in local recurrence rates, it is currently considered standard for treatment of CRC in Germany. The grading of tumor regression was first introduced by *Dworak et al.* (Dworak et al. 1997) and reflects response to RCT. However, response rates



vary significantly, which raises the question of how to adequately predict a patient's response to therapy. Accurate prediction would allow for the selection of appropriate treatment regimens for patients and therefore optimize multimodal treatment of CRC. Besides prediction, forecasting the course of disease, prognosis, is of great importance. Among the most important prognostic markers for CRC patients is nodal status as survival rates drop from 88.3% in node-negative to 69.1% in node-positive rectal cancer patients (Kanemitsu et al. 2012). The nodal status also determines whether adjuvant chemotherapy is applicable as it is only indicated for stage III patients (Schmiegel et al. 2008). K-ras mutation status is an example of a biomarker that is both prognostic and predictive as it indicates a patient's prognosis and eligibility for treatment with anti-EGFR-therapy. Unlike patients with a k-ras mutation, patients with a tumor wild-type for k-ras show better chances of survival and benefit from treatment with anti-EGFR-antibody Cetuximab (Van Cutsem et al. 2011). Currently, both new prognostic and predictive markers are being investigated. Survivin, for example, is able to inhibit the activation of caspase-3 and 7, thus preventing apoptosis (Shin et al. 2001), and is regarded as a possible new prognostic marker. Low expression of survivin in patients with locally advanced rectal cancer leads to better survival rates while significantly reducing the risk of distant metastases (Rödel et al. 2002). *Sprenger et al.* imply that the transmembrane glycoprotein CD133 might be both a new prognostic and predictive marker as it is believed to make statements about metastasis and survival in rectal cancer patients treated with preoperative RCT. Patients with an increased amount of CD133-positive cancer cells were thought to demonstrate higher resistance to preoperative RCT as their disease-free survival was lower, while also showing higher residual tumor stages and less tumor regression (Sprenger et al. 2013a). The Transvalid trials A and B of the German Rectal Cancer Study Group aim at further promoting the concept of 'personalized medicine' by submitting biomaterial of CRC patients to genomic analyses prior to treatment. By testing the validity of prognostic and predictive biomarkers such as survivin, doctors have the possibility of distinguishing between a 'low-risk' and a 'high-risk' patient and thus therapy regimens can be adapted according to the patient's risk in local and distant recurrence (Grade et al. 2012).

### **1.3 Lymph Nodes**

#### **1.3.1 The Definition of Lymph Nodes, Lymph Node Metastasis and Micrometastases**

Lymph nodes are small, usually round or kidney-shaped lymphoid organs, coated with a fibrous capsule, parts of which extend into the node, the trabeculae. Lymphoid fluid enters the node through afferent lymphatic vessels, passes through a system of sinuses, which subdivides the node into compartments, before exiting the node through the efferent lymphatic vessel at the hilus. While passing through the node, about 99% of circulating

antigens are removed from the lymph, possibly activating antigen-presenting cells and thus stimulating an immune response (Welsch 2010, Martini et al. 2006). The human body contains up to 700 lymph nodes (Welsch 2010). Many malignant neoplasms are accompanied by lymph node metastases, which cause the swelling of the nodes and are often the initial clinical symptom of cancer patients (Herold 2011). CRC shows both hematogenous and lymphogenic dissemination and approximately 40% of CRC patients develop lymph node metastases (Parsons et al. 2011). Scientists were aware of the existence of isolated tumor cells (ITC) as early as the beginning of the 19<sup>th</sup> century (Hermanek et al. 1999), and the American Joint Committee on Cancer first defined isolated tumor cells as single cells or a cluster of cells with a diameter of no more than 0.2mm. Cell clusters with a diameter of 0.2 to 2mm or an infiltrate of more than 200 non-cohesive cells are considered micrometastases (Sirop et al. 2011).

### **1.3.2 The Impact of Micrometastases on Prognosis**

Patients with stage II CRC have a five-year relative survival rate of roughly 90%, compared to the five-year survival rate of only 69.6% for stage III colorectal cancer patients (SEER Cancer Statistics Review 1975-2009). Despite the more favorable prognosis, about 20% of stage II CRC patients die from cancer recurrence (Liefers et al. 1998). Some suggest that undetected occult metastases are responsible for this relatively high recurrence rate. Thus, the role of micrometastases as a potential prognostic factor especially for stage II patients becomes increasingly important. Micrometastases can be detected using conventional H&E histology, immunohistochemistry (IHC) or reverse transcriptase polymerase chain reaction (RT-PCR). By comparing studies that used either IHC or RT-PCR to detect micrometastases, *Sirop et al* showed that the different methods varied in efficiency as the detection rate of micrometastases increased from 24.7% after using IHC to 36.6% after using RT-PCR (Sirop et al. 2011). The exact relevance of micrometastases for prognosis is still unclear but a reverse correlation seems apparent: *Bilchik et al.* observed a recurrence rate of 22% in CRC patients with micrometastases compared to only 6% in patients without micrometastases (Bilchik et al. 2010). *Märkl et al.* evaluated 44 cases with routinely diagnosed micrometastases and, with regard to overall survival, found a similar negative outcome for patients with micro- and macrometastases. They could not show a prognostic difference between the presence of micrometastases or of isolated tumor cells, but they could show a strong trend for negative outcome in the presence of isolated tumor cells (Märkl et al. 2013a). *Sprenger et al.*, on the other hand, compared disease-free survival rates of patients solely with micrometastases to node-negative patients and observed a similar positive outcome in both cohorts (76% vs 86%). However, patients in this trial underwent neoadjuvant RCT, which probably accounts for the discrepancy of the results compared to *Bilchik* and *Märkl* and, according to *Sprenger et al.*, this might indicate that the presence of micrometastases

reflects a patient's response to radiation and chemotherapy (Sprenger et al. 2013b). Despite these data the concept of micrometastases and their impact on prognosis is not generally accepted: In a survey performed by *Short et al.*, 42% of the 602 clinicians questioned about the prognostic relevance of micrometastases were unsure and 7% did not believe in it at all. Only 15% used IHC on lymph nodes regularly (Short et al. 2012).

### **1.3.3 The Importance of Nodal status for Therapy**

The presence of lymph node metastases in patients with CRC is decisive in determining the tumor stage: If no lymph node metastases are present, tumor stage depends mainly on the depth of tumor infiltration and can be classified as UICC stage 0, I, or IIA-C. As soon as at least one regional lymph node is infiltrated, the tumor is classified as UICC stage IIIA-C, depending on the number of nodes affected (Wittekind and Meyer 2010). A different tumor stage ultimately leads to different approaches in therapy: The decision to apply adjuvant chemotherapy requires R0-resection of the tumor and is based on tumor stage, which relies on the nodal status. According to treatment guidelines, adjuvant chemotherapy is not indicated for stage I colon cancer patients and since studies have not been able to prove significant survival benefit for stage II colon cancer patients receiving adjuvant chemotherapy (Gill et al. 2004), it is only recommended for patients with high-risk stage II colon cancer, such as T4-tumors or patients presenting with tumor perforation (Schmiegel et al. 2008).

### **1.3.4 The Importance of Nodal Status for Survival and Prognosis**

Studies have shown that the 5-year disease specific survival after curative resection is 94.1% for stage II and 79.1% for stage III colon cancer patients as well as 88.3% for stage II and 69.1% for stage III rectal cancer patients, demonstrating the impact of nodal status on survival (Kanemitsu et al. 2012). *O'Connell et al.* examined possible factors that have an impact on recurrence rates in CRC patients. In their study, 80% of all patients with cancer recurrence were stage III colon cancer patients while the remaining 20% with recurrence were stage II patients. They also observed that unlike stage III patients, stage II patients survived longer after tumor recurrence occurred (O'Connell et al. 2008). This demonstrates the impact of nodal status on both survival and prognosis. To ensure adequate staging, the UICC recommends that a minimum of 12 lymph nodes in CRC patients should be evaluated (Nelson et al. 2001). Controversies remain about the number of lymph nodes that should be retrieved and their prognostic value. Several studies have shown that an increased number of harvested lymph nodes in CRC patients were correlated with better prognosis, such as *Kotake et al.*, who compared survival rates of stage II and III CRC patients on the basis of the number of lymph nodes retrieved and show that a larger number of nodes retrieved correlated with a decreased risk in death and therefore a better prognosis in both stage II and III colorectal cancer patients (Kotake et al. 2012). Although the connection of increased

node sampling and better prognosis is widely accepted, the reason for this connection is still debatable. The fact is, however, although there is a benchmark on the number of nodes to be examined, there is no recommendation or guideline on how to standardize the process of finding and harvesting lymph nodes, even though studies have shown different degrees of efficiency of pathological methods for lymph node harvest (Denham et al. 2012).

## **1.4 The Pathological Workup of Lymph Nodes**

### **1.4.1 The Pathological Workup of Lymph Nodes**

Studies demonstrate that the percentage of hospitals complying with the recommended minimum of harvesting at least 12 lymph nodes in CRC specimens has increased over the years (from 15% in 1996-1997 to 38.9% in 2004-2005 in the U.S.) (Bilimoria et al. 2008) and will most likely continue to rise. However, according to UICC criteria, that number is still insufficient to guarantee adequate tumor staging. One of the reasons why hospitals fail to meet the benchmark of a 12 lymph node yield might be the fact that there are several different pathological methods for harvesting lymph nodes, such as manual nodal dissection, fat clearing, methylene blue staining or acetone compression, which vary significantly in their efficacy of finding lymph nodes. In the following section, each of the above-mentioned methods will be described briefly.

### **1.4.2 Manual Nodal Dissection and Fat Clearance Methods**

Manual nodal dissection is the technically simplest yet most challenging method of harvesting lymph nodes from colon or rectal cancer specimen. As described in a study conducted by *Jass et al.* the rectal cancer specimen is fixed with buffered formaline (4-10%) and sliced into 2 to 5mm sections. The nodes are retrieved by inspection and palpation. According to *Jass et al.*, about 20 to 30 minutes are required per specimen (Jass et al.1986). The difficulty in finding small lymph nodes by using only sight and palpation has already been pointed out by *Gilchrist and David*, who first introduced fat clearance as a new technique to harvest lymph nodes from cancer specimens more thoroughly. They used a sophisticated method, which is based on the injection of red lead into the superior rectal artery, enabling an average harvest of 52.1 nodes per specimen (Gilchrist and David 1938). Based on this now historic approach, several other fat clearing techniques have been developed over the years. *Cawthorn et al.* suggest a mixture of ethanol and xylene, by which they were able to retrieve a median of 23 nodes per case (Cawthorn et al. 1986). *Brown et al.* used a slightly different approach, termed 'complete submission of the mesentery'. After manually dissecting the lymph nodes, the entire remaining mesenteric tissue was treated by elution in a mixture of alcohol and acetone, subsequent manual compression with a rolling pin and elution in xylene. The remnants were completely encapsulated and embedded in paraffin for histology. An average of 20.9 nodes per case was found with gross dissection and a median of 68.6

additional nodes was found after complete submission of the tissue (Brown et al. 2004). Another approach to enhance the visualization of lymph nodes in fatty tissue is the use of Carnoy's solution, a fixative first described in 1933 by Cutler and Zollinger, which was initially used to treat cysts and fistulae (Kumar et al. 2013). Carnoy's solution, a mixture of chloroform, ethanol and glacial acetic acid, facilitates lymph node retrieval by decolorizing nodes, thus enabling the harvest of more (22 vs 8) and smaller nodes than using standard formaldehyde fixation (Luz et al. 2008).

### **1.4.3 Methylene Blue Injection**

To further improve and facilitate lymph node harvest, another pathologic technique was introduced: methylene blue injection, first described by *Hermanek et al.* Originally, methylene blue was injected into the superior rectal artery to check for defects in the mesorectal fascia after TME to ascertain the thoroughness of the surgery (Hermanek et al. 2003). *Märkl et al.* have taken up this method to increase lymph node yield in CRC specimens, proceeding by injecting 15 to 20ml methylene blue solution (50mg diluted with 0.9% saline, ratio 1:3) into the superior rectal artery to contrast the lymph nodes. The specimen is fixed in 10% formalin for 24 hours. Next, it is cut into 5 to 7mm thick slices, before the whole mount technique is used to embed representative areas. The remaining fat is dissected and visible lymph nodes are harvested using sight and palpation. Finally, the tissue is embedded in paraffin, stained with hematoxylin and eosin, and the slides are examined for metastases. Using methylene blue injection, *Märkl et al.* were able to harvest an average of 27 nodes per case (Märkl et al. 2007).

### **1.4.4 Acetone Compression**

Along with the aforementioned techniques, acetone compression is another, relatively recently developed method for best possible lymph node yield, first introduced by *Basten et al.* The basic idea behind this technique is to accelerate and simplify the process of harvesting lymph nodes by decreasing the amount of fatty tissue to about 10% of the initial weight while still ensuring the same quality of histological sections as conventional methods do. The combination of perforating the tissue with a nail roll, soaking it in acetone and finally compressing it with a squeezing machine results in the removal of most of the fatty tissue. The remaining tissue, the pellet, still contains lymph nodes, nerves and blood vessels. The pellet is placed in tissue capsules before being embedded in paraffin, sectioned and mounted on glass slides for histopathologic evaluation (Basten et al. 2010). Acetone compression allows complete embedding of the entire mesorectal tissue without previous manual examination, requiring a relatively short processing time and also guaranteeing the harvest of an increased number of lymph nodes as compared with manual dissection or the

fat clearance method (Gehoff et al. 2012a). Table 1.3 shows an overview of historic and current histopathological methods for lymph node retrieval in CRC specimens.

**Overview of Historic and Current Histopathological Lymph Node Retrieval in CRC:**

<b>Method</b>	<b>Year of description</b>	<b>Relevant papers regarding this method</b>
Manual node dissection	1986	Fat Clearance Method Versus Manual Dissection of Lymph Nodes in Specimens of Rectal Cancer, Jass et al. 1986
Fat clearance method using red lead	1938	Lymphatic Spread of Carcinoma of the Rectum, Gilchrist and David 1938
Fat clearance method using xylene	1986	Clearance Technique for the Detection of Lymph Nodes in Colorectal Cancer, Cawthorn et al. 1986
Complete submission of the mesentery (fat clearance method using acetone and xylene)	2004	Efficacy of Manual Node Dissection of Lymph Nodes in Colon Cancer Resections, Brown et al. 2004
Methylene blue injection	2007	Methylene Blue Injection into the Rectal Artery as a Simple Method to Improve Lymph Node Harvest in Rectal Cancer, Märkl et al. 2007
Carnoy's solution	2008	Carnoy's Solution Enhances Lymph Node Detection: an Anatomical Dissection Study in Cadavers, Luz et al. 2008
Whole mesorectal compartment embedding (WME)	2010	Preoperative Chemoradiotherapy Does Not Necessarily Reduce Lymph Node Retrieval in Rectal Cancer Specimens- Results from a Prospective Evaluation with Extensive Pathological Work-Up, Sprenger et al. 2010
Acetone compression	2010	Acetonkompression, Basten et al. 2010; Optimal Lymph Node Harvest in Rectal Cancer (UICC Stages II and III) after Preoperative 5-FU-based Radiochemotherapy. Acetone Compression is a New and Highly Efficient Method, Gehoff et al. 2012a

**Table 1.3**

## 1.5 Hypotheses

The nodal status of patients with CRC has a crucial impact on tumor stage, therapeutic decisions and the patient's prognosis. However, the efficiency of lymph node yield varies, especially in patients treated with preoperative RCT.

This study focuses on the macropathological procedure Acetone Compression for the comprehensive retrieval of lymph nodes from rectal cancer specimens. The main hypotheses are:

1. Does Acetone Compression alter the morphology of lymph nodes?
2. Can Acetone Compression be used to investigate the impact of preoperative RCT on lymph node sizes and numbers?
3. Does the application of Acetone Compression affect the pathological staging of lymph nodes? How does the efficiency and performance of Acetone Compression vary compared to previous studies?

The three hypotheses are interrelated and can only be addressed cohesively. To address the hypotheses, collections of rectal cancer specimens worked-up either with Acetone Compression, Manual Dissectioning or with Whole Mesorectal Embedding are digitally measured. The resulting morphological descriptors for each lymph node are considered in relation to the mode of preparation, preoperative treatment, clinical response and to the characteristics of the respective patients.

## 2. Materials

### 2.1 Devices

<b>Device</b>	<b>Name</b>	<b>Manufacturer</b>
Arbor press	Quantum Arbor Press, DDP2	Stürmer Werkzeuge Maschinen KG, Hallstadt, Germany
Autostainer for IHC	BenchMark Ultra	Ventana Medical Systems, Tucson, AZ, USA
CCD photo scanner with transillumination unit	Scanjet G4050	Hewlett Packard, Palo Alto, CA, USA
Cold plate	OTS 40	Medite GmbH, Burgdorf, Germany
Dehydration machine	Shandon Excelsior ES Tissue Processor	Thermo Fisher Scientific GmbH, Schwerte, Germany
Drying cabinet	UNE 400	Memmert GmbH, Schwabach, Germany
Embedding center	TES 99	Medite GmbH, Burgdorf, Germany
Film Coverslipper	Tissue-Tek Film	Sakura Finetek Germany GmbH, Staufen, Germany
Freezer, -20°C	Liebherr "Premium" Product line	Liebherr Gruppe, Biberach an der Riss, Germany
Fridge, 4°C	Liebherr "Premium" Product line	Liebherr Gruppe, Biberach an der Riss, Germany
Instruments for Gross Examination	"Aesculap" Surgical Scissors, Forceps, Probes	B. Braun AG, Melsungen, Germany
Magnetic stirrer and hot plate	MR Hei-Standard	Heidolph Instruments GmbH, Schwabach, Germany
Microscope	Eclipse 80i with Plan Fluor Objectives (1x, 4x, 10x, 20x, 60x)	Nikon, GmbH Germany, Düsseldorf, Germany
Pipetts	Eppendorf Research Plus	Eppendorf AG, Hamburg, Germany
Rotation microtome	HM 355 S	MICROM International GmbH, Walldorf, Germany
Scale, digital, De=0.1g	Kern-PCB6000-1	Satorius GmbH, Göttingen, Germany
Staining machine, HE	HMS 760X	MICROM International GmbH, Walldorf, Germany
Staining machine, PAS, EvG	COT 20	Medite GmbH, Burgdorf, Germany
Whole Slide Scanner	ScanScope XT	Aperio, Vista, CA, USA



## 2.2 Consumables

Description	Name	Manufacturer
Blades for microtome	Typ A35, Typ 130S	Feather
Embedding cartridges	Universal embedding cartridge	Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany
Glass slides (76x26x1mm)	StarFrost	Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany
Glass slides (76x26x1mm)	StarFrost	Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany
Medical Examination gloves	Nitra-Tex	Ansell Healthcare Europe, Brussels, Belgium
Paraffin	Sasol-Wax	Sasol, Hamburg, Germany
Tissue Dye for Grossing	CDI Tissue Marking Dyes	Cancer Diagnostics Inc, Morrisville, NC, USA

## 2.3 Chemicals

Chemical	Manufacturer
Acetone 99.5%	Carl Roth GmbH und Co. KG, Karlsruhe, Germany
Eosin 0.5% solution	Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany
Ethanol 100% (fully denatured)	ChemLogistics GbR, Düren, Germany
Isopropyl alcohol 100%	ChemLogistics GbR, Düren, Germany
Mayer's Hemalum solution	Merck KGaA, Darmstadt, Germany
Periodic Acid	Merck KGaA, Darmstadt, Germany
Schiff's Reagent	Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany
Xylene 100%	ChemLogistics GbR, Düren, Germany

## 2.4 Primary Antibodies

Name	Target Protein	Clone	Buffer	Dilution	Manufacturer
CK pan	Cytokeratines	AE1+AE3	CC1	1:100	Zytomed Systems
CK20	Type I Cytokeratine, 20	Ks20.9	CC1	1:200	Medac
CD31	PECAM-1, Endothelium	JC70	CC1	1:100	Cellmarque/Medac
D2-40	Podoplanin, Lymphatics	D2-40	CC1	1:40	Signet/DCS

"CC1": Antigen Retrieval Buffer "cell conditioning 1" by Ventana Medical Systems, tris-buffered, pH=8.0

## 2.5 Secondary Antibodies

Secondary antibodies were part of ready-made kits. The kit includes a polymer, which is conjugated to anti-mouse and anti-rabbit antibodies and to either horse raddish peroxidase to produce DAB by oxidation or to alkaline phosphatase to produce a naphtol red dye by hydrolysis. The polymer method increases the sensitivity of IHC-staining as several enzymes

Description	Manufacturer
Universal DAB Detection kits	Ventana Medial Systems Inc, Tucson, AZ, USA
ultraView Alkaline Phosphatase Red Detection kits	Ventana Medial Systems Inc, Tucson, AZ, USA

are recruited to the site of a bound primary antibody.

