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**ON
TREATMENT AND PROGNOSIS
IN
EPIDERMOID ANAL CANCER**

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Bedömning

Man ser inte genast skillnad
på småsten och gråsparvar
i den nysådda, svarta åkern.

Några flyger och sätter sig i nyponsörnet
– de är gråsparvar.

Andra blir kvar och trippar i åkern
– de är också gråsparvar.

Andra återigen ligger stilla kvar i åkern
– de är troligen stenar.

Anna Rydstedt
Ur "Dess kropp av verklighet", 1976

(With permission)

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ABSTRACT

Epidermoid anal cancer is an uncommon, malignant disease that is diagnosed in approximately 100 patients annually in Sweden. The incidence is increasing worldwide. Over the past 30 years, a dramatic change in the therapeutic approach has occurred leading to non-surgical treatment with radiotherapy and chemotherapy being primary options. Inter-individual variation in treatment response exists and a proportion of patients require radical surgery for cure. Treatment regimens in current use yield good survival rates, but treatment related morbidity and the need for surgery still represent a problem. Reliable prognostic markers are lacking.

The aims of this thesis are to extend present knowledge on treatment, including surgery, and to explore different potential prognostic markers for the benefit of epidermoid anal cancer patients. The basis for this thesis was a consecutive, population-based cohort of 308 anal cancer patients from Stockholm-Gotland, collected 1985-2000. Therapeutic results were reviewed and prognostic markers explored.

It is concluded that results comparable to those in clinical trials are possible to achieve in an unselected, population-based series. Adherence to pre-determined treatment protocols, centralised management of patients within a multidisciplinary team and close follow-up may be key elements in the treatment of epidermoid anal cancer patients. In locally advanced cases, treatment results appear to be superior when neoadjuvant platinum-based chemotherapy is added to radiotherapy.

Assessment of treatment response after initial radiotherapy is of great importance, as further irradiation adds morbidity, but no survival benefits in poor responders. Salvage surgery can result in >50 per cent long-time survivors, but is associated with considerable post-operative morbidity. Clinicopathological parameters should be used for prognostic information with caution; however, stage appears to be of prognostic importance.

Archived tumour material was investigated, using immunohistochemistry, with respect to potential prognostic markers. Tumour budding appears to be of prognostic importance. Furthermore, Cyclin A expression, reflecting proliferation rate, may be a marker of radiosensitivity and a valuable prognostic marker in epidermoid anal cancer. Markers related to proliferation merit further investigation.

Future challenges include definition of optimal treatment regimens with regard to locally advanced tumours, but also smaller lesions. Access to prognostic and predictive markers may lead to a more individualised therapy.

ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AIN	Anal intraepithelial neoplasia
APR	Abdominoperineal resection
ATZ	Anal transitional zone
CDK	Cyclin dependent kinases
CR	Complete response
CRT	Chemo-radiotherapy
CT	Computerised tomography
EAUS	Endoanal ultrasound
Gy	Gray (unit for radiation dose)
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
IHC	Immunohistochemistry
MRI	Magnetic resonance imaging
RT	Radiotherapy
STD	Sexually transmitted disease
TNM	Tumour Node Metastasis
UICC	International Union Against Cancer

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals as indicated below:

- I. Epidermoid anal cancer: A review of a population-based series of 308 consecutive patients treated according to prospective protocols.
Nilsson PJ, Svensson C, Goldman S, Ljungqvist O, Glimelius B.
Int J Radiat Oncol Biol Phys 2005; 61: 92-102.
- II. Salvage abdominoperineal resection in anal epidermoid cancer.
Nilsson PJ, Svensson C, Goldman S, Glimelius B.
Br J Surg 2002; 89: 1425-1429.
- III. Tumor budding detected by laminin-5 γ 2-chain immuno-histochemistry is of prognostic value in epidermoid anal cancer.
Nilsson PJ, Rubio C, Lenander C, Auer G, Glimelius B.
Ann Oncol 2005; 16: 893-898.
- IV. Prognostic significance of Cyclin A in epidermoid anal cancer.
Nilsson PJ, Lenander C, Rubio C, Auer G, Ljungqvist O, Glimelius B.
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INTRODUCTION

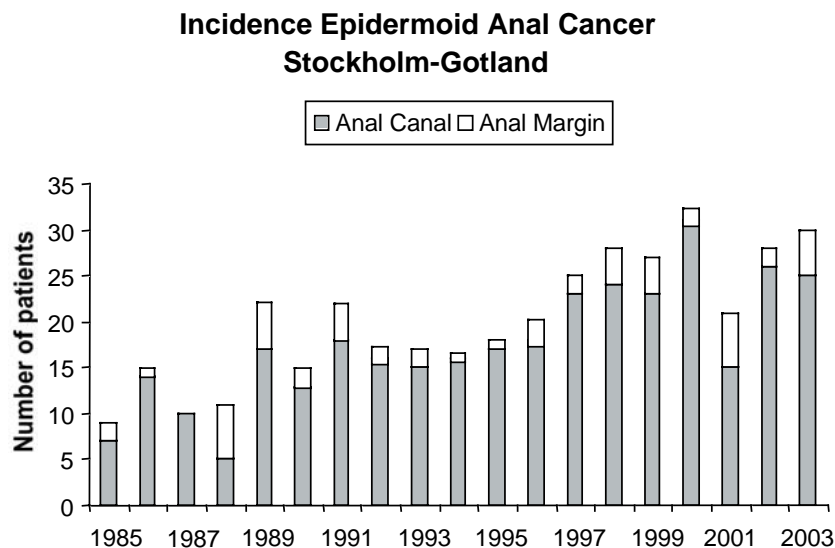
Epidermoid anal cancer is an uncommon malignant disease. In Sweden, approximately 100 new cases are diagnosed each year, and the incidence of anal cancer has continuously increased since the 1970s [1, 2]. In the Stockholm-Gotland Health Care Region (population 1.9 million) an increase in the number of patients diagnosed with anal cancer has been observed over a number of years (fig. 1). A rising incidence is also observed in other countries, e.g. USA where it is estimated that almost 4000 new patients will be diagnosed in 2005 [3, 4].