## 2.6 Software

Name	Manufacturer
Image J, Version 1.45s on Java 1.6.0_20 (32bit)	National Institutes of Health, Bethesda, MD, USA, <a href="http://rsb.info.nih.gov/ij/">http://rsb.info.nih.gov/ij/</a>
'R', language for statistical programming, Version 2.13.1	R Foundation for Statistical Computing, Vienna, Austria, <a href="http://www.R-project.org">http://www.R-project.org</a>
Microsoft Office 2007	Microsoft, Redmond, WA, USA

## 3. Methods

### 3.1 Patient Population

	<b>WME</b>	<b>AC</b>	<b>MD</b>
Cases	51	138	131
Lymph nodes	1,759	3,624	3,140
Sex (m/f)	14/37	43/95	47/84
Age, mean	63.2 ( $\pm$ 10.5)	67.7 ( $\pm$ 12.5)	67.9 ( $\pm$ 10.6)
T1+ T2	11 (21.5%)	48 (34.8%)	40 (30.5%)
T3+ T4	32 (62.7%)	70 (50.7%)	82 (62.6%)
N+	14 (27.5%)	43 (30.1%)	54 (41.2%)
M1	5 (9.8%)	10 (7.2%)	9 (6.9%)
Neo treatment	51 (100%)	85 (62%)	68 (52%)
ypT0	8 (15.7%)	13 (15.3%)	4 (5.9%)

**Table 3.1: Overview of the Study Population's Clinical Parameters ("N+": node-positive cases, "M1": distant metastasis, "neo treatment": application of preoperative RCT, "ypT0": pathological remission after preoperative RCT).**

Table 3.1 constitutes an overview of the clinical parameters of the patient population in this study, which consists of 320 cases of patients with rectal carcinoma UICC stage I to IV. The lymph nodes were retrieved from the surgical specimens using three different techniques of pathological workup: 51 cases were examined using whole mesorectal embedding (WME), 138 cases were examined using acetone compression (AC) and 131 cases were examined using manual dissectioning (MD). The 51 patients of the WME group were treated at the University Hospital of Göttingen, Germany, as part of the CAO/ARO/AIO-2004 study. Histopathological examination of this group was also performed at the University Hospital in Göttingen. The 269 patients in the AC and MD group were treated at six different hospitals in Kassel, Germany; the histopathological examination was performed in one laboratory: The Institute of Pathology Nordhessen, Kassel. Patients in the AC group were treated between 2009 and 2012; patients in the MD group were treated between 2005 and 2012. The median age of patients in the WME group was 63.2 ( $\pm$ 10.5) years, in the AC group 67.7 ( $\pm$ 12.5) years and in the MD group 67.9 ( $\pm$ 10.6) years. 43 patients in the AC group were female (31%) and 95 male (69%). 47 patients in the MD group are female (36%) and 84 male (64%). Depending on their tumor stage, the patients either received primary surgical treatment or preoperative radiochemotherapy (RCT) followed by surgery. All patients in the WME group (100%), 85 (62%) of the patients in the AC group and 68 (52%) in the MD group received neoadjuvant radiochemotherapy. After pretreatment with RCT, 13 (15.3%) of the patients in

the AC group and 4 (5.9%) of the patients in the MD group were diagnosed with pathological complete response (ypT0), i.e. histologically no vital tumor cells were present anymore. 48 patients (34.8%) in the AC group were diagnosed with T1 or T2 tumors, and 70 patients (50.7%) were diagnosed with T3 or T4 tumors. In the MD group, 40 patients (30.5%) were classified as T1 or T2 and 82 patients (62.6%) were classified as T3 or T4. 14 patients (27.5%) of the WME group, 43 (30.1%) of the AC group and 54 (41.2%) of the MD group showed lymph node metastasis in the investigated specimens. Distant metastases were clinically reported in 10 patients (7.2%) of the AC group and 9 patients (6.9%) of the MD group.

### **3.2 Definition of Lymph Nodes**

#### **3.2.1 The Histological Structure and Function of Lymph Nodes**

Lymph nodes are small lymphoid organs, spread throughout the entire human body. Typical locations for aggregations of lymph nodes are the neck, the axilla or the groin. Lymph nodes can be regarded as collecting basins for the intercellular fluid from an adjacent organ or body region. Each node has multiple feeding vessels and one draining vessel. Lymph fluid coalesces via lymphatic vessels in the thoracic duct, which mainly collects lymph of the lower extremities, abdomen, thorax, left arm and left side of the head, and the right lymphatic duct, which mainly collects lymph from the right arm, right side of the head and the neck, emptying into the left and right subclavian vein and transporting lymph fluid back to the blood stream.

Lymph nodes are usually round or kidney-shaped and covered with a dense fibrous capsule. Parts of the capsule, the trabeculae, extend into the node. Several lymphoid vessels, the vasa afferentia, perforate the node's capsule and emit lymphatic fluid into the sinuses. The lymph fluid passes through a system of sinuses, consisting of the subcapsular marginal sinus, the cortical sinus and finally the medullary sinus, which drains into the efferent lymphatic vessel. The efferent lymphatic vessel exits the node at the hilus, accompanied by a venous and an arterial vessel. The interior of a node is lined with reticular connective tissue and can be subdivided into cortex, subcortical zone and medulla.

The cortex mostly contains B-cells, arranged as lymphoid follicles, which can present as primary, secondary or tertiary lymphoid follicles. Primary lymphoid follicles consist of reticular cells, follicular dendritic cells (fDCs) and naïve B-cells that have not been presented with antigens yet. Secondary lymphoid follicles contain a marginal wall with naïve B-cells and a germinal center with activated B-cells, follicular dendritic cells, T-cells and macrophages. FDCs collect antigens in a major histocompatibility II (MHC II) independent manner and present them to adjacent B-cells. B-cells with a matching receptor are activated and proliferate within the germinal center, which is histologically distinguishable. The proliferating B-cells undergo somatic hypermutation to increase the affinity of the B-cell receptor. Cells

with unsuitable receptors undergo apoptosis, which causes a recognizable subdivision of the germinal center: Proliferating cells form a darker half of the germinal center, while predominant apoptosis causes the other half of the germinal center to appear lighter.

The paracortical zone of the lymph nodes is located in between and underneath the lymphoid follicles and contains mostly T-cells, MHC II-positive dendritic cells and high endothelial venules. Most of the T-cells exit the blood stream by penetrating the venules and remain in the paracortical zone where they can be activated by antigen-presenting dendritic cells. The medulla of the node contains medullary cords, where plasma cells and macrophages are located.

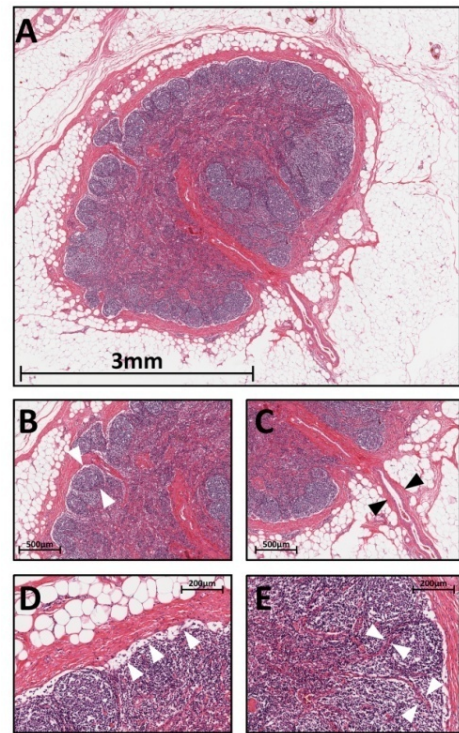
The composition of lymphoid fluid is equivalent to tissue fluid when entering the node. Since the main functions of lymph nodes are to preserve a physiological intravascular pressure and guarantee an adequate immune response, the majority of antigens are removed from the lymphoid fluid during passage through the nodes while immunoglobulins and lymphocytes are added. Analogical neoplastic cells detached from a solid tumor may be displaced to regional lymph nodes via lymphatic vessels of the affected organ. Given the flow of the lymph, these neoplastic cells are likely to arrive at and be stuck in the marginal sinus from where they may start infiltrative growth and give rise to lymph node metastases. As any tissue, lymphocytes themselves may also undergo malignant transformation. Depending on the cell of origin and their respective maturation state, distinct forms of lymphoma result.

Thus, the swelling of lymph nodes is a prominent clinical symptom, which may either reflect a physiological reaction of the lymphatic tissue to an infectious agent or it may be a symptom of malignancy. If the latter cannot be ruled out with clinical examinations, needle biopsy or bioptic sampling of a lymph node may be indicated to determine the origin of the swelling by histopathological analysis. Conversely, comprehensive lymph node examination is an integral part of the pathological staging of solid tumors (Welsch 2010 chapter 6, p.243-246; Lüllmann-Rauch 2009 chapter 13, p.311-313).

### **3.2.2 Criteria for Determination of Lymph Nodes**

In the comprehensive examination of lymph nodes it is important to define the histological criteria of what constitutes a lymph node. Lymph nodes are dynamic structures which expand and diminish on demand. Given the aforementioned microstructure of lymph nodes, four criteria for the identification of lymph nodes were determined (Figure 3.1, Table 3.2). To qualify as a lymph node, at least two of the four criteria had to be recognizable: the shape of the lymph node, which is typically round or kidney-shaped, the histological structure of the node with lymphoid follicles, the presence of a capsule and the presence of blood vessels such as venules or afferent and efferent lymphatic vessels in the lymph node. These criteria distinguish lymph nodes from lymphoid inflammatory infiltrate.

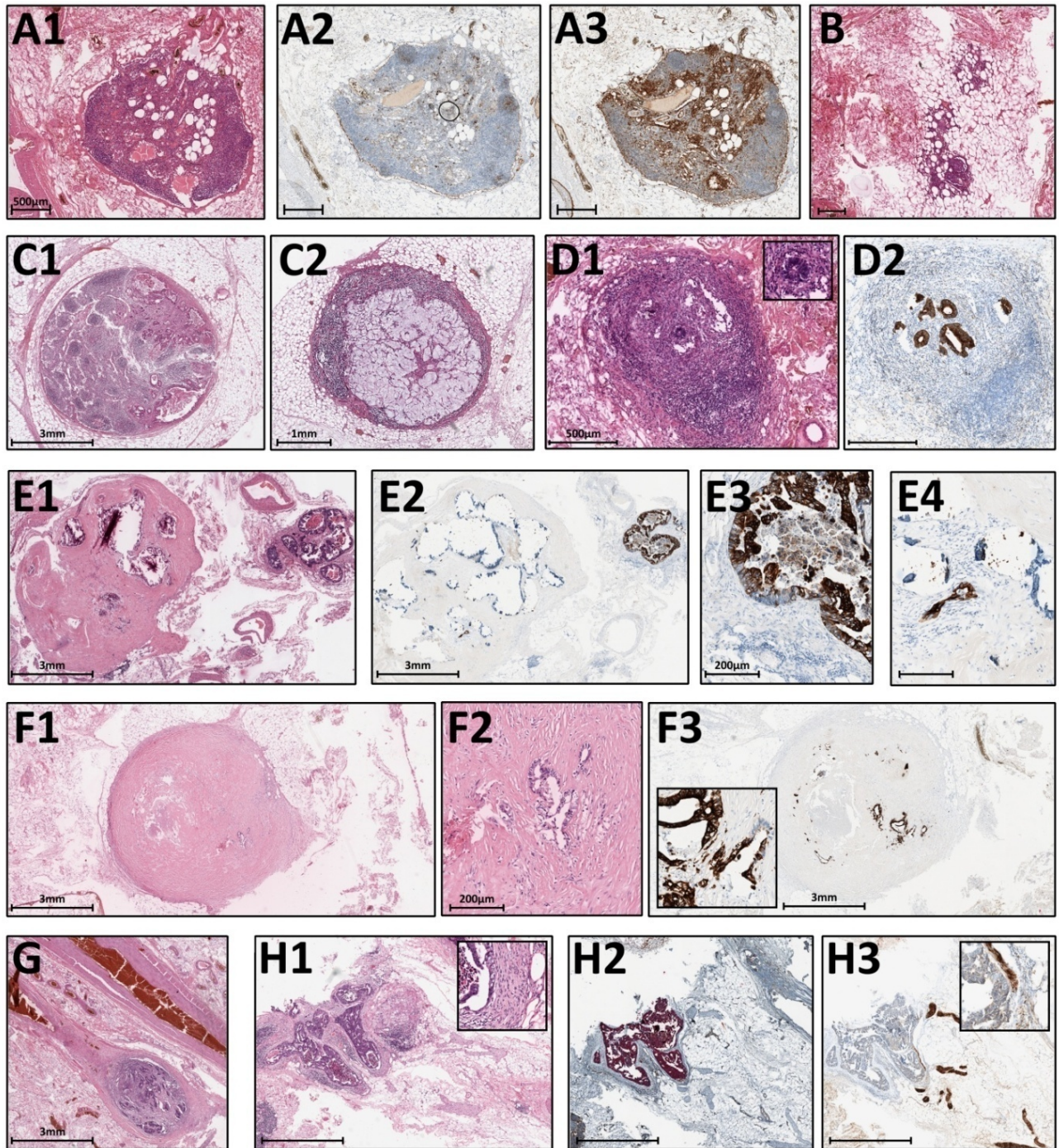
Criterion	Typical feature
1. Shape of the node <b>(A)</b>	Round or kidney-shaped
2. Histological structure	Lymphocytes, lymphoid follicles <b>(B)</b> , medullary cords <b>(E)</b>
3. Capsule and marginal sinus <b>(D)</b>	Collagen fibers (type-I collagen), fibroblasts, elastic fibers
4. Hilum vessels <b>(C)</b> , lymph vessels	Endothelia, valves, elastic fibers



**Table 3.2 The Criteria for Identification of Lymph Nodes (as shown in Figure 3.1)**

**Figure 3.1 Typical lymph node. B:** Lymphoid follicles. **C:** Hilum vessels. **D:** Capsule with marginal sinus. **E:** Medullary cords.

Lymphoid infiltrate may mimic lymph nodes by forming follicular structures and by their being located close to blood vessels. However, these infiltrates are not part of the lymphoid fluid transportation system and thus not the target of metastatic spread. The four criteria (Table 3.2) were also found to be present in tiny lymph nodes (<1mm), yet are never mimicked simultaneously by lymphoid infiltrate. Figure 3.1 illustrates an archetypical lymph node featuring all four criteria. In questionable cases, the four criteria can be determined by means of immunohistochemistry (IHC). Figure 3.2 A shows a representative tiny lymph node after retrieval with acetone compression. The lymphatic vessels (Figure 3.2, A2) may be contrasted by using IHC-staining against podoplanin, while blood vessels (Figure 3.2, A3) show a positive reaction to CD31-staining. In contrast, lymphocytic infiltrate (Figure 3.2, B) does not show any of these features.



**Figure 3.2 A selection of figures of lymphoid structures stained with IHC**

**A1-3:** The morphology is preserved during AC and the defining structures may be verified by immunohistochemistry (A1, 20x, method: AC): marginal sinus and feeding lymphatic vessels (A2, IHC: podoplanin/ D2-40), hilum artery (A3, IHC: CD31). **B:** An example of lymphatic aggregates not showing any of the 4 defining structures (20x).

**C1:** Macrometastasis with glandular architecture of the malignant infiltrate (10x, method: MD). **C2:** Macrometastasis with extensive extracellular mucus (20x, method: MD). **D1, D2:** Micrometastasis detected after AC (D1, HE; D2, IHC: CK20; 50x).

**E1-4:** The regression of malignant infiltrate after neoadjuvant chemoradiotherapy. **E1:** Node with vital infiltrate (right) next node with sclerosis and remnants of infiltrate (10x). **E2:** IHC for CK20 contrasts vital tumor cells in both nodes (10x). Details for right node (E3, 100x) and left node (E4, 100x). **F1:** Lymph-node showing fibrotic and sclerotic tissue (10x). Higher magnification reveals remnants of vital tumors cells (F2, 100x), IHC for CK20 contrasts more infiltrates (F3, 10x) and isolated tumor cells that separate from the the glandular structures (magnified insert).

**G:** Angioinvasion in a venous vessel after AC. The vessel is obstructed by malignant infiltrate. The accompanying artery is seen

next to the vein. **H1-3:** Perineural invasion after AC. Malignant infiltrates adjacent to nervous strands (H1, HE, 20x). Double-IHC for panCK (red) and CD31 (brown) demonstrated extensive perineural invasion (center); isolated CK-positive tumor cells are present in a CD31-positive vessel (top). The peripheral nerves are S100 positive (I3).

### **3.3 The Histopathological Workup of CRC Specimens**

In the following paragraphs (3.3.1 - 3.3.6) gross examination, dissectioning, embedding, and histological and immunohistochemical staining are described, which were performed by certified pathologists at the Institute of Pathology Nordhessen in Kassel (Acetone Compression, Manual Dissectioning) and at the University Hospital Göttingen (Whole Mesorectal Embedding).

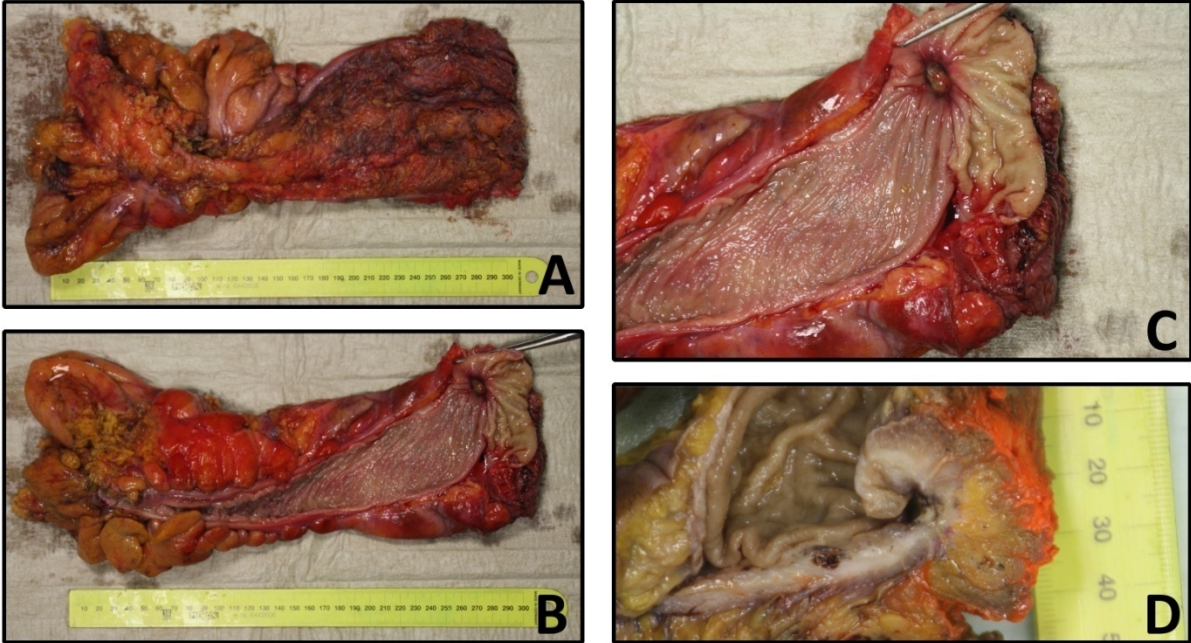
#### **3.3.1 The Dissection of CRC Specimens**

Gross examination and dissectioning were performed according to standard protocol. Cases with AC and MD retrieval of lymph nodes were examined at the Institute of Pathology Nordhessen in Kassel. The cases with WME retrieval were examined at the University Hospital in Göttingen. Except for the differences in lymph node preparation, the dissectioning of the rectum was performed identically. First, the colorectal cancer specimen was macroscopically inspected, and the quality of the total mesorectum excision (TME) was judged. The mesorectum is regarded as a "continuity of the mesosigmoid" (Hoorens et al. 2009, p.252) and its complete removal is an important factor in the risk evaluation of local recurrence (Heald et al. 1982). This was already recognized by the phase II CORE study, which suggested assessing the thoroughness of the surgery based on a grading system ranging from Grade 1 (incomplete/poor) to Grade 3 (complete/good) (Maughan and Quirke 2003). The MERCURY study, performed in 2002, compared Magnetic Resonance Imaging (MRI) prior to surgery and post-operative pathologic assessment of rectal cancer patients in predicting successful surgical resection (MERCURY Study Group 2006). These studies established criteria for the assessment of the TME quality, which are now diagnostic standard, the so-called M.E.R.C.U.R.Y criteria: The good quality of the TME features an intact mesorectum with only few irregularities. No defect is deeper than 5mm and there is no coning towards the distal margin. If the specimen shows a moderate amount of mesorectum with irregularities on the surface of the mesorectum, the quality of the TME is considered moderate. There is moderate coning, and the lamina muscularis propria is not visible. If the quality of the TME is assessed as poor, only little mesorectum remains and there are defects as far as the lamina muscularis propria. The TME is classified as incomplete if the muscular layer is visible and the specimen shows perforation. Additionally, the circumferential resection margin (CRM) should be assessed. The CRM represents the area of the specimen closest to the deepest infiltration of the tumor and a positive CRM is defined as the tumor being located less than 1mm from the CRM (Hoorens et al. 2009). Statements about the tumor involvement of the CRM are required since a positive CRM has proven to be a



significant risk factor in the local recurrence of rectal cancer (Quirke and Dixon 1988). To evaluate the CRM, the mesorectum is marked with xylene-resistant ink. The specimen is longitudinally opened by cutting from proximal to distal. Cutting through the tumor is avoided by careful palpation. Now the distance of the tumor from the oral and aboral resection margin can be measured. Subsequently, the tumor area is sliced into transverse sections. The size of the tumor area, the depth of infiltration and the distance of the tumor from the resection margin are measured. The depth of infiltration as far as it is macroscopically visible is documented. The specimen is cut into pieces of 2 to 4mm in size, and several blocks are selected for histology. The region of deepest tumor infiltration is also selected for histology to verify the depth.

The lymph nodes in the sliced tumor regions that are visible upon macroscopic inspection, as well as large, palpable nodes from the whole specimen are manually retrieved and embedded in paraffin. The remaining fatty tissue is removed from the rectum and lymph nodes can be retrieved using one of the three following techniques: manual dissection by slicing, palpation and inspection; fat clearance by elution in a mixture of solvents overnight and subsequent manual dissection or acetone compression by combined elution in pure acetone and mechanical compression. An experimental approach is the whole mesorectal compartment embedding (WME) of the entire fatty tissue without pretreatment of the specimen. The pathological assessment of the colon cancer resection specimen is handled in a similar manner, with the exception of the quality assessment of the TME as this is a procedure only performed on carcinoma of the rectum.



**Figure 3.3:** An example of the rectal cancer specimen of case #116. **A, B:** opened specimen, front and back. **C:** tumor region **D:** tumor region close-up with depth of infiltration visible

### 3.3.2 Tumor Regression Grading

Tumor regression is regarded as a reflection of the treatment response of the tissue to preoperative radiochemotherapy. It is assessed by the microscopic examination of glass slides containing sections of the tumor area and can be classified according to different systems. Currently, the most commonly used system is the one established by *Dworak et al.* (Dworak et al. 1997) and ranges from Grade 0 (no regression) to Grade 4 (complete regression):

- **Grade 0:** no regression
- **Grade 1:** dominant tumor mass with obvious fibrosis and/or vasculopathy
- **Grade 2:** dominantly fibrotic changes with few tumor cells or groups (easy to find)
- **Grade 3:** very few tumor cells (difficult to find microscopically) in fibrotic tissue with or without mucous substance
- **Grade 4:** no tumor cells, only fibrotic mass (total regression or response)

### 3.3.3 Advanced Lymph Node Retrieval: WME and Acetone Compression

51 of the 320 cases in this study were examined using the whole mesorectal embedding technique (WME) for the complete workup of the mesorectal fatty tissue. It may be regarded as reference standard since it allows the retrieval of virtually all lymph nodes without additional tissue alteration. On the other hand, it is too time and resource consuming to be used in daily pathological practice. The WME-cases analyzed here are part of a study mentioned earlier (Sprenger et al. 2010). In brief, the specimens were opened longitudinally along the rectal lumen and fixed in 5% formalin for 72 hours. No additional preconditioning of the fatty tissue was performed. For macroscopic grossing, the specimen was sliced into 5mm cross-sections. For paraffin-embedding, the cross-sections were again divided into 2.5mm slices. To avoid causing a misleadingly high number of lymph nodes, care was taken not to embed both halves of the cut lymph nodes (Sprenger et al. 2010). 138 of the remaining 269 cases were examined, using acetone compression (AC) according to the procedure published by *Basten et al.* (Basten et al. 2010, Gehoff et al. 2012a). AC is a recent advancement in techniques used to enhance lymph node retrieval by means of using solvents and mechanical procedures. The use of acetone was described by *Brown et al.* in 2004: acetone in combination with alcohol was used as a solvent for mesenteric fat. The mesenteric fat was washed daily with a graduated series of alcohol and acetone over several days to dehydrate the tissue before soaking it in xylene for another day (Brown et al. 2004). The use of pure acetone in combination with mechanical compression was first developed and described by *Basten et al.* in 2010 and applied in a slightly altered manner in the present

study: the fatty tissue was carefully dissected and weighed after macroscopic inspection of the specimen. It was inspected for palpable and visible lymph nodes, which were manually harvested. After the fatty tissue was perforated with a needle roller, it was soaked in acetone for 12 hours. The acetone used was disposed of, and the tissue was perforated again before elution in acetone for another 4 to 6 hours. The tissue was twitched into pieces of 3 to 5cm in size and placed in a "brazen cylindrical tube with multiple small perforations" (Gehoff et al. 2012a, p.205). The tissue was then manually compressed with the help of an arbor press (Quantum Arbor Press). While the tissue was being compressed, fat and acetone leaked out of the perforations in the tube, thus resulting in a reduction of the initial tissue weight of up to 95% (fatty tissue with a weight of 300g prior to AC can weigh as little as 20g after compression). Despite the considerable weight reduction, the remaining tissue, the so-called pellet, still contained fully-preserved lymph nodes, vessels and nerve structures. As usual, it was encapsulated usually with 1g of tissue per capsule and transferred to routine embedding. *Basten et al.* used heated acetone at a temperature of up to 56 degrees for elution. However, the use of heated acetone has proven not only to make cutting the tissue blocks difficult but also to result in IHC stainings of low quality due to "more unspecific background staining" (Gehoff et al. 2012a, p. 207). These drawbacks can be compensated for by using acetone at room temperature as was done in this study.

#### **3.3.4 Embedding and Manufacturing of Paraffin Blocks**

In order to proceed with the histopathological evaluation, it is necessary to embed the examination material. Embedding takes place overnight in a dehydration machine (Shandon Excelsior ES Tissue Processor), where the material is incubated with 4% formaldehyde, 70% isopropyl, 96% isopropyl, 100% isopropyl, xylene and finally paraffin. By elution of the material in alcohol of increasing concentrations, water and tissue fluid are gradually removed and replaced with a wax such as paraffin, which strengthens and preserves the tissue. After embedding the material in paraffin with help of an embedding center (TES 99), paraffin blocks are manufactured, which are then cut using a microtome (HM 355 S). The paraffin block is inserted into the microtome and sections of 1 to 1.5µm size are cut. The sections slide from the microtome blade onto the surface of a water bath, which is heated to room temperature to prevent them from wrinkling. Each section is captured on a glass slide and put on the surface of a second water bath. The section expands and is captured again on a glass slide with a refined surface that enables strong adhesion ("StarFrost"). Once they are dry, they are available for histochemical and immunohistochemical stains. After sectioning, paraffin blocks are stored in cardboard boxes for a period of at least ten years.

### 3.3.5 Histological Staining

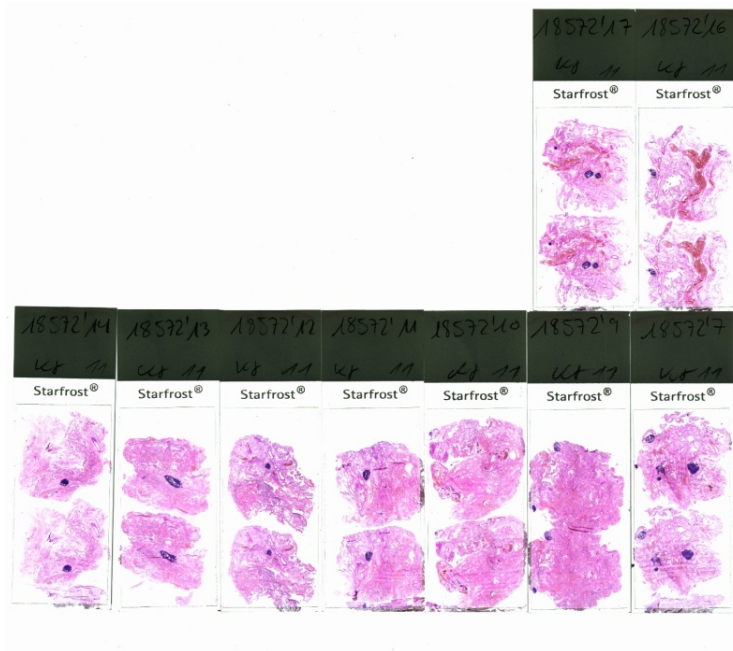
After the paraffin sections are mounted onto glass slides, they are stained for further histopathological examination. Prior to being staining, the sections have to be rehydrated and the paraffin must be removed as it would prevent the sections from taking on color. A descending alcohol series is performed:

<b>Substance</b>	<b>Duration</b>
Xylene	10 minutes
Xylene	5 minutes
100% Isopropyl	1 minute
100% Isopropyl	1 minute
96% Isopropyl	1 minute
90% Isopropyl	1 minute
70% Isopropyl	1 minute
Distilled water	Until the staining process starts

Since the different components of tissue vary in their electric charge, it is essential to use a stain that dyes structures that are both basophilic (such as the DNA) and acidophilic (such as the cell nucleus). The most common stains used in histology are hematoxylin and eosin (H&E). Hematoxylin, a basic substance, allows the staining of anionic structures that adopt red color, while eosin, an acid substance, allows the staining of cationic structures that adopt blue color (Lüllmann-Rauch 2009). H&E was performed in the staining machine (HMS 760X for HE stains or COT 20 for PAS stains) by incubating the sections in the following substances:

<b>Substance</b>	<b>Duration</b>
Warm tap water	1X 1minute
Hemalaun	4X 1minute
Warm tap water	3X 1minute
1% Eosin	1X 1minute
96% Isopropyl	2X 1minute
100% Isopropyl	2X 1minute
Xylene	4X 1 minute

Staining takes place in aqueous solution. Afterwards, the sections are dehydrated again. The slides were sealed using a coverslipper machine (Tissue-Tek Film).



**Figure 3.4** Example of H&E staining: glass slides from case # 50

### 3.3.6 Immunohistochemical Staining

Immunohistochemical staining allows for the identification and visualization of structures that can be bound by specific antibodies. IHC is used, for example, to verify the presence of DNA mismatch repair enzymes, such as MLH1 or MSH2 in CRC patients and thus exclude a DNA mismatch repair deficiency typical for HNPCC. IHC can be performed using a direct or an indirect approach. The indirect approach involves two antibodies, a primary antibody that binds to an epitope of the antigen in question, and a secondary antibody that reacts with the fc-fragment of the primary antibody. Visualization is achieved by coupling an enzyme either to the primary antibody (direct IHC) or to the secondary antibody (indirect IHC). When the appropriate substrate is added, the enzyme will catalyze the formation of a chromophore at the side of the antigen-antibody complex. IHC was performed by preparing 1 $\mu$ m sections as described above. After the removal of paraffin with xylene, the sections are incubated with alcohol in decreasing concentrations. While soaking in 50% Isopropyl, hydrogen peroxide was added. This 'peroxide-block' results in blocking of the activity of endogenous peroxidase and thus prevents non-specific background-staining of the sections. Immunohistochemical staining takes place in immunostainers (Optimax or BenchMark Special Stains). Formalin fixation not only preserves material but may also cause the cross-linking of proteins, thus hampering their immunoreactivity. The so-called antigen retrieval is prerequisite to IHC: heat-incubation at a certain pH-value or enzymatic digestions 'unmask' a given epitope by removing such cross-links. Antigen retrieval was generally performed by heat-incubation in a water bath. Temperature, incubation time and buffer/pH were optimized for each antibody. Detection was performed ultraView Universal DAB Detection kits (Ventana Medial Systems Inc, Tucson, USA, vgl. <http://www.ventana.com/documents/ultraViewDABbrochure.pdf>),

which rely on the oxidation of 3,3'-Diaminobenzidine (DAB). The secondary antibodies and the horse raddish peroxidase (HRP) are bound to a polymer to increase the recruitment to the primary antibody. HRP oxidizes DAB, which makes it insoluble in an aqueous solution. DAB-precipitates form at the antigen-antibody complex yielding a brownish-black stain.

Double-IHC was achieved by performing the staining procedure again and using ultraView Alkaline Phosphatase Red Detection kits (Ventana Inc), which use the enzyme Alkaline Phosphatase (AP) to hydrolyze Naphtol Red into an insoluble red azo dye. The sections were counterstained with hematoxylin before they are covered and forwarded to histopathologic assessment.

### **3.4 Morphometric Analysis of Lymph Nodes**

In the following two paragraphs (3.4 and 3.5) microscopical examination and morphometric analysis of the lymph nodes as well as statistical analysis are described, which were performed by Rebecca A. Reineke under supervision of PD Dr. med. P. Middel and Dr. med. A. Scheel.

To ensure standardized conditions, the lymph nodes of all 320 cases were evaluated again based on the aforementioned morphological criteria (Table 3.2). Every glass slide of each case was microscopically examined using an Eclipse 80i microscope (Nikon, Japan) and Fluor Objectives (1x, 4x, 10x, 20x, 40x, 60x). The number of lymph nodes and lymph node metastases was noted on the respective glass slide. Each lymph node was checked for integrity. For positive lymph nodes, the amount of tumor infiltration (0 to 25%, 26 to 50%, 51 to 75%, 76 to 100%), extracapsular growth and the morphologic structure of the malignant tissue were documented. Additionally, several morphological parameters were recorded: the extent of extracellular mucus (no mucus, 0 to 50% of the tumor area, 51 to 100% of the tumor area) and the presence of inflammatory infiltration and necrosis were evaluated.

The slides were subsequently digitalized using a charge-coupled device-scanner with transillumination adapter (Scanjet G4050). The device allows for the simultaneous scanning of 3 rows of 7 glass slides, i.e. 21 slides per run. As a compromise to accuracy and speed, the resolution of the Scanjet G4050 scanner was set to 200 dots-per-inch (dpi). At this setting, one inch (25.4mm) is represented by 200 pixels, i.e. 7.87 pixels per millimeter. The optical properties were verified using a reference scale bar of 1mm length (Nikon, Japan). The bar measured 8 pixels (px) at 200dpi resolution (figure 3.5), thus the measurement accurately matched the theoretical prediction. At the highest resolution of 7200dpi, the 1mm bar measured 287px. Given the theoretical length of 283.46px, the maximum deviation caused by the scanner was calculated to be 1.25%. The morphometric analysis was performed using 'Image J' analysis software (<http://rsb.info.nih.gov/ij/>). The parameters

recorded per node are: area, perimeter, bounding rectangle with height and width, fit ellipse with minor and major radius, Feret diameter, aspect ratio, roundness and solidity (Table 3.3).

Parameter	Definition, Comments
Area	Calibrated to square millimeters (mm <sup>2</sup> )
Perimeter	Length of the outside boundary of the selection
Bounding rectangle, width	Width of the minimum bounding rectangle (i.e.. the smallest rectangle fitting around the selection)
Bounding rectangle, height	Height of the minimum bounding rectangle
Fit ellipse, major radius	Primary radius of the ellipse best fitting around the selection
Fit ellipse, minor radius	Secondary radius of the ellipse best fitting around the selection
Feret diameter	Largest 'caliper' diameter of the selection
Aspect ratio	Ratio of the primary and secondary radius of the fit ellipse, $\frac{[\text{Major Axis}]}{[\text{Minor Axis}]}$
Roundness	$4x \frac{[\text{Area}]}{\pi x [\text{Major Axis}]^2}$
Solidity	$\frac{[\text{Area}]}{[\text{Convex Area}]}$

**Table 3.3 Overview of the Morphometric Parameters Estimated for Each Lymph Node**

Image J is an open-source software which was developed at the National Institute of Health in Bethesda, USA. It provides a highly customizable environment for image analysis in the JAVA programming language. Here, the lymph nodes were indicated as regions of interest using either the Freehand selection tool or the Elliptical selection tool, depending on the shape of the respective node (Figure 3.5). A script was used to perform scale-adjusted measurements.

### 3.5 Statistical Analysis

Data were collected using Excel Version 2007 (Microsoft, Redmond, USA). Two tables were created: Table 1 contained one row per case and provides a summary of each case and the case-related information. Table 2 contained one row per lymph node and was used to record the morphometric parameters including number of nodes, number of metastasis and mode of lymph node retrieval. The redundancy was used to verify the values.

Statistical analysis was carried out using 'R' statistical programming language version 2.13.1 ([www.r-project.org](http://www.r-project.org)). R is available under the GNU general public license (<http://www.r-project.org/COPYING>). The following functions are used:

The data were divided into subgroups using `split()` of package `[base]`. `split` allows separation of a table or of columns of a table by one or several criteria. For example, `split(AC[,30], AC[,12]>0)$"TRUE" -> AC_nodes` will operate on object 'AC' (a dataframe containing all cases treated with acetone compression) and separate column 30

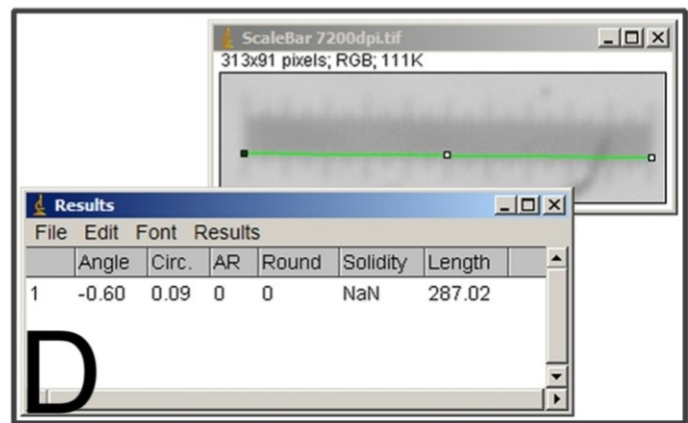
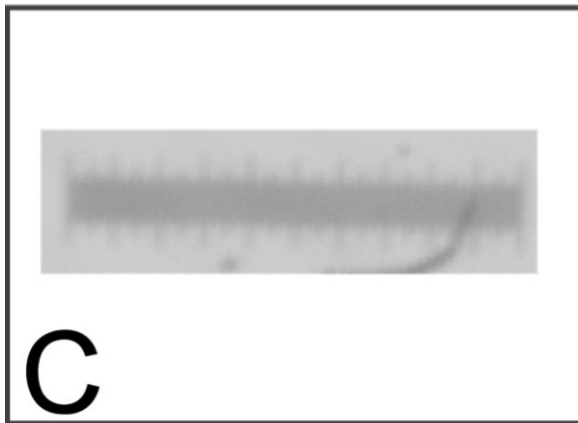
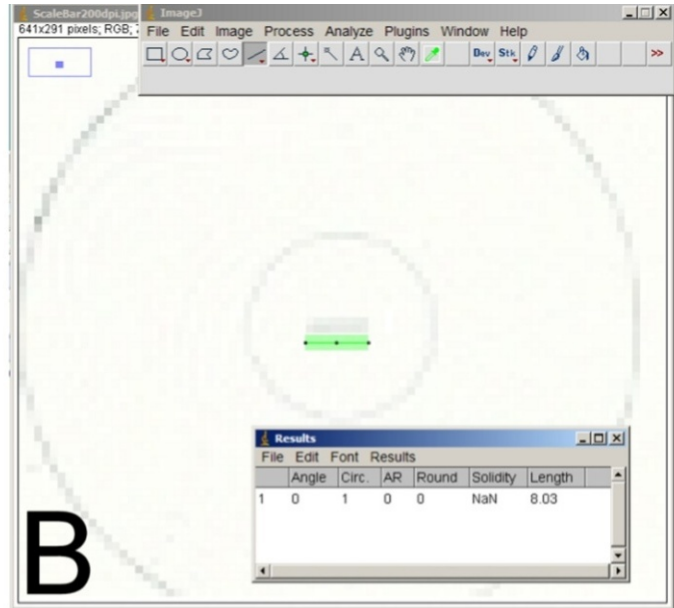
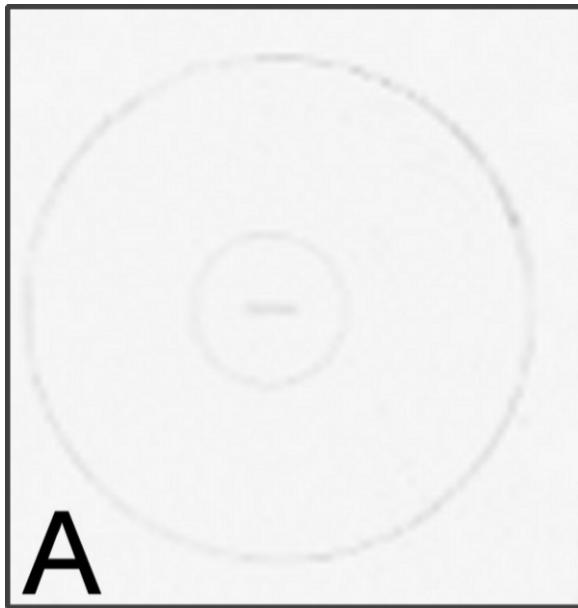
(Number of lymph nodes for each case) according to column 12 (Type of treatment: 0= primary operation, 1= neoadjuvant chemoradiotherapy). The suffix `$"TRUE"` extracts values fulfilling criterion `AC[,12]>0` from the results of the function. Thus, a vector containing the number of nodes of cases treated with chemoradiotherapy and acetone compression is output and `mean(AC_nodes)` will calculate the average number. Likewise, changing the suffix to `$"FALSE"` will output cases treated with primary operation.

Statistical functions were provided with the package `[stats]` while graphical functions for drawing diagrams were provided by package `[graphics]`. Statistical testing was performed with Student's t-test (`t.test()`) by assuming normal distributions. Significance levels were set to  $\alpha = 5\%$ .

Correlations were investigated using `cor()` set to Pearson's (`cor(x, y, method="p")`). Linear regressions was calculated with `lm()` and 2D plots with regression lines were plotted using `plot()` and `abline()` using intercept and slope from `lm()`.

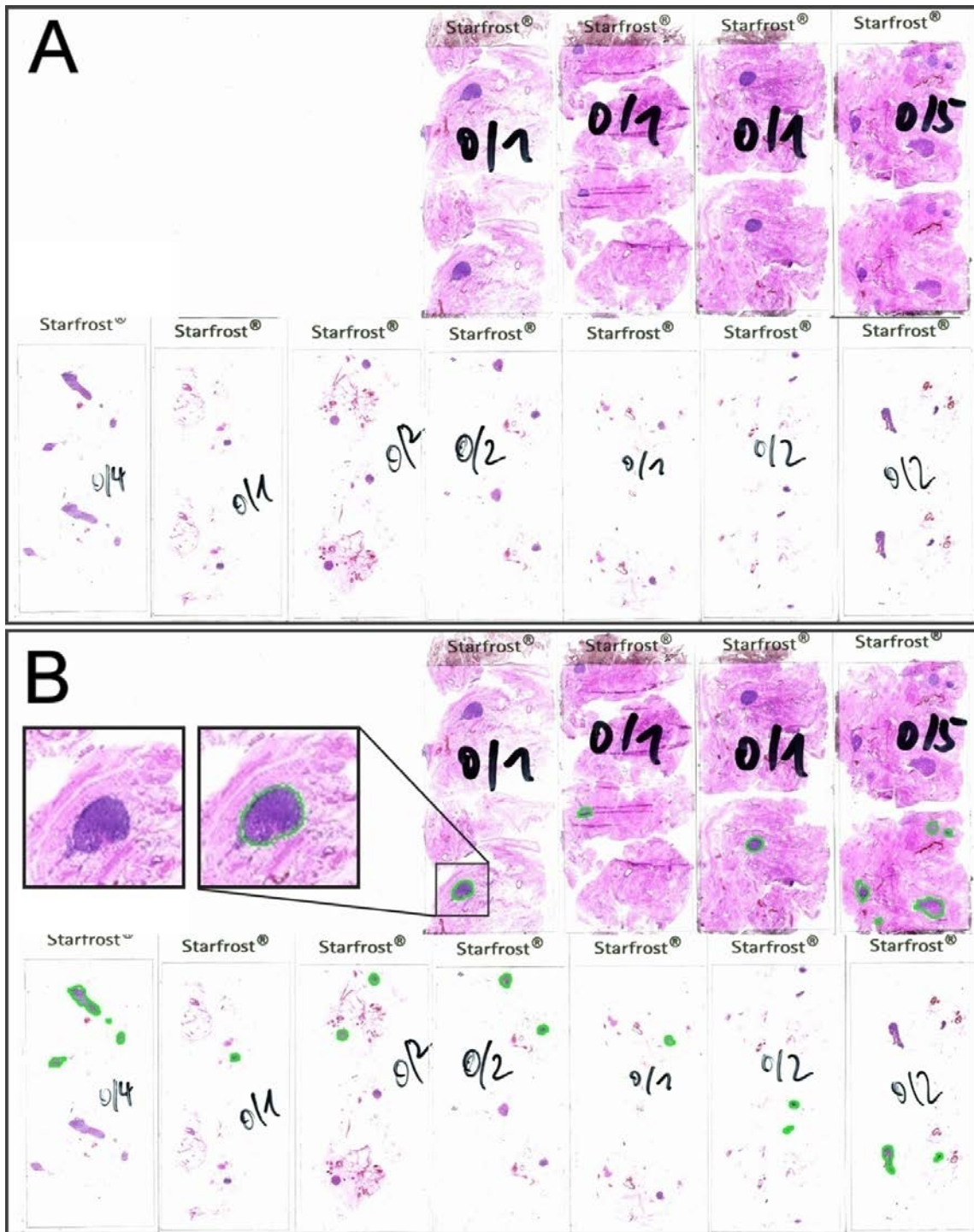
Kernel density estimation (`density()`) of the lymph node size was calculated using a Gaussian kernel and a rule-of-thumb estimation of the bandwidth (`bw = "nrd0"`). The output was displayed using `plot()` and subgroups were added using `lines()`.





**Figure 3.5 The verification of the scanning device**

A precision scale bar of 1.0mm length was digitized at 200dpi (A) and 7200dpi (C). The bar is in the center of the double circle. At 7200dpi the subdivisions into 100 $\mu$ m sections are visible (ticks). Quantification with ImageJ yields a length of 8 pixel at 200dpi (B) and 287 pixel for 7200 dpi (D). (Original scans were upscaled for printing, A 12x, C 3x).



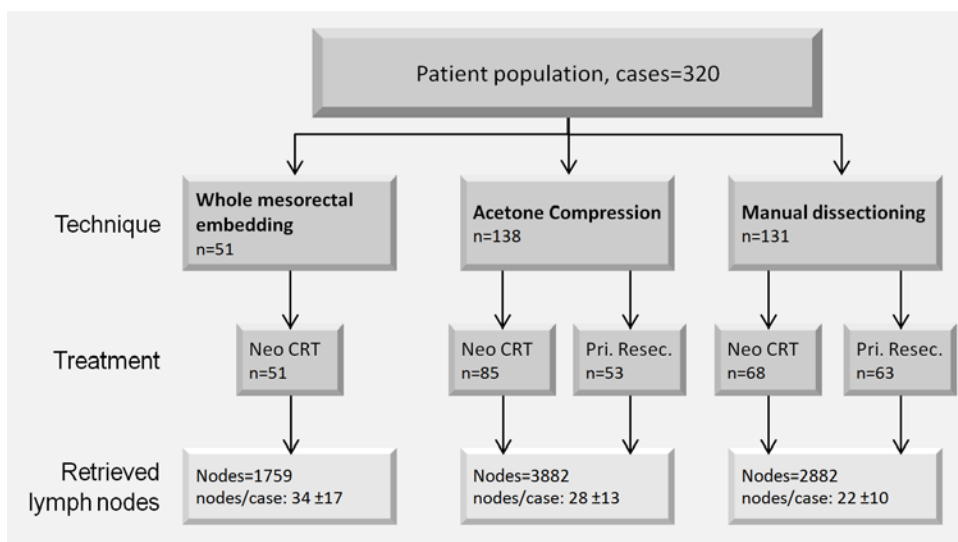
**Figure 3.6 Lymph node morphometry with ImageJ**

**A:** Digitized glass slides of case #27 featuring 7 slides with manually dissected nodes (lower row) and 4 slides with additional nodes after acetone compression (upper row). Note that no fatty tissue is visible after compression, the pink color represents the compacted cell membranes, vessels and matrix. **B:** Lymph nodes were indicated as Region-Of-Interests using the ImageJ free-hand tool and are highlighted green. Inserts: Magnifications of indicated region with and without manually drawn outline indicated by green line (**A, B:** Scan in original size at 200dpi, 1mm = 8 pixel. Hematoxylin and eosin staining, two sections per slide. Labels of the slides were removed by cropping to exclude patient IDs used for diagnostic purposes. Inserts: Upscaled 3x).

## 4. Results

### 4.1 Summary and Study Population

8,523 lymph nodes of 320 rectal cancer patients treated between 2005 and 2012 were analyzed in this study. The patients were treated at six different hospitals in Kassel, Germany or at the University Clinic in Göttingen, Germany. Depending on their tumor stage, the patients either received radiochemotherapy followed by surgery or primary surgical treatment. The clinical parameters of the patients are summarized in Table 3.1. The surgical specimens of the patients were prepared using one of three techniques available for lymph node retrieval: 51 cases were prepared with whole mesorectal embedding (WME), 138 cases were prepared using acetone compression (AC) and 131 cases were prepared using conventional manual dissectioning (MD). Each case was microscopically examined and digitalized, and the morphometric parameters of the lymph nodes were assessed. The evaluation of the lymph node morphometry allows the comparison of the efficiency of the different lymph node retrieval techniques as well as the investigation of the impact of preoperative radiochemotherapy on lymph node size and numbers. Figure 4.1 shows a flowchart of the 320 cases of the patient population subdivided by technique used for lymph node retrieval as well as the mode of treatment (preoperative CRT or primary surgery). The figure also shows the total number of nodes harvested with each technique and the mean number of nodes harvested per case (Figure



4.1).

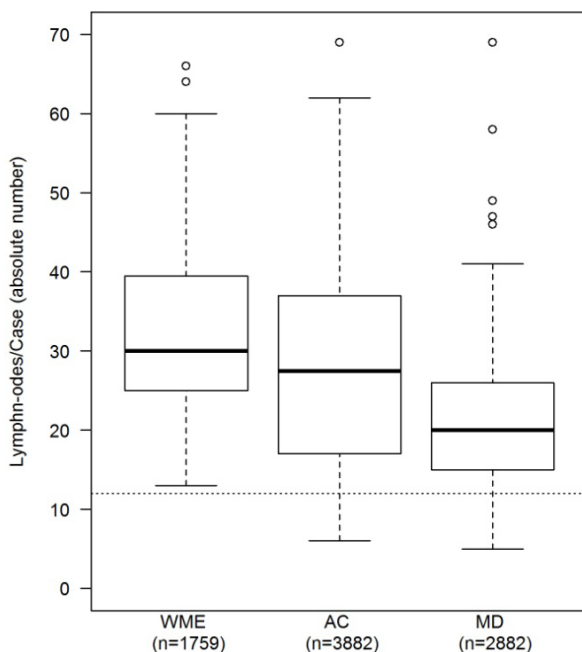
**Figure 4.1 Overview of the study population:** the population is subdivided by technique used for lymph node retrieval (WME, AC or MD) and each group is further subdivided by treatment (RCT or primary surgery). For each group, the total number of nodes harvested and the mean number of nodes per case is shown

## 4.2 Lymph Node Yield

### 4.2.1 Lymph Node Yield according to Preparation

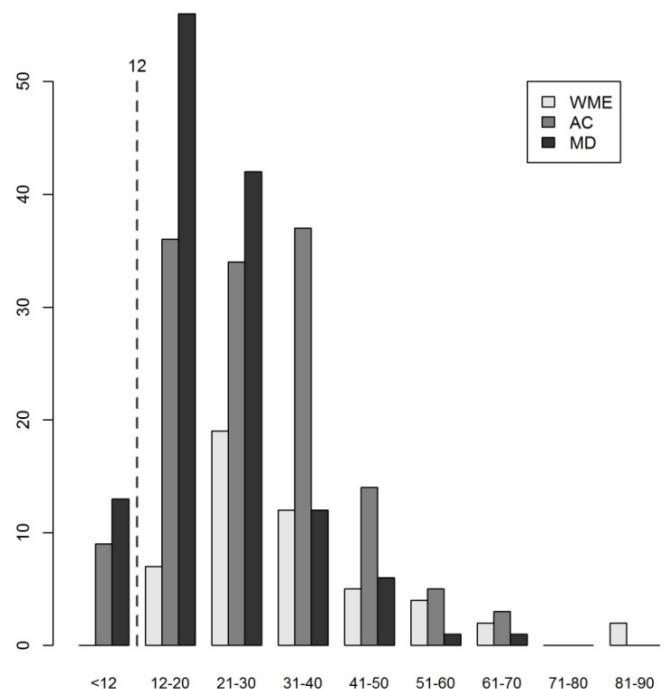
To test the efficiency of AC, the number of retrieved lymph nodes was assessed and compared to the number of lymph nodes found with MD or WME. Additionally, possible influence of different pathologists using MD was investigated.

The total number of lymph nodes harvested was compared between WME, AC and MD (Figure 4.2). Using WME, the harvest of 1,759 lymph nodes in 51 cases was achieved, resulting in the greatest number of retrieved lymph nodes with an average of 34 ( $\pm 17$ ) nodes per case. In the 138 cases examined with AC, a total of 3,882 of lymph nodes were harvested. 257 nodes in the AC group were manually detected prior to AC and 3,625 nodes were detected after subsequent acetone compression, yielding an average of 28 ( $\pm 13$ ) lymph nodes per case. Manual dissection led to the harvest of the smallest number of lymph nodes with a total of 2,882 nodes in 131 cases, i.e. 22 ( $\pm 10$ ) nodes per case on average (Figure 4.2). The differences in the lymph node harvest between the three groups were statistically significant (WME vs AC  $p=0.017$ , AC vs MD  $p<0.01$ ).



**Figure 4.2** The total number of lymph nodes per case according to preparation:

The number of lymph nodes harvested per case according to the three methods used (WME, AC and MD). WME: 34 ( $\pm 17$ ), AC 28 ( $\pm 13$ ) and MD: 22 ( $\pm 10$ ) nodes/case.



**Figure 4.3** The number of lymph nodes harvested according to preparation:

The dashed line marks the 12-lymph node threshold according to the UICC. The histogram shows the number of cases with a certain number of retrieved nodes (ranging from <12 nodes to 81 to 90 nodes per case) according to the techniques used for lymph node harvest (WME, AC and MD).

The UICC-recommended minimum of harvesting 12 or more nodes per case was met in all of the WME cases (100%). AC succeeded in delivering a minimum of 12 nodes in 129 of 138 cases (93.5%), and MD in 118 of 131 cases (90%). In Figure 4.3, a histogram shows the number of cases with a certain number of nodes harvested (ranging from less than 12 nodes per case to 81 to 90 nodes per case) according to preparation.

When comparing the numbers of lymph nodes found with MD, a difference was noted regarding the extent of the lymph node yield of the pathologists responsible. The pathological workup of the 131 cases examined with MD took place at the Institute of Pathology Nordhessen in Kassel, and was performed by certified pathologists. The lymph node harvest of four different pathologists (pathologists 1 to 4), who examined 69 of the MD specimens, was compared (Table 4.1). A significant difference in the numbers of lymph nodes harvested between pathologist 1 and 2 was noted ( $p= 0.0048$ ). Pathologist 1 examined 16 cases using MD and found a minimum of 12 nodes in all 16 cases with a mean number of 23.6 nodes per case ( $SD\pm 6.4$ ). Pathologist 2 examined 17 cases with a mean number of 17.3 nodes per case ( $SD\pm 5.5$ ) and failed to meet the benchmark of harvesting at least 12 nodes in 2 of the 17 cases. The lymph node yield obtained through pathologists 1, 3 and 4 were comparable though differences in standard deviation (SD) and cases with less than 12 nodes examined were noticed: Pathologist 3 examined 17 cases and harvested a mean number of 19.2 nodes per case ( $SD\pm 6.2$ ). One of the cases examined by pathologist 3 did not meet UICC criterion. Pathologist 4 examined 19 cases with a mean number of 21.7 nodes per case ( $SD\pm 9.45$ ) and harvested fewer than 12 nodes in 2 cases (Table 4.1).

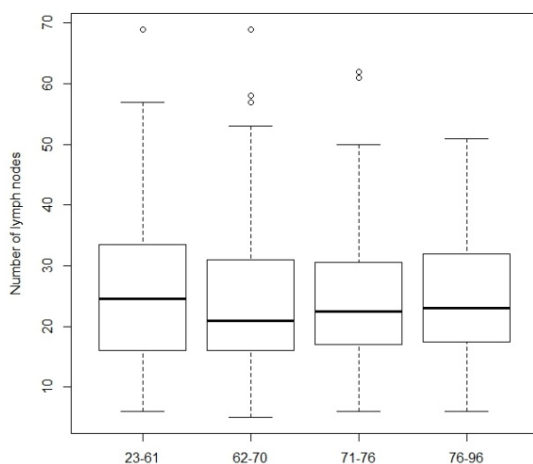
Pathologist	Number of cases	Mean number of nodes harvested	SD	Number of cases with <12 nodes
1	16	23.6	6.4	0
2	17	17.3	5.5	2
3	17	19.2	6.2	1
4	19	21.7	9.45	2

**Table 4.1: The Lymph Node Yield Obtained through Conventional Manual Dissection of Four Different Certified Pathologists at the Institute of Pathology Nordhessen, Kassel**

#### 4.2.2 Lymph Node Yield according to Patient Characteristics

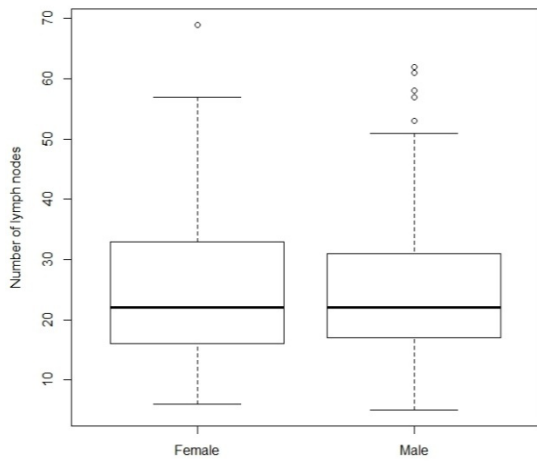
To address potential biases in the data which might affect the apparent performance of the different retrieval techniques, the lymph node yields were placed in the context of different patient characteristics:

As several studies have observed a relation between certain patient traits and lymph node yield, the number of lymph nodes harvested in this study was also evaluated according to a number of patient characteristics, including age, gender, the amount of fatty tissue prior to AC and the presence of positive lymph nodes. The mean patient age of the study population is 63.2 years in the WME group, 67.7 years in the AC group and 67.9 years in the MD group. To evaluate the association between patient age and the number of lymph nodes found, AC and MD cases were subdivided into quartiles (Q1 to Q4) according to age. Q1 included patients from 23 to 61 years of age, Q2 consisted of patients from 62 to 70 years of age, Q3 contained patients from 71 to 76 years of age, and Q4 included all patients older than 76 years of age. The differences between the quartiles were not significant (Figure 4.4). To assess a possible distinction in the lymph node harvest of male (n=179) and female patients (n=90) in the AC and MD groups, the number of lymph nodes harvested was evaluated according to gender (Figure 4.5). Again, no significant difference was found. In the AC group, the weight of the fatty tissue of the specimens prior to and after acetone compression was measured and documented. A moderate linear correlation was noted between the total number of lymph nodes retrieved and the weight of the fatty tissue prior to compression. Pearson's correlation coefficient was  $r=0.417$  (Figure 4.6). No correlation was found between the total number of lymph nodes per case and the number of positive lymph nodes (Figure 4.7). No increase in positive lymph nodes was detected if the overall number of lymph nodes was high.



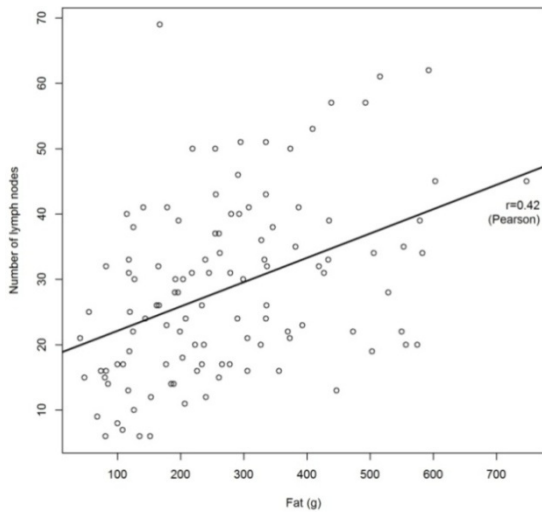
**Figure 4.4 The number of lymph nodes retrieved according to age:**

The AC and MD group were subdivided into quartiles according to age. No significant difference in the number of nodes harvested was found between the quartiles.



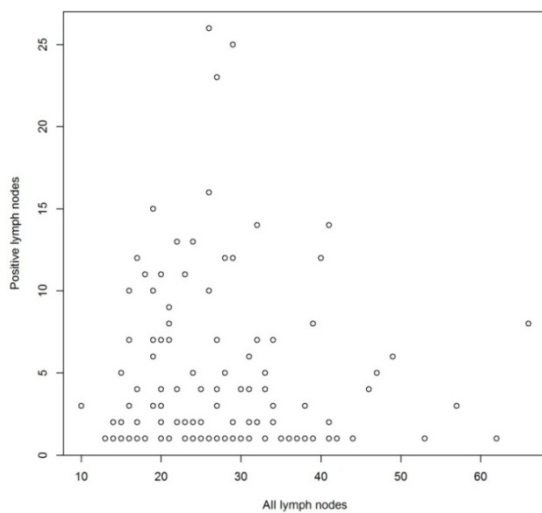
**Figure 4.5 The number of lymph nodes retrieved according to gender:**

The number of lymph nodes harvested was assessed according to gender. No significant difference between the male (n=179) and the female (n=90) patients was found.



**Figure 4.6 The number of lymph nodes retrieved according to weight of fatty tissue prior to AC:**

A linear correlation was found between the number of nodes harvested and the weight of the uncompressed fatty tissue (Pearson's correlation coefficient:  $r=0.417$ ).



**Figure 4.7 The number of positive lymph nodes retrieved according to the total number of nodes harvested:**

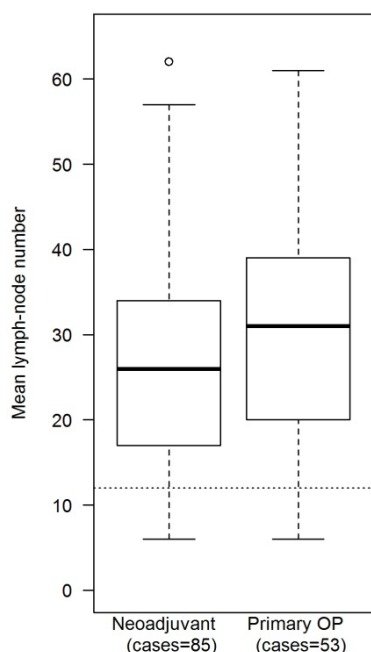
There was no increase in the number of positive nodes harvested if a greater number of nodes were found overall.

### 4.2.3 Lymph Node Yield according to Treatment

The lymph node yields were considered in relation to clinical treatment to test the influence of preoperative RCT:

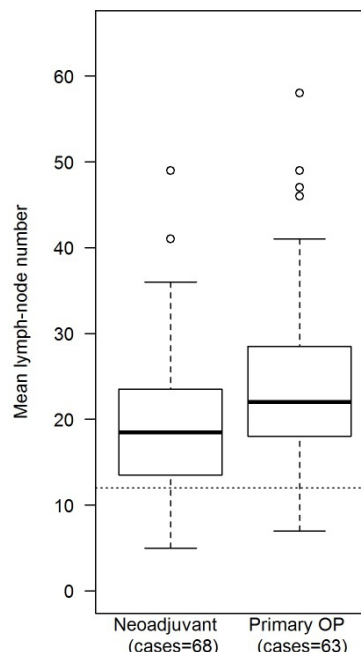
All 51 patients in the WME group were treated with neoadjuvant radiochemotherapy. In the AC group, 85 patients (62%) and in the MD group 68 patients (52%) received preoperative RCT. The lymph node yield in patients who received preoperative RCT and patients who were primarily operated was compared: in the AC group, the administration of neoadjuvant RCT did not have a significant impact on the extent of the lymph node yield. An average of 27 ( $\pm 12$ ) nodes was harvested in patients treated neoadjuvantly, and an average of 30 ( $\pm 15$ ) nodes was found in patients treated with primary surgery ( $p=0.13$ ) (Figure 4.8).

In the MD group, neoadjuvant RCT resulted in a significantly smaller number of nodes harvested compared to the lymph node harvest in patients who were primarily operated ( $19\pm 8$  vs  $25\pm 12$ ,  $p=0.0032$ ) (Figure 4.9).



**Figure 4.8** The lymph node yield according to treatment in the AC group:

The mean number of nodes according to treatment (neoadjuvant RCT or primary surgery). No significant difference in the number of nodes found was noted between the two groups ( $p=0.13$ ).



**Figure 4.9** The lymph node yield according to treatment in the MD group:

A significant difference in the mean number of nodes found by MD was noted between patients who received RCT and primarily operated patients ( $p=0.0032$ ).



### 4.3 Lymph Node Morphometry

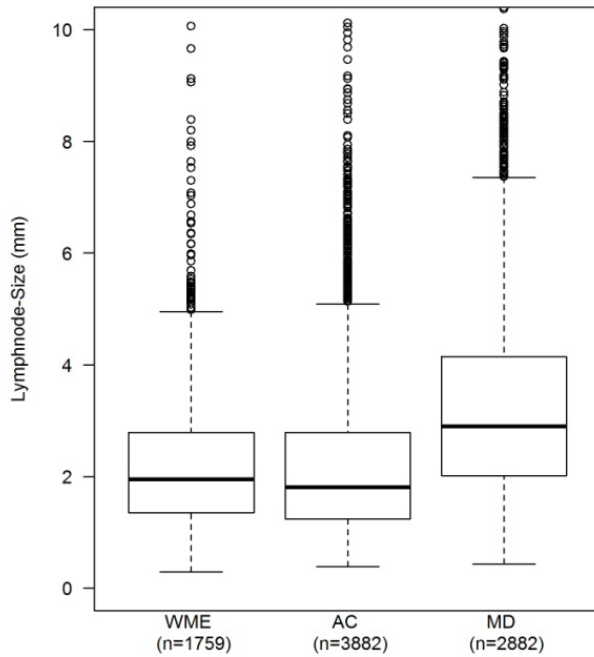
#### 4.3.1 Lymph Node Morphometry according to Preparation

To address the main question if the lymph nodes are morphologically altered by AC, morphometry of AC and WME cases was compared (hypothesis 1). Since WME does not involve treatment with solvents or mechanical stress, it may serve as reference standard for lymph node morphology.

Additionally, the lymph node diameters of cases worked-up with either AC, MD or WME were compared to assess the impact of these methods to the sizes of the nodes.

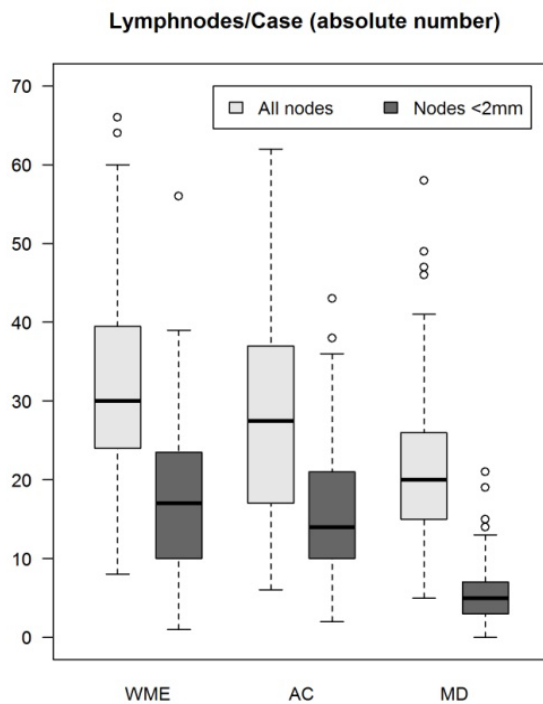
Next, morphometric characteristics of the lymph nodes were assessed. First, the average lymph node sizes were evaluated for each technique. It was found that WME resulted in the harvest of the smallest nodes as the mean size (in mm) of lymph nodes in the WME group was 2.25 ( $\pm 1.3$ ). The lymph nodes harvested with AC showed a mean size of 2.27 ( $\pm 1.6$ ). While the difference in lymph node size between the WME and the AC group was not statistically significant ( $p=0.105$ ), the nodes harvested with MD were found to be significantly larger with a mean size of 3.36mm ( $\pm 2.0$ mm,  $p<0.01$ ). Special attention was paid to the harvest of small lymph nodes, i.e. nodes with a maximum diameter of 2mm or less. In the WME group, 920 (52%) of all nodes found were smaller than 2mm. AC resulted in the harvest of the greatest number of nodes smaller than 2mm (2,099 nodes, 58%), whereas MD led to the harvest of the smallest proportion of nodes with a diameter less than 2mm (791 nodes, 25%) (Figure 4.11). In comparison to WME and AC, MD resulted in the harvest of lymph nodes with the largest diameter (3.36mm). The 257 nodes in the AC group found prior to compression showed a mean diameter of 3.38mm, which is comparable to the mean diameter of nodes harvested through MD.

Next, morphologic descriptors were compared between nodes found with WME and AC. Unlike the nodes found with AC and MD, the nodes found with WME were not subjected to solvents or mechanical procedures and can be regarded as a reference standard. The evaluated morphologic descriptors were comparable for the WME and the AC nodes: roundness (0.69, 0.68), aspect ratio (1.58 for both groups), mean area (3.47mm<sup>2</sup>, 3.43mm<sup>2</sup>) as well as the mean perimeter (6.3mm, 6.1mm). In Figure 4.10, the lymph node sizes according to preparation are depicted as a boxplot. Similar to a histogram, kernel density estimation (KDE) was used in Figure 4.12 as a non-parametric way to illustrate lymph node sizes according to preparation. The curves in the figure illustrate the distribution of the sizes of the lymph nodes harvested with each of the three methods. Nodes harvested with WME and AC are represented by the light grey (AC) and the black graph (WME) and show a much more similar distribution in sizes than the MD group.



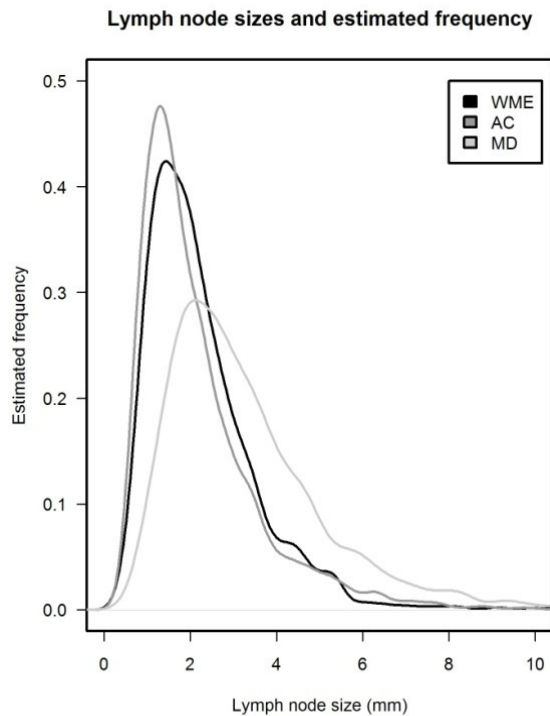
**Figure 4.10 Lymph node size according to preparation:**

WME resulted in the harvest of the smallest nodes with a mean size of 2.25mm; the nodes found with AC were only slightly larger (2.27mm). Lymph nodes found by MD were significantly larger with a mean size of 3.36mm ( $p < 0.01$ ).



**Figure 4.11: The proportion of small nodes found according to preparation:**

The proportion of nodes found by each technique, including the nodes harvested with a diameter smaller than 2mm (dark grey boxes). WME and AC led to the harvest of a severely greater proportion of small nodes (52% and 58%) than MD (25%).



**Figure 4.12 Lymph node size according to preparation:**

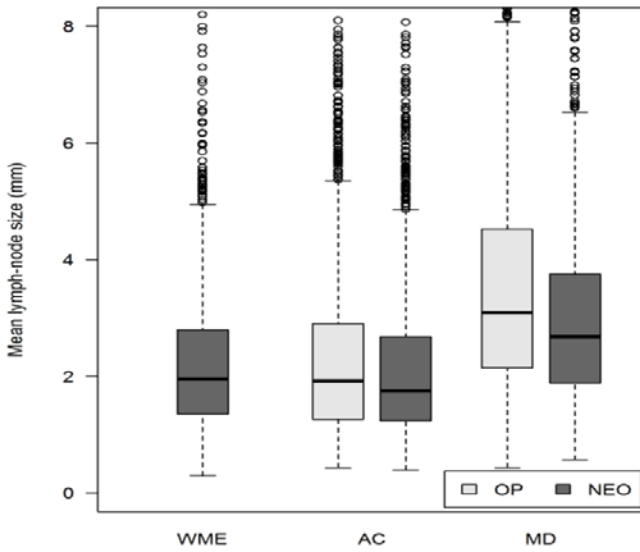
Kernel density estimation (KDE) was used to depict the distribution of the sizes of the lymph nodes harvested with each technique.

#### 4.3.2 Lymph Node Morphometry according to Treatment

To address the impact of preoperative RCT on lymph node morphology, the morphometric descriptors as well as the numbers of lymph nodes per case were considered in relation to the clinical treatment (hypothesis 2):

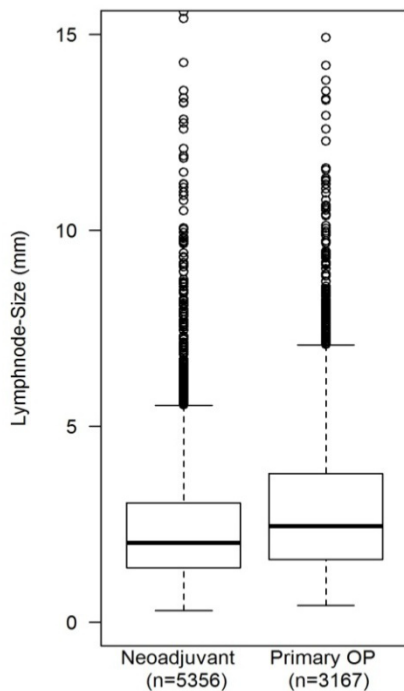
Preoperative RCT has been reported to be responsible for the shrinkage of lymph nodes in CRC specimens (Sprenger et al. 2010), a finding that was confirmed in this study: A significant difference in size was observed after comparing the mean lymph node size in pretreated specimens (n=5,356; mean size 2.42mm  $\pm$ 1.5) to the mean lymph node size after primary surgery (n= 3,167; mean size 3.00mm  $\pm$ 2.1, p<0.001) (Figure 4.14). Neoadjuvant RCT causing the shrinkage of lymph nodes was also confirmed after subdividing the groups according to preparation and treatment: In the AC group, the mean lymph node size was 2.4mm after primary surgery and 2.2mm after neoadjuvant treatment, differences in size that are statistically significant (p<0.001). The mean lymph node size in the MD group after primary surgery was 3.6mm, and 3.0mm after neoadjuvant treatment. (Figure 4.13).

**Mean lymph-node size, Neoadjuvant vs. Primary OP**



**Figure 4.13 Lymph node size according to treatment and preparation:**

The AC and MD group were subdivided according to treatment and lymph nodes in patients treated with preoperative RCT were found to be significantly smaller than nodes found in primarily operated patients in both the AC and the MD groups ( $p < 0.001$ ).



**Figure 4.14 Lymph node size according to treatment:**

Nodes harvested in patients treated with preoperative RCT were found to be significantly smaller than nodes found in patients treated with primary surgery (2.42 vs 3.00,  $p < 0.001$ ).

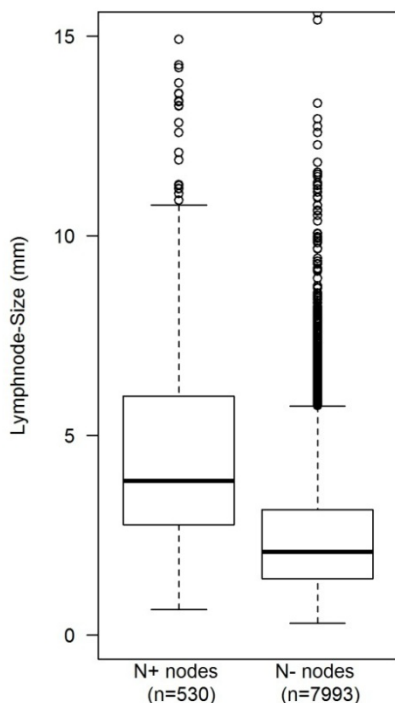
### 4.3.3 Morphometry of Lymph Node Metastases

To test if small lymph nodes may be tumor-infiltrated, the status of lymph nodes (unaffected or infiltrated) was placed in the context of lymph node size:

530 of the 8,523 nodes examined in this study were affected by metastases (6.22%).

Positive lymph nodes were found to be distinctly larger. Lymph node metastases showed a

mean diameter of 4.74mm ( $\pm 2.9$ ), whereas negative nodes showed a mean diameter of 2.49mm ( $\pm 1.6$ ) (Figure 4.15). Small nodes can be affected as well, and a distinction is made between macro- and micrometastases. Micrometastases are infiltrated lymph nodes smaller than 2mm in greatest diameter (Sirop et al. 2011). 52 of the positive nodes assessed in this study can be classified as micrometastases. In the WME group, 4 of the 35 metastases found were micrometastases (11.4%). In the AC group, 257 lymph nodes were found prior to acetone compression and 35 showed metastatic involvement, 6 (17%) of which were micrometastases. 3,625 nodes were harvested after acetone compression, and 100 of these nodes were metastases, 18 of which were smaller than 2mm (18%). Using MD, 24 lymph node metastases (6.4%) smaller than 2mm were found. With the use of AC, the greatest amount of micrometastases was found (18% vs 11.4% in the WME group and 6.4% in the MD group). A larger amount of micrometastases were found in patients treated neoadjuvantly as compared to primarily operated patients. Of the 52 micrometastases examined, 31 (59.6%) were found in patients treated with preoperative RCT, the remaining 21 (40.4%) were found in patients treated with primary surgery. In 6 cases of the AC group, all metastases found were smaller than 2mm and they were all harvested in patients treated with preoperative RCT. In 2 of these 6 cases, the nodes were found prior to compression, in the remaining 4 cases the nodes were found after acetone compression had been applied.



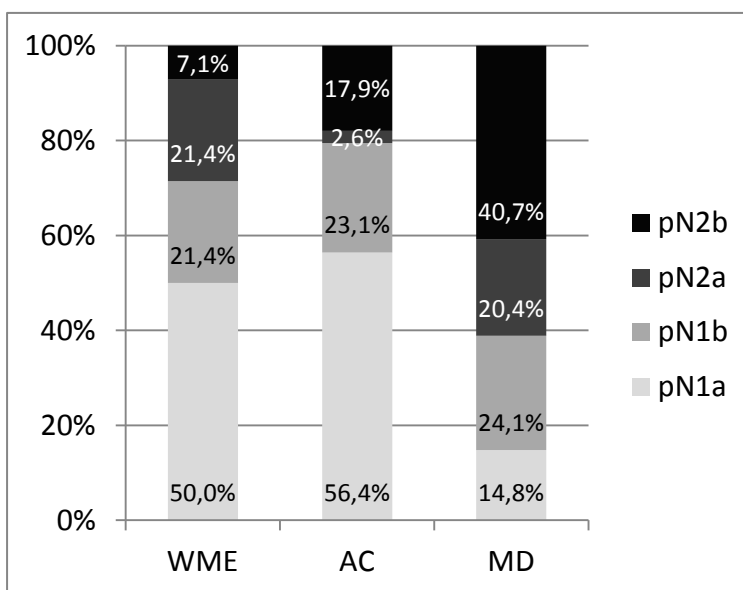
**Figure 4.15 Lymph node size according to nodal status:**

Infiltrated nodes were found to be distinctly larger than negative nodes (4.74mm vs 2.49mm).

#### 4.4 Nodal Stage according to Preparation

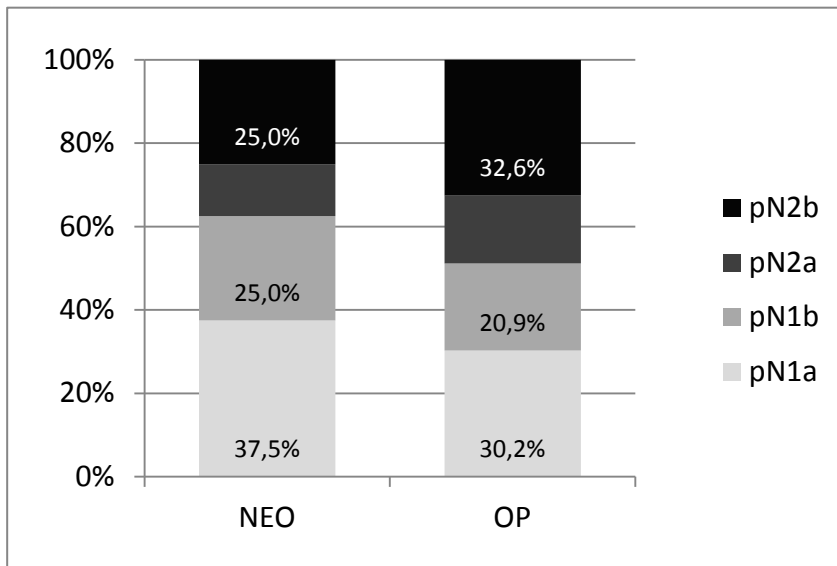
To investigate if AC and WME affect the pathological lymph node staging, the tumor stages were considered in relation to the employed retrieval method (hypothesis 3):

The pathological nodal status according to preparation was evaluated, and a distinct distribution of the different nodal stages among the three groups was noted (Figure 4.16). In the WME and the AC group, the majority of cases contained only one lymph node metastasis and was classified as pN1a (50% in the WME group and 55.8% in the AC group), whereas in the MD group, only a small fraction of cases were classified as pN1a (14.8%) or pN1b (24.1%). With the use of MD, 20.4% of cases were classified as pN2a and the majority of cases (40.7%) was classified as pN2b (more than 7 lymph node metastases). In the WME group, there was an equal number of cases classified as pN1b or pN2a (21.4%), and only 7.1% of the cases were classified as pN2b. In the AC group, 20.9% of the cases were classified as pN1b, 7% as pN2a and 16.3% of the cases were classified as pN2b. Additionally, the nodal status of the specimens was also evaluated according to treatment. It was noted that there was a distinct distribution of pN1a cases between the RCT and the surgical group since a larger number of patients treated with preoperative RCT were classified as pN1a (37.5%) than patients treated with primary surgery (30.2%). The fraction of patients classified as pN1b or pN2a is comparable between the RCT and the surgical group (25% vs 20.9% and 12.5% vs 16.3%), but a distinctly greater number of patients in the surgical group presented with 7 or more metastases (32.6%) than patients in the RCT group (25%) (Figure 4.17). In Figure 4.18, nodal stage of patients in the AC group according to treatment is depicted. A severely larger proportion of patients treated with preoperative RCT was classified as pN1a than patients who were primarily operated (65.2% vs 45%). In the RCT group, only 13% of patients were classified as pN2b, whereas in the surgical group, 15% were classified as pN2a and 20% were classified as pN2b (Figure 4.18).



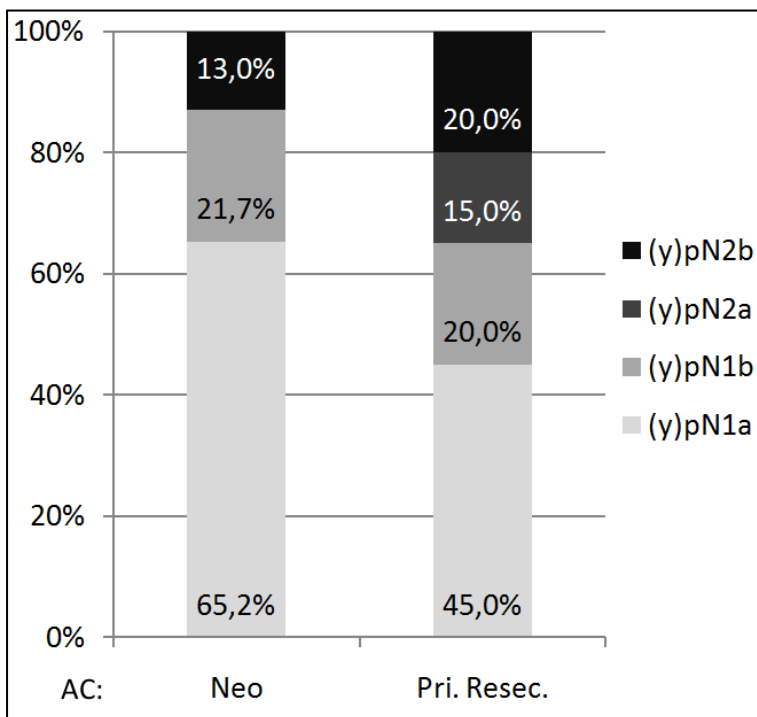
**Figure 4.16 The nodal stage of the node-positive cases according to preparation:**

A distinctly greater proportion of cases with only one lymph node metastasis (pN1a) was found in the WME and AC group compared to the MD group. In the MD group, the majority of cases presented with 7 or more positive nodes (pN2b, 40.7%).



**Figure 4.17 The nodal stage of the node-positive cases according to treatment:**

A greater proportion of cases with only one positive lymph node (pN1a) was found in patients treated with RCT compared to primarily operated patients (37.5% vs 30.2%).



**Figure 4.18 The nodal stage in the AC group according to treatment:**

A greater proportion of pN1a cases was found in patients treated with preoperative RCT than in patients who were primarily operated (65.2% vs 45%).

Patients in the MD group were also subdivided according to year of treatment. 89 patients in the MD group were treated between 2005 and 2009. 37 of these patients (41.6%) were node-positive and 3 (8%) were classified as pN1a. 42 patients in the MD group were treated between 2010 and 2012. 17 of these patients (40.5%) were node-positive and 5

(29.4%) were classified as pN1a. A variance in nodal stage was noted between the older cases and the patients treated more recently as a greater proportion of patients treated more recently were classified as pN1a (29.4% vs 8%). A similar amount of patients was treated with preoperative RCT in both groups (51.7% vs 47.8%) (Table 4.2).

	<b>2005 to 2009</b>	<b>2010 to 2012</b>
Number of cases	89	42
Neoadjuvant treatment	46 (51.7%)	22 (47.8%)
Node-positive	37 (41.6%)	17 (40.5%)
pN1a	3 (8%)	5 (29.4%)

**Table 4.2 Patients in the MD Group Subdivided by Year of Treatment**

#### **4.5 Lymph Node Size and Tumor Regression Grading (TRG) according to Preparation**

To test if lymph node sizes and lymph node yield are interrelated to the response to preoperative RCT, the parameters were considered in relation to the regression grade (hypothesis 2):

The tumor regression grading (TRG) of the specimens was microscopically assessed and classified according to Dworak (Dworak et al. 1997). TRG is regarded as patient response to preoperative RCT, ranging from TRG 0 (a specimen showing no regression) to TRG 4 (no viable tumor cells left, total regression). 73 of the patients in this study showed tumor regression 0 to 2, and 46 patients showed tumor regression 3 or 4. A greater amount of tumors showing TRG 0 to 2 were node-positive (25, 34.1%) than tumors with TRG 3 or 4 (9, 19.5%) (Table 4.3). These two groups (TRG 0 to 2 and TRG 3 or 4) were further subdivided according to the technique used for lymph node preparation. TRG 0 to 2 was observed in 48 cases in the AC group and in 25 cases in the MD group. TRG 3 or 4 was observed in 29 cases in the AC group and in 17 cases in the MD group. In the MD group, a significantly larger number of cases showing TRG 0 to 2 were node-positive than cases showing TRG 3 or 4 (52% vs 11%). In the AC group, a similar amount of cases showing TRG 0 to 2 or TRG 3 or 4 were node-positive (25% vs 24%). The lymph nodes in the MD group showing TRG 0 to 2 or TRG 3 or 4 were larger than the nodes with TRG 0 to 2 or TRG 3 or 4 in the AC group (the mean size of 3.03mm and 2.73mm vs 2.24mm and 2.05mm).

Additionally, a relation between TRG and the presence of micrometastases was noted. In patients with a higher grade of tumor regression (TRG 3 or 4), 9 of 42 metastases could be classified as micrometastases (21.4%), whereas in patients with TRG 0 to 2 only 10 of 142



(7%) metastases were smaller than 2mm. It was also noted that more micrometastases found using AC showed tumor regression 3 or 4 than micrometastases found through MD (35.3% vs 12%) (Table 4.4).

	Tumor regression 0 to 2	Tumor regression 3 or 4
Cases	73	46
Nodal-positive cases	25 (34.1%)	9 (19.5%)
pN+ (mic)	1 (4%)	3 (33.3%)
pN1a	12 (48%)	4 (44.4%)
Positive nodes (all)	142 (5.68/case)	42 (4.66/case)
Micrometastases	10 (7%)	9 (21.4%)
Mean size positive nodes	4.7mm ( $\pm 2.7$ )	4.64mm ( $\pm 3.07$ )

**Table 4.3: Nodal Status and the Presence of mi in Patients who Underwent Preoperative RCT Subdivided by TRG**

	Tumor regression 0 to 2		Tumor regression 3 or 4	
	MD	AC	MD	AC
Cases	25	48	17	29
Nodal-positive cases	13 (52%)	12 (25%)	2 (11%)	7 (24%)
pN+ (mic)	0	1 (8.3%)	0	3 (42.9%)
pN1a	3 (23%)	9 (75%)	0	4 (57%)
Positive nodes	102	40	25	17
Micrometastases	5 (4.9%)	5 (12.5%)	3 (12%)	6 (35.3%)

**Table 4.4: Nodal Status and the Presence of mi in Patients who Underwent Preoperative RCT Subdivided by TRG and Technique for Lymph Node Harvest**

#### 4.6 The Efficiency of Acetone Compression

Several technical parameters were recorded to investigate the efficiency and reproducibility of AC (hypothesis 3):

AC obtains sufficient lymph node harvest by combining elution in acetone with the manual compression of mesorectal fatty tissue. Manual compression results in the draining of most of the acetone and fat and thus a reduction of the initial weight. Prior to AC, the mean weight of the fatty tissue was 273g ( $\pm 151$ g) and 24.6g ( $\pm 19.4$ g) after AC, resulting in a weight reduction of the remaining tissue of 91% ( $\pm 3.5$ %). As mentioned earlier, the greatest morphometric descriptors were comparable between the lymph nodes harvested with WME and AC (roundness, aspect ratio, mean area and the mean perimeter) (Table 4.5). The integrity of all nodes was assessed, and 87% ( $\pm 7$ %) of the nodes found with WME and 75% ( $\pm 15$ %) of the nodes found with MD were untruncated. In the AC group, 64% ( $\pm 25$ %)

of the nodes harvested were untruncated. The efficiency of AC is based on the decisive weight reduction of the fatty tissue and, in this study, resulted in a workload of 25 ( $\pm 14$ ) capsules per case with an average of 1.5 ( $\pm 1.6$ ) nodes per capsule.

<b>Morphologic descriptor</b>	<b>AC</b>	<b>WME</b>
Roundness	0.68	0.69
Aspect ratio	1.58	1.58
Mean area (mm <sup>2</sup> )	3.43	3.47
Mean perimeter (mm)	6.1	6.3

**Table 4.5: Morphologic Descriptors of Lymph Nodes**

## 5. Discussion

### 5.1 Overview

Lymph nodes and their swelling in inflammatory and neoplastic diseases were observed by ancient Greek and Roman physicians during the beginnings of humoral pathology (Crivellato et al. 2007). With the advent of cellular pathology, the mechanisms of metastatic spread were revealed and the prognostic value of lymph nodes was recognized. Ever since, the best mode of lymph node retrieval and the most significant way to interpret them have been a matter of debate. With the introduction of highly efficient neoadjuvant irradiation combined with chemotherapy, this matter is as important as ever. Several different approaches to lymph node retrieval are employed by pathologists and have been described in this study, in which a more recent method for comprehensive lymph node retrieval from fatty tissue, the so-called acetone compression (AC), was analyzed and compared with two other methods: whole mesorectal embedding (WME) and manual dissectioning (MD). The findings of this study have made it possible to describe the efficiency of the different methods as well as the impact of preoperative radiation on lymph node morphometry.

### 5.2 Lymph Node Yield in CRC Specimens

#### 5.2.1 The Impact of Lymph Node Yield in CRC Specimens

The histological lymph node assessment of CRC resection specimens is of crucial importance for a number of reasons. Besides enabling physicians to determine the tumor stage, the nodal assessment also has an impact on their ability to specify the patient's prognosis and determine the course of treatment. The presence of a single lymph node metastasis causes a shift in the tumor stage from stage II to stage IIIA (Wittekind and Meyer 2010) and consequently, the 5-year disease-specific survival rate drops from 94.1 to 79.1 in colon cancer patients and from 88.3 to 69.1 in rectal cancer patients (Kanemitsu et al. 2012). The nodal status is one of the most important prognostic factors in CRC patients, and decisions on therapy rely on it. In colon cancer patients, the administration of adjuvant chemotherapy is only indicated in node-positive stage III patients (Schmiegel et al. 2008). Further therapeutic decisions, such as the resection of liver metastases in CRC patients, are also based on the presence of positive lymph nodes (Fong et al. 1999).

#### 5.2.2 The 12-lymph-node-minimum

To ensure adequate nodal assessment, a sufficient number of lymph nodes must be harvested and examined, a factor which has been recognized by the UICC. The harvest of

12 to 15 negative lymph nodes has been found to accurately reflect nodal negativity, resulting in the UICC's decision to recommend the harvest of a minimum of 12 nodes per patient (Compton et al. 2000). This recommendation is based on the findings of two studies. *Scott et al.* compared the lymph node yield obtained through manual dissection and additional fat clearance methods in 41 rectum and 62 colon specimens and showed that by administering additional fat clearance methods, 12.4 additional nodes per specimen could be harvested (Scott et al. 1989). *Ratto et al.* conducted a study that involved the investigation of lymph node yield and the survival rates of 801 CRC patients and were able to demonstrate an association between node-positivity and negative outcome, higher rates of local recurrence and distant metastasis (Ratto et al. 1999).

While the UICC does not encourage the retrieval of additional nodes, several lines of evidence indicate that a comprehensive lymph node investigation may yield further relevant information. Besides allowing accurate nodal assessment, harvesting a high number of lymph nodes has also been reported to improve patient survival. *Tepper et al.* found an association between longer relapse time and better chances of survival in node-negative rectal cancer patients when a large number of nodes were harvested (Tepper et al. 2001). *Kotake et al.* showed an association between better chances of survival and a greater lymph node count in both stage II and stage III CRC patients (Kotake et al. 2012).

### **5.2.3 Techniques for Lymph Node Retrieval**

In view of the importance of sufficient lymph node retrieval, a number of techniques for the improved histopathological workup of CRC resection specimens have been introduced over the years: fat clearance techniques such as the application of Carnoy's solution, methylene blue injection or whole mesorectal embedding. In the present study, lymph node yield obtained through conventional manual dissection, whole mesorectal embedding and acetone compression was assessed and compared. WME is the most extensive of all three methods and since- other than MD or AC- it is a technique that does not involve use of solvents or mechanical compression, it can be regarded as a reference standard. WME proved to be the most comprehensive technique as it resulted in the harvest of 34 ( $\pm 17$ ) nodes per case. AC also presented as a method of high accuracy with a mean number of 28 ( $\pm 13$ ) nodes per case, whereas MD led to the harvest of a significantly smaller number of nodes ( $22 \pm 10$ ). Those cases that had at least 12 nodes examined were evaluated and WME was successful in meeting the UICC benchmark in all 51 cases (100%), and AC in 93.5% of the cases. In the MD group, only 90% of all cases had 12 or more nodes examined. The failure of MD to guarantee thorough lymph node harvest is even more noteworthy in view of the fact that the numbers of lymph nodes found manually in this study were comparatively high: In a study involving 221 rectal cancer patients, *Cawthorn et al.*

found a mean number of 10.5 nodes per case through MD (Cawthorn et al. 1986). Märkl et al. compared conventional MD and methylene blue injection in 669 CRC patients and found a mean number of 13 nodes with MD (Märkl et al. 2013b), and Jass et al. harvested a mean number of 18.7 nodes per case with MD after comparing the thoroughness of fat clearance and MD in 20 rectal specimens (Jass et al. 1986).

#### **5.2.4 Lymph Node Size**

Whereas most studies involving lymph node yield in CRC based their decision to count nodes solely on the size of the node in question (Märkl et al. 2007, Brown et al. 2004), nodes in this study were identified according to four histological criteria (the shape of the node, histological structure, the presence of a capsule and the presence of blood vessels). Of these four criteria, at least two had to be present, ensuring a distinction between lymphoid inflammatory infiltrate and actual lymph nodes. Aside from a distinction in the numbers of nodes found with each technique, there was also a significant variation of the mean lymph node size among the three groups as the lymph nodes found with WME and AC were significantly smaller than the nodes harvested manually (the mean size in mm in the WME and AC groups: 2.25 and 2.27 vs 3.36 in the MD group). WME and AC also led to the harvest of the greatest proportion of very small nodes (with mean sizes below 2mm). 920 (52%) of the nodes found with WME and 2,099 (58%) of the nodes found with AC were smaller than 2mm. In the MD group, only 791 (25%) of the harvested nodes were smaller than 2mm. If a similar size distribution can be assumed to occur in all groups, MD seems to miss every second small node.

#### **5.2.5 Infiltrated Nodes**

Infiltrated lymph nodes have been reported to be larger than negative nodes (Cserni 2002), a finding that was confirmed in this study. Positive nodes were significantly larger than negative nodes in all cases (4.74mm vs 2.49mm,  $p < 0.001$ ). It is interesting to note that the infiltrated nodes show comparable sizes between the three retrieval techniques (means, WME=4.77mm, AC= 4.69mm and MD=4.76mm). Nevertheless, a considerable amount of infiltrated nodes in this study were found to be smaller than 2mm (52 of 530 positive nodes) and thus can be classified as micrometastases (mi) (Sirop et al. 2011). Compared to WME and MD, AC led to the harvest of the greatest number of mi (18% vs 11.4% and 6.4%). The presence of mi was recognized as early as 1999, not only for CRC but for other forms of cancer as well (Hermanek et al. 1999). However, their prognostic significance is still uncertain, especially in stage II CRC patients. Liefers et al. found stage II CRC with mi to have a lower five-year survival rate than stage II patients without mi and since there are still as many as 20% of stage II CRC patients that die of recurrent disease (Liefers et al. 1998), it has been suggested that an oversight of mi during histological assessment might be the

reason for this poor outcome (Märkl et al. 2013a). However, there are also experts questioning the prognostic significance of mi, especially in irradiated rectal cancer patients. Most of the mi in this study was found in patients treated with preoperative RCT (59.6% vs 40.4%), and the presence of mi in irradiated specimens was associated with better tumor regression. This finding confirms the assumption that mi might be regarded as a sign of treatment response to RCT (Sprenger et al. 2013b). The role of mi in CRC, both in pretreated and nonirradiated patients, remains controversial and should be further examined in future studies.

#### **5.2.6 Lymph Nodes and other Parameters**

As shown by the results of this study, lymph node yield obtained through different pathologic workup methods varies significantly, and WME and AC have proven to be much more thorough in harvesting lymph nodes than MD. Although increased survival rates due to the harvest of a larger number of nodes per patient has been demonstrated in a number of studies, the reason for this phenomenon is still unknown. Some believe certain patient- or tumor-related characteristics to be responsible for the variation in lymph node yield among patients. *Chou et al.* examined clinical and pathologic factors in 153,483 patients with CRC stages I to III and observed that "for every 10-year incremental increase in age, there was an average reduction of 9% in lymph node harvest", resulting in an average of 9.3 lymph nodes found in rectal cancer patients older than 70 years (Chou et al. 2010, p. 2565). *Kanemitsu et al.* evaluated the lymph node yield in 4,538 colon and rectal cancer patients and found an association between a higher lymph node number for both younger age (<60 years) and the female sex in rectal cancer patients (Kanemitsu et al. 2012). The factors of age, gender and tumor-stage were related to the number of lymph nodes investigated in this study. None of the reported associations were reflected by the 320 cases investigated, i.e. the total number of nodes and the number of positive nodes were independent of age and gender, and no relation to tumor stage was found. However, in the AC cases, a correlation between the weight of the fatty tissue prior to compression and the total number of lymph nodes harvested was found (Pearson's correlation coefficient was  $r=0.417$ ). It has also been suggested that a more favorable tumor-host interaction results in a higher lymph node count since more and larger nodes can be regarded as "the expression of an enhanced immunological defense" of the body towards the tumor (Märkl et al. 2012, p. 1420), and an increase of nodes in size facilitates the harvest. Other factors considered to be associated with a higher lymph node count are the presence of the MSI phenotype (Eveno et al. 2010), the size and location of the tumor or more advanced pT stages (Shia et al. 2012).

### 5.2.7 Achieving the 12-lymph-node-minimum

Since a sufficient lymph node yield is necessary for accurate staging and thus treatment decisions, many think the number of harvested nodes might be a surrogate parameter for the hospital's quality of medical care (Denham et al. 2012). Although this has not been proven with certainty so far, the fact is that there are still a large number of hospitals that do not meet the benchmark of harvesting at least 12 nodes: When comparing lymph node yield in CRC patients, *Lagoudianakis et al.* found that of the 454 CRC patients, only 41.6% had 12 or more nodes examined (Lagoudianakis et al. 2011). *Baxter et al.* found that only 37% of the CRC patients had at least 12 nodes examined (Baxter et al. 2005), and even though colon cancer patients have been reported to have more lymph nodes retrieved than rectal cancer patients (Chou et al. 2010), *Bilimoria et al.* still found that as many as 60% of the hospitals across the U.S. failed to meet the 12-node-minimum in colon cancer patients (Bilimoria et al. 2008). To a certain degree, variance in lymph node yield within one institution seems to be a common phenomenon. *Parkash et al.* compared the lymph node yield of pathologists from two affiliated institutions and found that, in terms of counting lymph nodes on glass slides, "there was no slide on which all pathologists agreed on all occasions" (Parkash et al. 2010, p. 42). The discrepancy in the lymph node yield among pathologists working at the same institution was observed in this study as well. The manual lymph node harvests of four pathologists were compared, and a significant difference in the number of harvested nodes was found for one pathologist, while the other three yielded comparable results. In view of the fact that the numbers of manually retrieved nodes in this study were comparably high, it appears likely that the variance of lymph node yield among different pathologists might be even higher within other institutions. Even though such variances in the lymph node yield within one institution do not necessarily imply poor quality in medical care, they could be overcome through the introduction of guidelines and more standardized procedures in daily pathological workup.

### 5.2.8 The Concept of Stage Migration

One of the most frequently-discussed reasons for the association between harvesting more lymph nodes and better chances of survival is the concept of stage migration, also known as the 'Will Rogers Phenomenon' (Märkl et al. 2013b). Stage migration implies "spurious understaging because too few nodes are removed/examined and possibly small lymph nodes with metastases missed" (Parkash et al. 2010, p. 47). Some experts think this is the reason for the higher lymph node count causing improved survival rates (Scott et al. 1989). However, the data from this study refute this concept: WME and AC resulted in a significantly higher lymph node yield than MD but they also contained a large number of cases with only one lymph node metastasis present (pN1a, 50% and 55.8%). In the MD

group, on the other hand, only 14.8% of the cases were classified as pN1a, whereas the majority of the cases were classified as pN2b (40.7%). Additionally, based on the present results, no correlation between the total number of nodes per case and the number of positive lymph nodes was found. There was no increase in the number of positive lymph nodes noted when the overall number of lymph nodes was high. If stage migration were in fact the reason for more lymph nodes causing better chances of survival, a higher rate of nodal-positive cases would be expected. Neither is this the case in our study nor in other studies (Märkl et al. 2013a, for example), in which it was demonstrated that advanced lymph node retrieval techniques or the ultrastaging of lymph nodes does not increase the rate of nodal-positive cases. *Hogan and Winter* suggest the introduction of a 'nodal positivity constant' since they observed that the nodal positivity rate in colon and rectal cancer patients combined has remained mostly steady over the past years (40% in 1988-1990, 42% in 2006-2008) (Hogan and Winter 2012) and *Parsons et al.* showed that although lymph node yield in colon cancer patients has been increasing over the years, the rate of nodal-positivity has not (Parsons et al. 2011). In view of the anatomic differences and the fact that colonic cancer is not treated with neoadjuvant RCT, lymph node yield in colon and rectal cancer patients is only comparable to a certain degree. However, the observation made by *Parsons et al.* further underlines the assumption that a greater lymph node yield does not necessarily result in the harvest of more positive nodes.

All in all, the impact of medical care, patient- and tumor-related factors as well as the quality of the histopathologic assessment of the lymph node yield and the chances of survival represent a multifaceted process involving a number of factors to varying degrees. As demonstrated by results of this study, the UICC's recommendation of harvesting at least 12 nodes per patient is feasible with a technique that allows comprehensive lymph node yield such as acetone compression. Even though the exact reasons are still unknown, it has been shown repeatedly that CRC patients exhibit increased survival rates when a greater number of lymph nodes are detected and when all departments involved (oncology, surgery and pathology) cooperate efficiently. It seems reasonable to demand that in future all CRC patients should have at least 12 nodes harvested and examined.

### **5.3 The Impact of Preoperative Radiation on Lymph Node Yield and Morphometry**

The administration of radiation and chemotherapy prior to surgery has become standard in the treatment of locally advanced rectal carcinoma in Germany based on the findings of the CAO/ARO/AIO-94 study and follow-up studies. The studies demonstrated the clinical advantages of preoperative RCT such as the improvement of sphincter-preservation or the lower rates of local recurrence (Sauer et al. 2004). Even though long-course radiotherapy has proven to be very effective by causing both nodal and tumor down-staging (Francois et



al. 1999), it also impedes the nodal assessment of CRC specimens since it is known to cause "lymphocyte depletion" as well as "atrophy and fibrosis of the stroma" (Baxter et al. 2005, p. 429). A number of studies on the impact of preoperative RCT on lymph node retrieval have been conducted over the years and it seems as if the majority of them indicate that RCT (both long-term as well as short-term radiotherapy) impairs the number and size of lymph nodes harvested from irradiated specimens. The results of this study show that with regard to lymph node size, there was a significant difference between the irradiated and the non-irradiated groups. In all cases, patients who received preoperative RCT had lymph nodes with a mean size of 2.42mm, whereas the lymph nodes of patients treated solely with surgery had a mean size of 3.00mm ( $p < 0.001$ ). After subdividing the groups according to the methods used for retrieval, both the AC group (mean lymph node size 2.4mm and 2.2mm) and the MD group (mean lymph node size 3.6mm and 3.0mm) showed smaller lymph nodes after treatment with preoperative RCT ( $p < 0.001$ ). In the WME group, all patients were treated with RCT, and the mean sizes of the nodes of the WME group and the irradiated AC group were comparable (2.25 vs 2.2,  $p = 0.108$ ).

With regard to the number of harvested lymph nodes after pretreatment, the present results demonstrate that preoperative radiotherapy does affect the number of retrieved lymph nodes if the nodes are harvested through manual dissectioning. The mean number of harvested nodes decreased from 25 to 19 nodes per case in nonirradiated and irradiated patients of the MD group ( $p = 0.0032$ ). In the AC group, on the other hand, the mean number of nodes harvested per case was not significantly affected by radiation compared to patients who were primarily operated (30 vs 27,  $p = 0.13$ ). Thus, factors other than pretreatment seem to influence the number of retrieved nodes within the AC group. This is not the case in the MD group. Here, pretreatment does cause a significant reduction of retrieved nodes and most cases not reaching the UICC-threshold are pretreated (Pretreated: 10/68 = 14.7%; primarily operated: 3/63 = 4.8%). In part, this distribution might be explained by the size reduction of the lymph nodes caused by preoperative radiation. Thus, it seems a more comprehensive technique might be better to work-up pretreated rectal cancer specimens.

Treatment response to preoperative radiation is microscopically evaluated based on the extent of tumor regression found in the resection specimen. In addition to reflecting treatment response, recent data indicate tumor regression grading (TRG) to be a prognostic factor as well. *Rödel et al.* evaluated the impact of TRG on survival in the context of the CAO/ARO/AIO-94 trial on rectal cancer and found an association between poor tumor regression and a higher risk of lymph node involvement and poorer chances of survival (Rödel et al. 2005). The association between poor tumor regression and lymph node involvement has been confirmed in the findings of this study as well since more nodal

positive cases (34.1%) showed poor regression (TRG 0 to 2) than TRG 3 or 4 (19.5%). The effect of RCT is reflected by a number of findings in the present study. After comparing the pathological nodal status according to treatment, a greater proportion of cases with only one lymph node metastasis (pN1a) was found in pretreated patients compared to patients who were primarily operated (37.5% vs 30.2%), which might be due to the downstaging effect of the pretreatment. 52 of the lymph node metastases found in this study can be classified as micrometastases and the majority of these nodes were found in patients treated with preoperative RCT (59.6% vs 40.4%). Additionally, an association between micrometastases and tumor regression was observed since patients with higher grades of tumor regression (TRG 3 or 4) showed more micrometastases than patients with poor tumor regression (TRG 0 to 2): 21.4% vs 7%. This association between tumor regression and the presence of micrometastases might support the idea that micrometastases be thought of as "regressive micrometastases" (Sprenger et al. 2013b, p.6) and thus mirrors a patient's response to treatment.

Despite several studies demonstrating the effect of RCT on lymph node yield, there are no recommendations regarding standardized, efficient lymph node yield in irradiated specimens (Sprenger et al. 2010). *Baxter et al.* found an average of 3 nodes fewer in irradiated specimens, and in 16% of the patients treated with RCT, no nodes were harvested at all (Baxter et al. 2005). *Rullier et al.* found preoperative RCT to decrease the number of lymph nodes harvested by as much as 24% (13 vs 17) and the number of lymph node metastases by 48% (1.2% vs 2.3%) (Rullier et al. 2008). *Govindarajan et al.* even question the entire concept of harvesting a minimum of 12 lymph nodes per patient and claim that it is not feasible in patients who underwent RCT, as they found an average of five nodes fewer harvested in irradiated than in non-irradiated patients (10.8 vs 15.5), and 63% of the patients in the RCT group had fewer than 12 nodes examined (Govindarajan et al. 2011). According to *Sprenger et al.* the "radiation-related reduction of lymph node size might be the main reason for a reputedly reduction of lymph node numbers in irradiated specimens worked up with conventional (manual) retrieval because of the apparent difficulty to detect lymph nodes smaller than 0.2cm" (Sprenger et al. 2010, p.101), an argument that is supported by the present results, which demonstrate both fewer small nodes (<0.2cm) detected by MD and a significant decrease in lymph node size after radiation. All in all, the data seem to indicate that MD finds fewer nodes after pretreatment because the nodes shrink and are less likely to be found manually. Thus, MD does not seem to be a reliable method for lymph node harvesting, especially in patients treated with preoperative RCT. The aforementioned studies all agree on RCT causing a decrease in lymph node yield, and they all used MD to find lymph nodes, except *Sprenger et al.*, who used WME and demonstrated the harvest of smaller but not necessarily fewer lymph nodes

after RCT. However, they also acknowledge that WME is too elaborate a method for routine processing (Sprenger et al. 2010). The results of the present study are consistent with the findings of a study conducted by *Gehoff et al.*, which showed that conventional MD results in a smaller lymph node yield in irradiated specimens than AC does, even if fat clearance methods are applied in addition to MD (Gehoff et al. 2012a). According to these results and the findings of the present study, AC seems to be a valid approach to ensure sufficient lymph node yield in CRC, both in irradiated and non-irradiated specimens, and the proposal of *Govindarajan et al.* to question the feasibility of the 12 lymph-node threshold in irradiated specimens (Govindarajan et al. 2011) no longer applies.

#### **5.4 The Efficiency of Acetone Compression**

The importance of adequate lymph node assessment in CRC patients has been proven sufficiently and optimizing lymph node harvesting by introducing a variety of new methods has been the aim of several studies over the years. MD was the standard in harvesting lymph nodes for a long time, but it has become increasingly obvious that this method has a number of shortcomings. MD is a method which greatly depends on the skill of the pathologist responsible. As the results of the present study show, its efficiency can vary significantly among different pathologists within one institution. Literature on lymph node numbers indicates an even higher variance between different institutions. MD resulted in the harvest of fewer lymph nodes than WME and AC (22 vs 28 and 34), and since MD failed to enable the harvest of small nodes (the mean size of nodes found with MD in this study was 3.36mm), it is not surprising that the 12-lymph node minimum recommended by the UICC could not be met in 10% of the cases examined manually. Additionally, MD resulted in the harvest of a significantly smaller number of nodes in patients treated with preoperative RCT (the mean number of nodes found in patients treated with primary surgery or RCT: 25 vs 19,  $p=0.0032$ ).

These limitations have been noted in other studies as well and led to the introduction of more advanced pathological workup techniques, including fat clearance methods, methylene blue staining or acetone compression. Fat clearance is based on the elution of fatty tissue with different chemicals (e.g. alcohol and xylene), depending on the type of clearing method used. The resulting decolourisation of the tissue facilitates localization of the lymph nodes and has proven to provide a greater lymph node yield than MD in a number of studies (Jass et al. 1986, Herrera et al. 1992, Brown et al. 2004). Despite resulting in a more efficient lymph node harvest than conventional MD, fat clearance methods show a number of limitations as well. *Abbassi-Ghadi et al.* compared efficiency, costs and the time exposure of different workup methods and found fat clearance to be rather expensive and time-consuming with a median preparation time of 79 hours (Abbassi-

Ghadi et al. 2012). Additionally, the solvents used for fat clearance, such as xylene or methyl salicylate, are considered to be harmful and require fume hoods as well as special rooms to work in. The disposal of the solvents used is also difficult as they cannot be simply drained into a sink (Jass et al. 1986). Märkl et al. were able to outline the advantage of methylene blue staining over manual dissection as the mean lymph node harvest in their study was 86% higher than the harvest of the unstained group (Märkl et al. 2007). Despite ensuring an effective lymph node yield, methylene blue injection still remains a rather elaborate method as it also requires at least 16 hours of preparation (Basten et al. 2010). In comparison to these methods, acetone compression seems to be a much more efficient technique for both a quick and sufficient lymph node harvest. By microscopically examining and digitalizing 8,523 lymph nodes from 320 rectal cancer specimens harvested with three different pathological methods, AC has proven to be almost as thorough in lymph node harvest as the more extensive WME method (the mean number of nodes harvested 34 vs 28), while being significantly more efficient than MD (28 vs 22). Acetone has been used by Brown et al. in combination with alcohol and xylene in terms of a fat clearance method (Brown et al. 2004) and was established and first introduced as a newly-developed method by Basten et al. in 2010 (Basten et al. 2010). AC was performed in this study according to this original protocol except for the slight alteration of using acetone at room temperature instead of heating it. The temperature was changed because of the findings of a study conducted by Gehoff et al., revealing difficulty in cutting the tissue blocks as well as the hampered quality of IHC staining when acetone was heated (Gehoff et al. 2012a). The use of non-heated acetone has shown presentable results in the present study. A robust compression was achieved, causing a weight reduction in the fatty tissue by 91% ( $\pm 3.5\%$ ). A mean number of 25 ( $\pm 14$ ) capsules per case was established, containing a mean number of 1.5 ( $\pm 1.6$ ) nodes per capsule. AC may result in a higher number of tissue blocks than other pathological workup methods but processing times are still reasonable. Gehoff et al. found AC to be "as effective as the WME method, but the AC method was much faster, with the total processing time within the range of conventional pathology workup" (Gehoff et al. 2012a, p. 211) and according to Basten et al., if the preparation of the specimen takes place in the morning, it can be fully diagnosed the following day. Additionally, AC seems to be much more cost-efficient and environmentally-friendly than techniques using solvents for fat clearance as the acetone used can be filtered or distilled and reused. It is a very feasible method that can be carried out by lab technicians and does not require special surgical preparation of the specimen beforehand (Basten et al. 2010). Although we did find a greater amount of lymph nodes to be truncated after the harvest with AC as compared to WME and MD, the tissue treated with acetone still contained blood vessels, nerve tissue and lymph nodes that showed morphometric characteristics comparable to those of the

lymph nodes found with the extensive WME method. Lymph nodes have been reported to have the tendency to shrink in the course of fixation and processing (Märkl et al. 2012) and the fact that the nodes harvested with AC are of a size similar to that of the nodes in the WME group (2.27 vs 2.25) also indicates that treatment with acetone does not impair the quality of the tissue. In a case report published by *Gehoff et al.*, AC not only resulted in the harvest of 21 additional lymph nodes in a rectal cancer resection specimen after MD, it also enabled the harvest of one positive node that had not been detected by the previous MD, thus greatly changing the diagnosis of the patient's tumor stage and subsequent course of treatment. *Gehoff et al.* also emphasize that AC allows for the detection of tumor cell deposits, small nests or nodules that represent residual lymph nodes and are equally as relevant for determining the tumor stage as macro- or micrometastases (Gehoff et al. 2012b). In addition to this, AC is not only of relevance in the lymph node assessment of CRC specimens but can also prove useful for the workup of fatty tissue in other forms of cancer such as neuroendocrine tumors (Scheel et al. 2013), thus bearing the potential for more widespread use in pathological workup. Consequently, even though WME has proven to be the most thorough of all methods, it is too elaborate to be suitable for everyday purposes. AC is much more practicable and seems to be an adequate alternative to routine histopathological assessment of CRC resection specimens that allows thorough lymph node yield even in patients treated with preoperative RCT.

## **5.5 Outlook and Limitations**

Despite the extensive assessment of the harvested lymph nodes of 320 rectal cancer patients, the present study presents with limitations as well. The study's design was retrospective, which restricts the comparability of the different cases. In addition, there are certain disparities regarding the patient cohort: the cases of the MD group were treated between 2005 and 2012, whereas the patients of the AC group were treated between 2009 and 2012. None of the patients of the MD group were enrolled in a clinical trial, whereas 15 patients of the AC group and all of the patients of the WME group were enrolled in the CAO/ARO/AIO-2004 study, which implies more standardized diagnostic and treatment proceedings. With treatment regimens changing and improving over time, the fact that some of the cases in the MD group were treated earlier than those in the AC group might account for the uneven distribution of pN1a and pN2b case. A shift in the pN-stages was seen after subdividing the MD cases by year of treatment: 89 patients in the MD group were treated between 2005 and 2009. 3 (8%) of the nodal-positive patients were classified pN1a. 42 patients in the MD group were treated between 2010 and 2012 and 5 (29.4%) of the nodal-positive patients were classified pN1a. For future evaluations it might be interesting to perform a prospective observational study and compare survival data of

patients according to preparation, number of nodes harvested and treatment to gain information about the impact of a more thorough lymph node harvest through acetone compression on patient survival.

## 6. Summary

In spite of the increasing awareness of this disease and the development of more accurate screening methods, colorectal cancer is still the third most common type of cancer in the world. Mortality rates remain high and incidence rates keep rising, especially in countries with growing adaptation to the western-type lifestyle. Of all the aspects regarding this disease, the pathological nodal status is of crucial importance as it determines the tumor stage, the course of treatment and is still considered to be among the patient's most important prognostic factors. The pathological nodal status can only be adequately estimated if a sufficient lymph node harvest has taken place. Over the years, a number of advanced methods for lymph node retrieval in CRC specimens have been developed (manual dissectioning, fat clearance methods, methylene blue staining, WME or acetone compression), each with various levels of efficiency. The optimal number of lymph nodes to be harvested is still under debate but the UICC recommends assessing a minimum of 12 nodes per patient. Administration of preoperative radiochemotherapy has been reported to cause lymph node shrinkage and even diminish the amount of nodes present in a specimen.

The present study evaluated the lymph node yield of 320 patients with locally advanced rectal carcinoma. The nodes were obtained through three different workup methods: whole mesorectal embedding, manual dissectioning and acetone compression. 8,523 lymph nodes were microscopically examined, digitalized and subjected to morphometry. The slides were stained with hematoxylin and eosin and in questionable cases, IHC staining with panCK was supplemented. To ensure differentiation of actual lymph nodes and lymphoid infiltrates, two of the following pre-determined criteria had to be recognizable in each node: the shape of the node, the presence of a capsule, follicular lymphoid tissue and the presence of blood vessels. By manually drawing the nodes during morphometry, information about the geometric characteristics were gathered.

Lymph nodes were found to be larger if infiltrated with malignant cells and smaller after preoperative radiation. Preoperative radiochemotherapy was also found to induce tumor regression of the primary tumor and of the lymph node metastases and was associated with the presence of micrometastases. In terms of the thoroughness of the three methods, acetone compression resulted in the harvest of significantly more and smaller lymph nodes than manual dissectioning (28 vs 22 nodes per patient and a mean size of 2.27mm vs 3.36mm), while proving to be almost as thorough as the more extensive and time-consuming WME method. AC also succeeded in reaching the UICC recommendation of harvesting a minimum of 12 nodes per patient to a satisfactory extent (in 93.5% of cases) and allowed for the harvest of a sufficient amount of lymph nodes (including very small

nodes) both in patients treated with preoperative radiochemotherapy and in patients treated with primary surgery (the mean number of 30 nodes per patient in nonirradiated specimens and 27 nodes per patient in irradiated specimens). Unlike other pathological methods available for lymph node yield, acetone compression is less time-consuming, does not require the contact with or disposal of harmful chemicals while obtaining reproducible results of a decent quality, suitable even for IHC staining or mutation analysis.

Despite the knowledge about the importance of adequate nodal assessment and the varying efficiency of the different pathologic methods, there is still a lack of guidelines regarding the standardization of lymph node harvest in CRC specimens. The results of this study suggest introducing acetone compression as a very practicable method suitable for adequate lymph node harvest in the daily routine pathological workup of CRC specimens, both in patients treated with and without preoperative RCT.



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[Stand 26.03.2013, 16.46]

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[Stand 25.3. 11:00]

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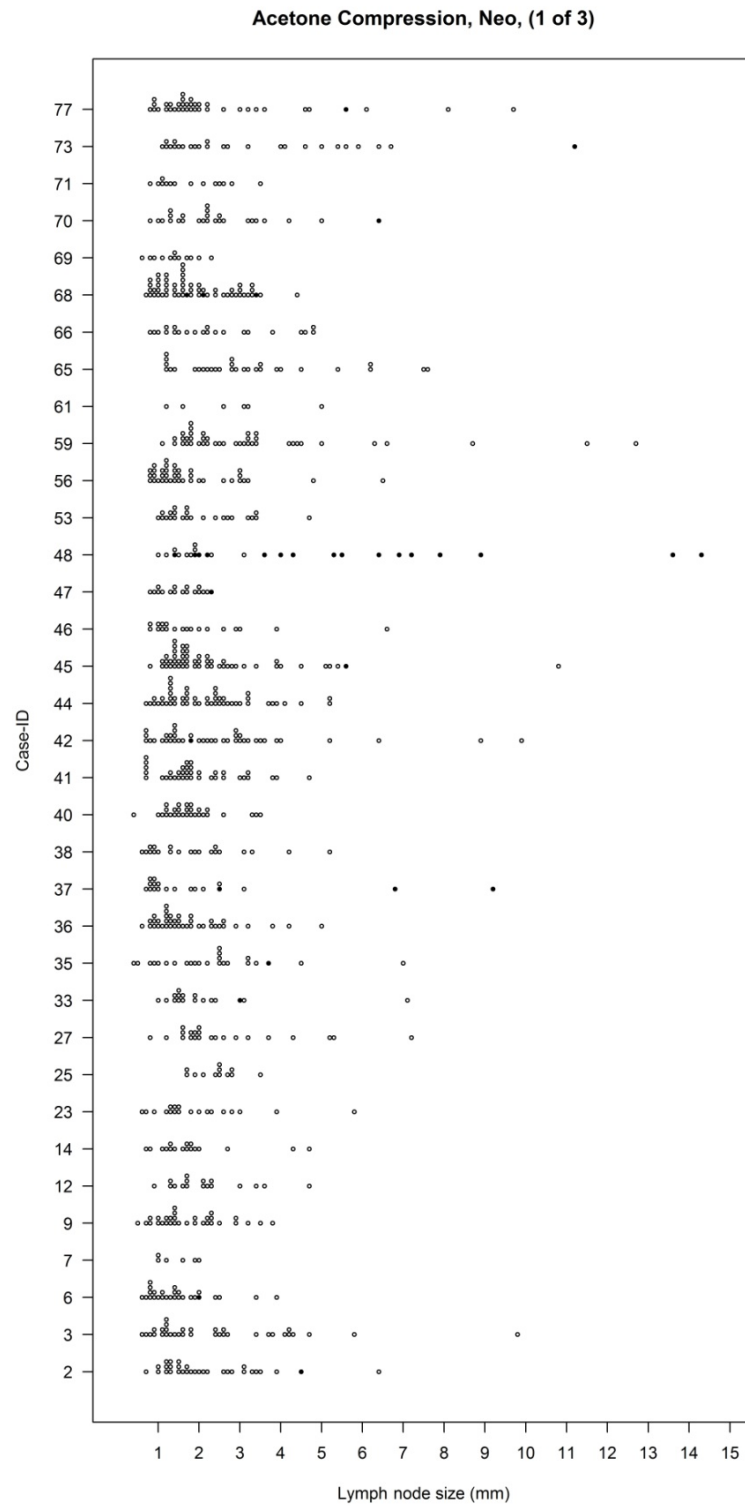
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[Stand 07.07.2013, 16.40]

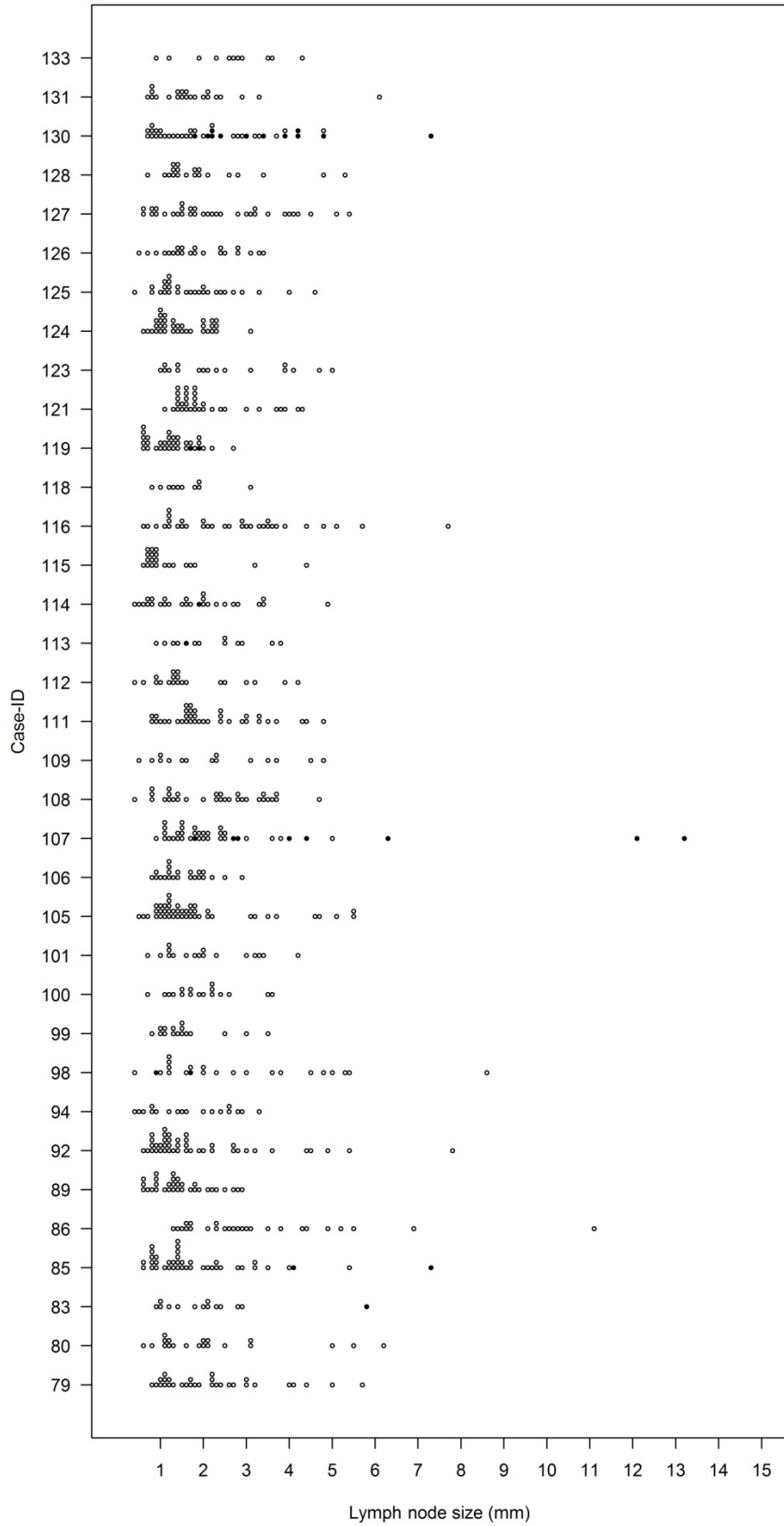
# 8. Appendix

## 8.1 Stripcharts for All Cases Subdivided by Treatment and Mode of Preparation

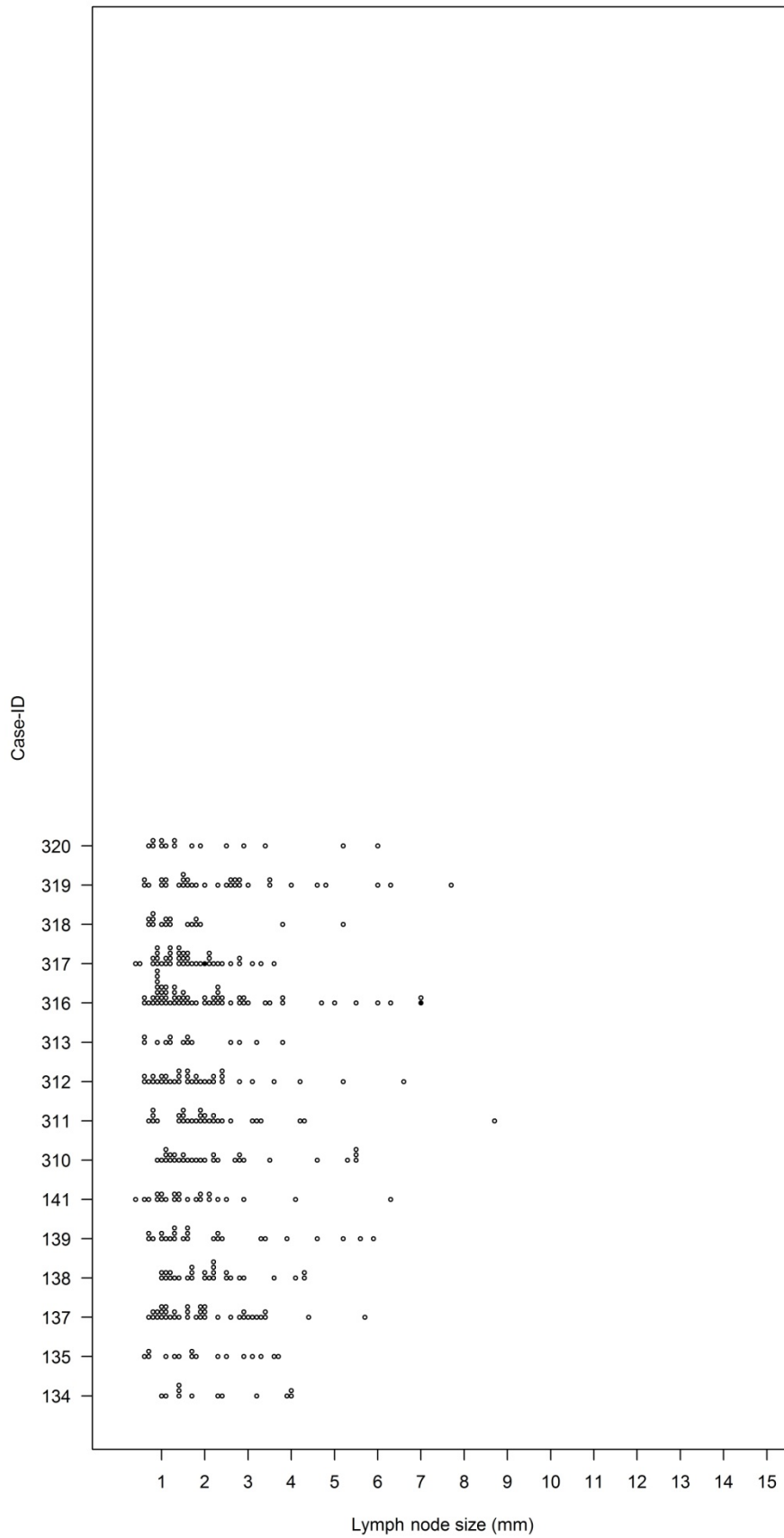
### 8.1.1 Stripcharts for AC Group, Patients Treated with Preoperative RCT (n=85)



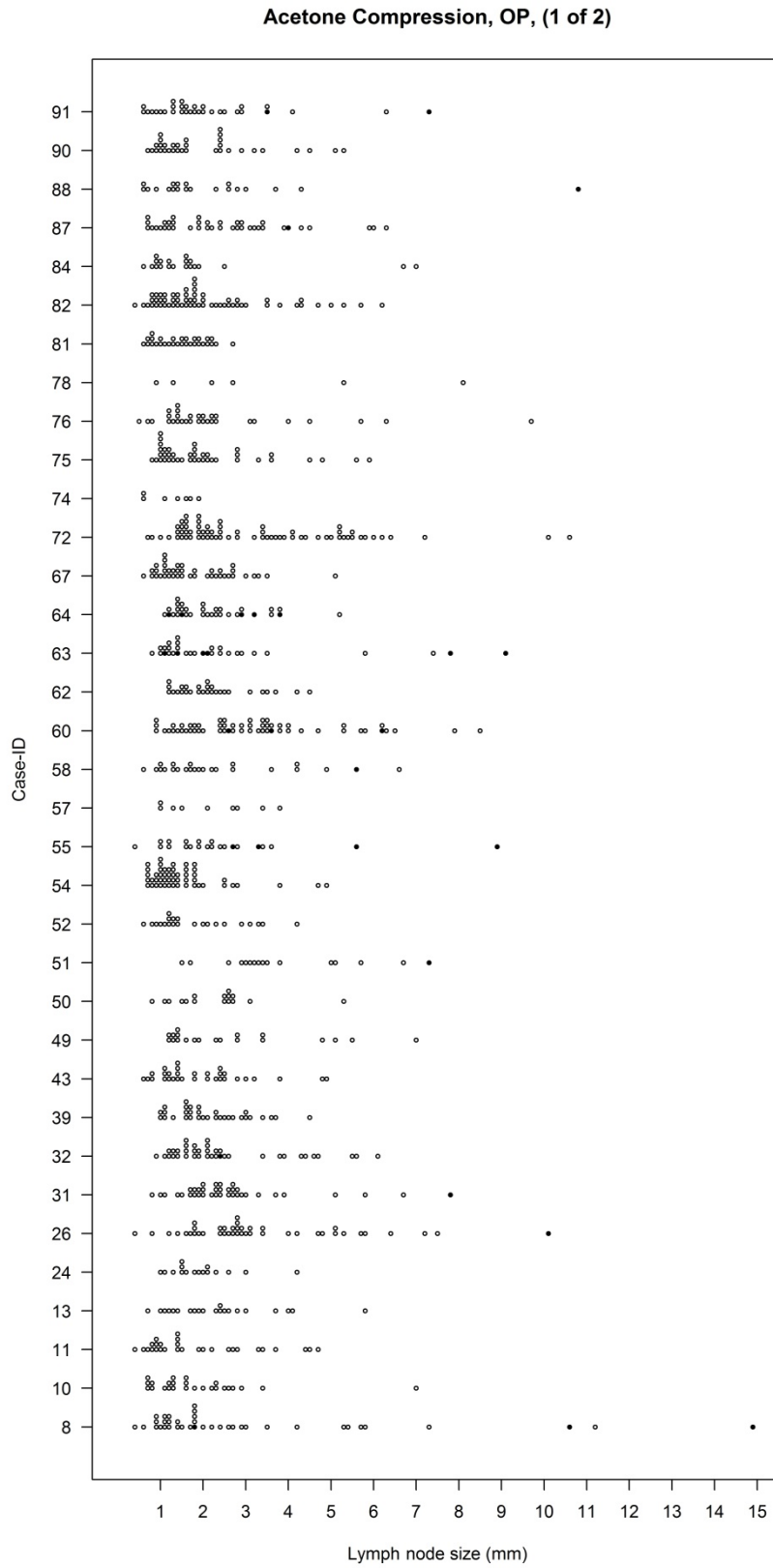
Acetone Compression, Neo, (2 of 3)



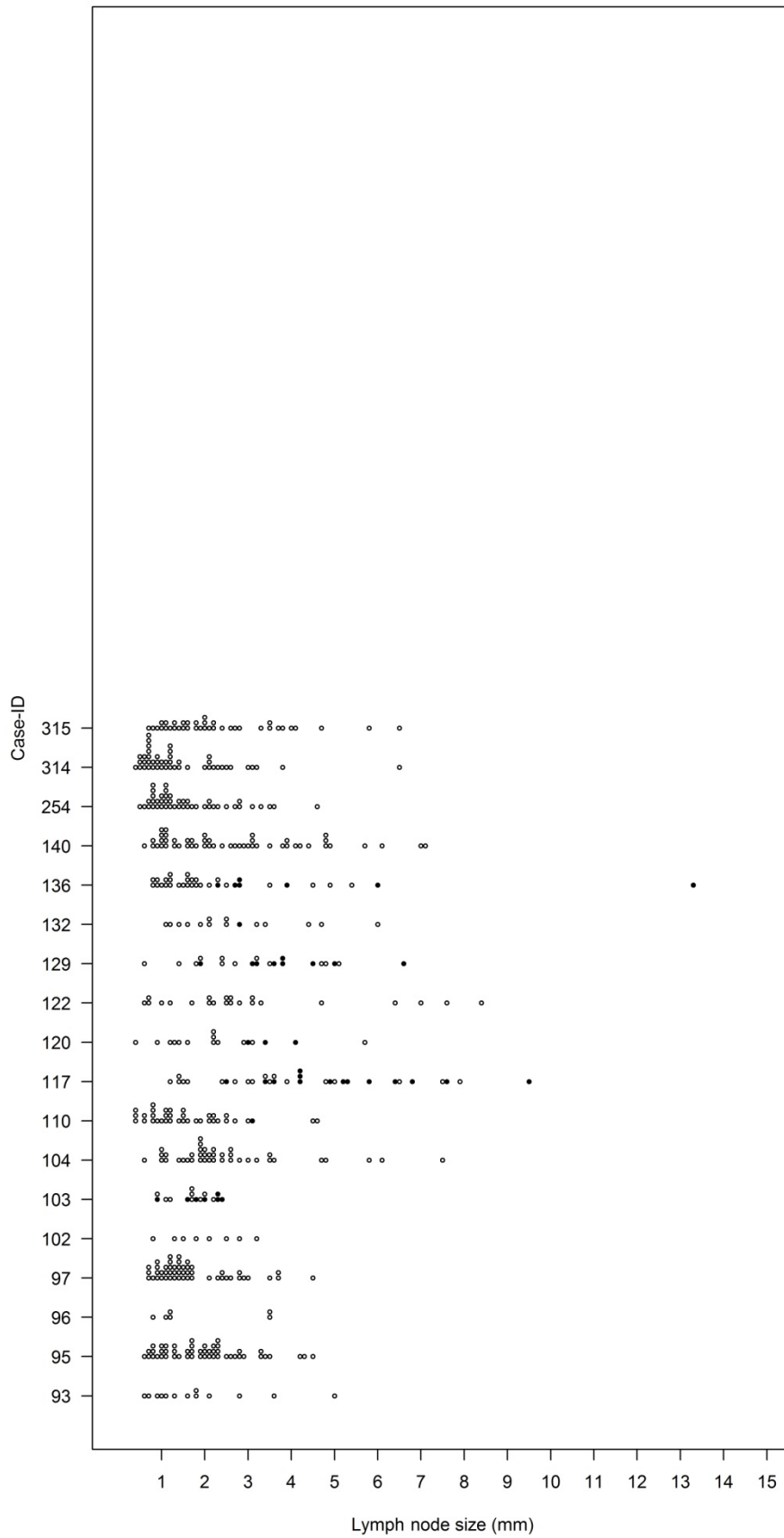
Acetone Compression, Neo, (3 of 3)



### 8.1.2 Stripcharts for AC Group, Patients Treated with Primary Surgery (n=53)



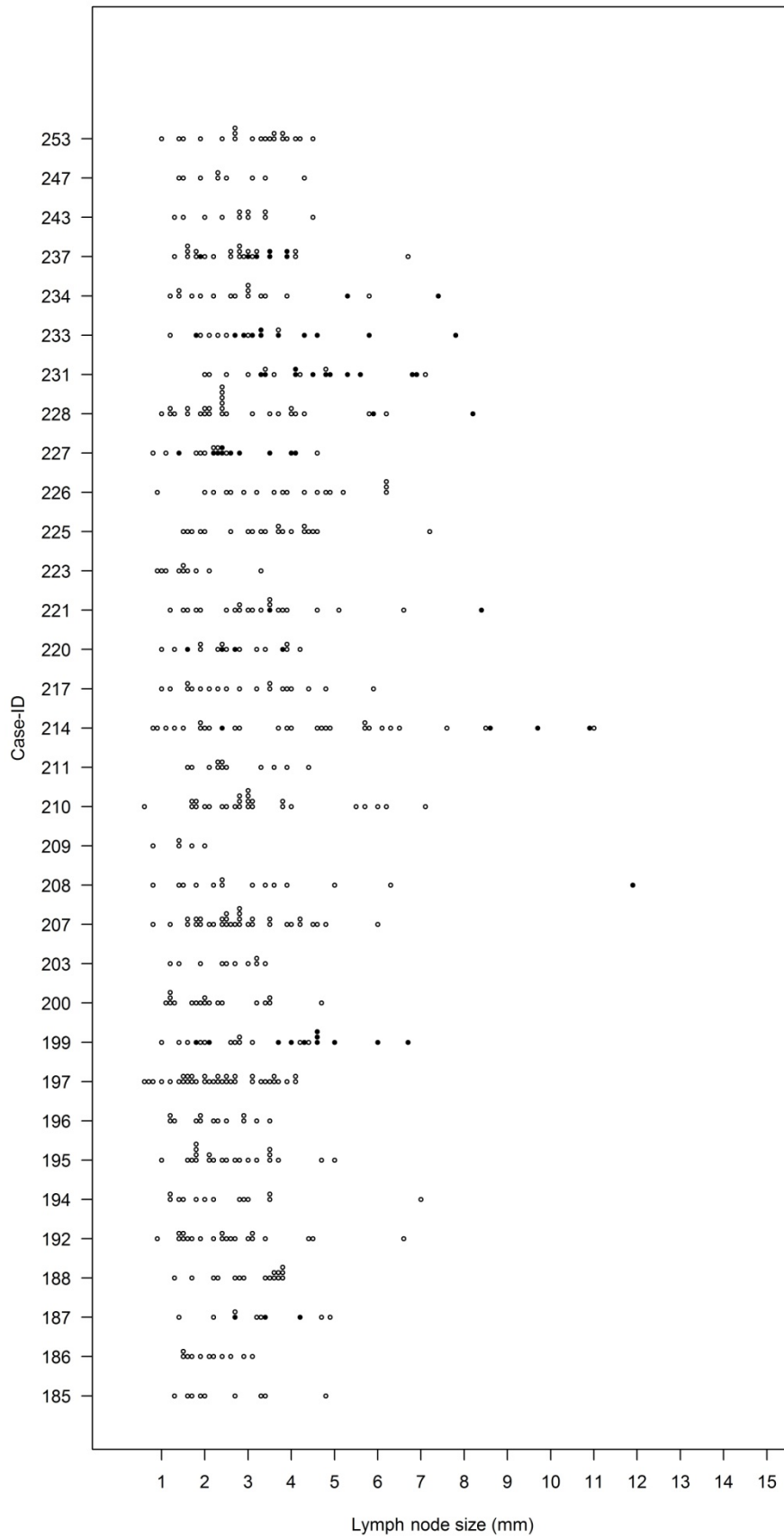
Acetone Compression, OP, (2 of 2)



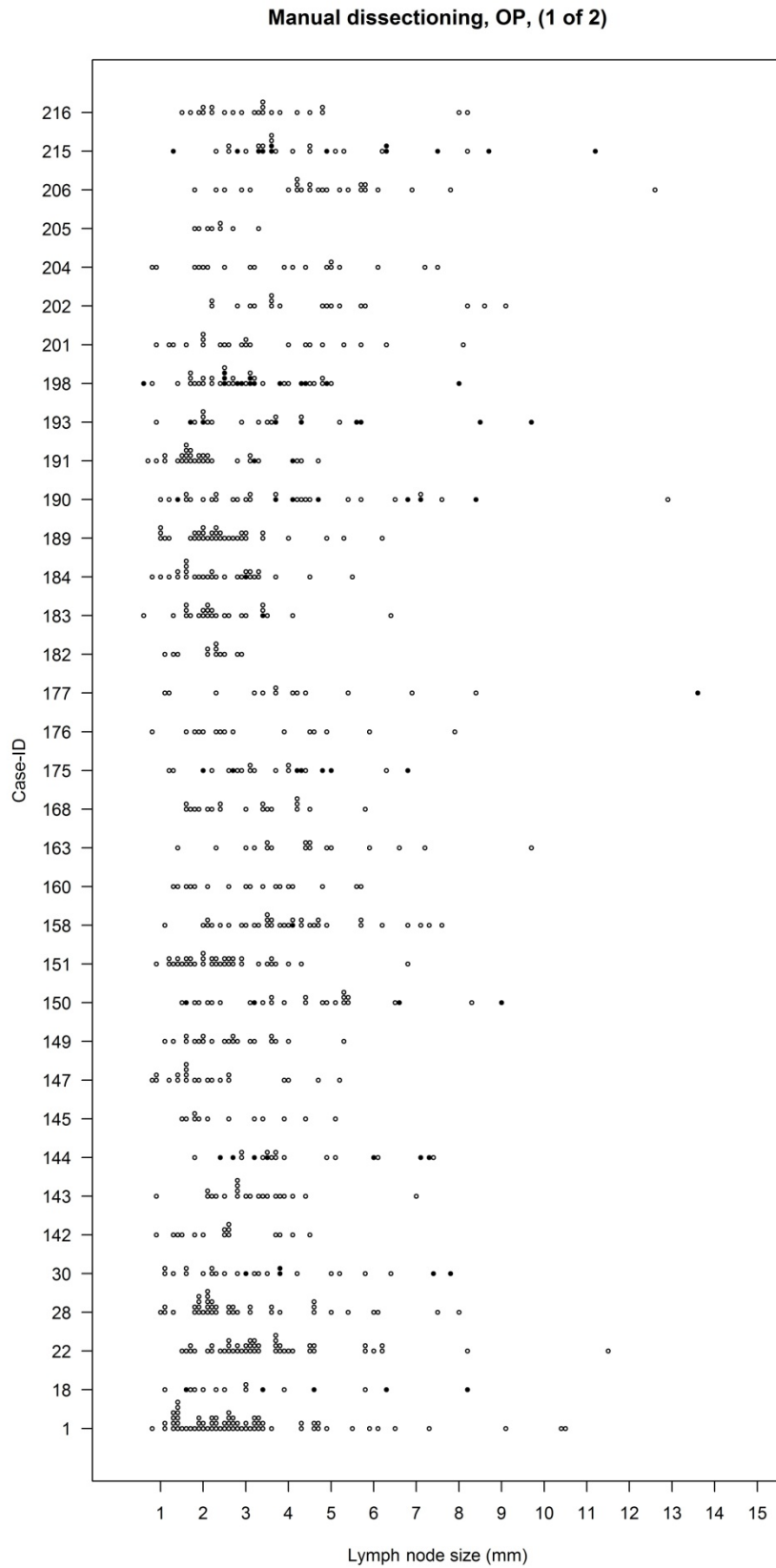




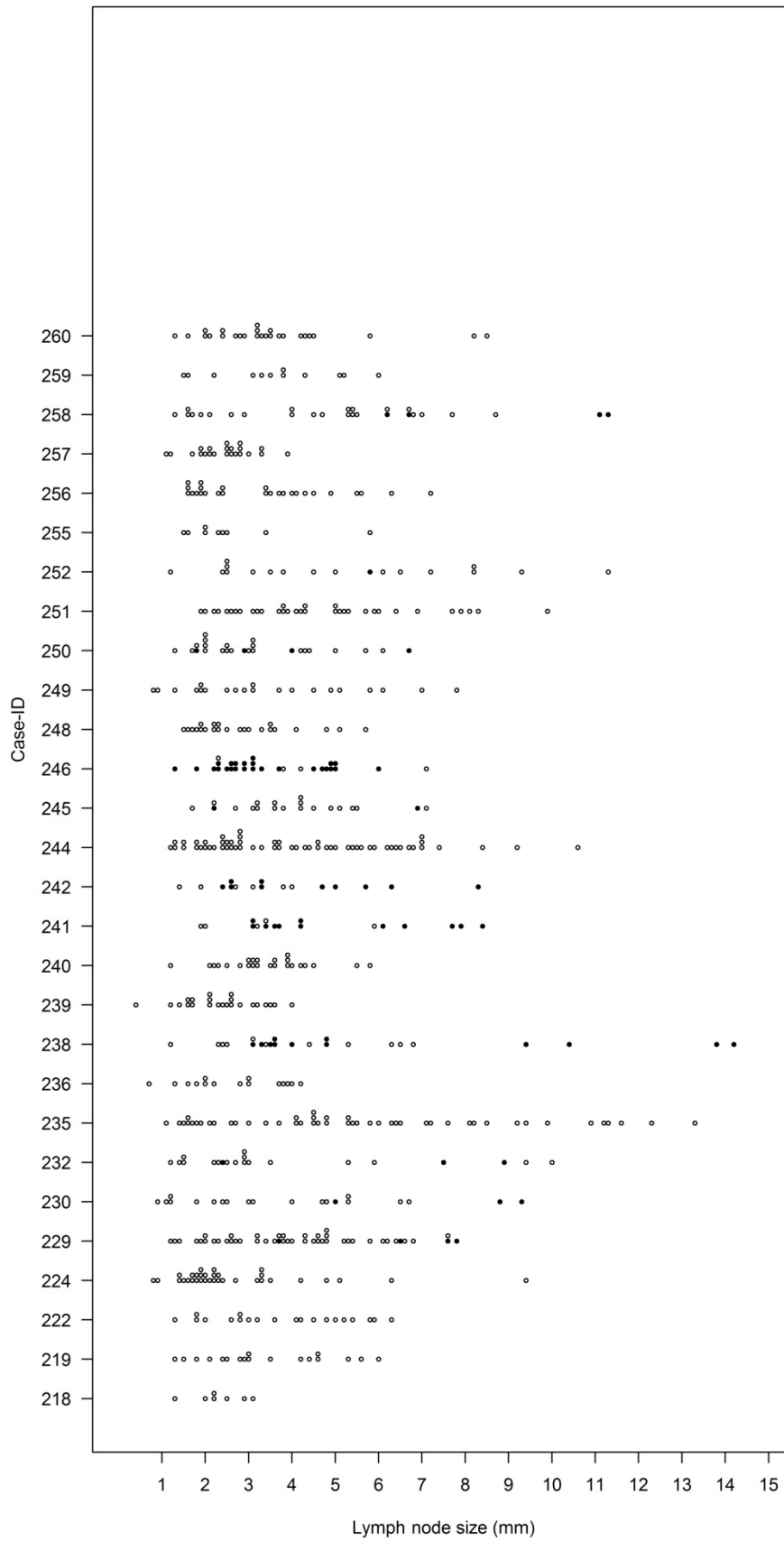
Manual dissectioning, Neo, (2 of 2)



### 8.1.4 Stripcharts for MD Group, Patients Treated with Primary Surgery (n=63)



Manual dissectioning, OP, (2 of 2)





WME, NEO, (2 of 2)