Anatomy

In 2002, Fenger made the following observation: “Few parts of the human body

have suffered such confusion with regards to definitions and terminology as the anal canal, and descriptions have appeared based on embryological, anatomical, historical, surgical, oncological and imaging studies. This has resulted in no less than four definitions of its extension, 10 different names for the line composed of the anal valves and sinuses and 14 names for the zone just above this line“ [5]. Following the anorectal nomenclature proposed by Wendell-Smith, the anal canal can be defined as the region between the two easily recognised and palpable landmarks; the intersphincteric groove and the anorectal ring [6] (fig. 2). Although a large inter-individual variation could be expected, the average length of the anal canal has been described as being 4.2 cm [7]. More recently, magnetic resonance

Figure 1



imaging (MRI) studies have shown the anal canal to be asymmetric, with longer lateral components and shorter distances in the anterior and posterior aspects [8].

The lymphatic drainage of the anal canal occurs in three general directions. The supradentate part drains primarily towards the inferior mesenteric nodes *via* the pararectal lymph nodes in the mesorectum. The infradentate part of the anal canal drains mainly to inguinal lymph nodes. Both areas may also drain through the middle or inferior rectal nodes towards the internal iliac lymph nodes. The anal margin drains exclusively to the inguinal nodes [9].

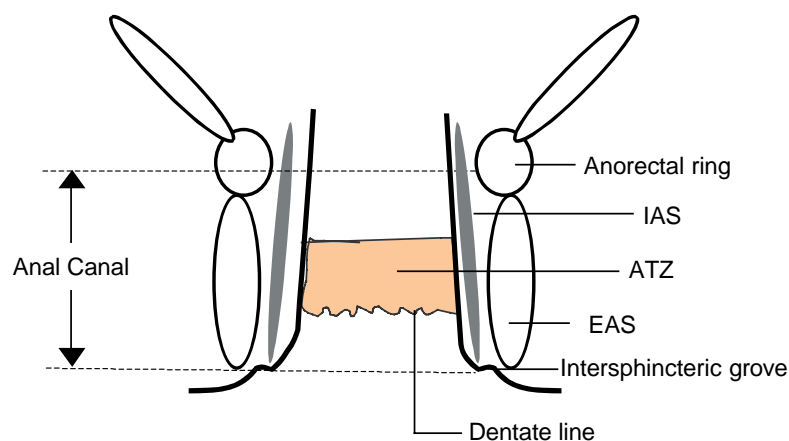
The histology of the anal canal mucosa changes as it descends. In the uppermost part of the supradentate area the mucosa is, like in the rectum, composed of columnar epithelium. The anal transitional zone (ATZ) has its extension for about 1 cm above the dentate line. The ATZ is characterised by multilayered epithelium with various cell types including columnar, cuboidal, umbrella-shaped and squamous epithelial cells. In this area endocrine cells and melanocytes can also be

found. Anal glands with an epithelial lining corresponding to that in the ATZ are present in the area. Below the dentate line the lining of non-keratinised squamous cell epithelium constitutes the anoderm. Apart from the squamous cells, melanocytes are found in the anoderm. At the anal verge, the perianal skin or anal margin begins and its extension is within a radius of 5 cm from the anus. This area is defined by the presence of skin appendages such as hair and apocrine glands [10].

Aetiology

As for the majority of malignant diseases, the exact pathogenetic mechanisms leading to an anal carcinoma are not known. Risk factors for developing an anal carcinoma that have been identified include lifetime number of sexual partners [11, 12], receptive anal intercourse [11-13] and smoking [13, 14]. There is also evidence suggesting that recipients of a solid organ transplant suffer an increased risk [15, 16]. In individuals with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), the incidence rate of anal cancer

Figure 2.



Schematic overview of anatomy of the anal canal.

IAS: internal anal sphincter. EAS: external anal sphincter.

ATZ: anal transitional zone. The dentate line divides the anal canal into a supradentate and an infradentate part.

is increased [17, 18]. In a Danish study the relative risk of epidermoid anal carcinoma was 31.2 in homosexual men compared to the general population and among the anal cancer patients 3 out of 4 were HIV-positive, whereas HIV-status was uncertain in the fourth [19]. Highly active antiretroviral therapy (HAART) is associated with beneficial effects on morbidity and mortality in HIV-positive individuals. HAART leads to increased CD4 lymphocyte counts, reflecting decreased immunosuppression. Whether the introduction of HAART will have an impact on the incidence rate of epidermoid anal cancer in HIV-positive patients remains unclear [20-22].

Human Papilloma Virus

Human papilloma virus (HPV) has been of particular interest in studies on the aetiology of epidermoid anal cancer. HPV is a small double stranded DNA virus for which humans are the only known reservoir. About 100 different strains of HPV have been identified. HPV is the most common sexually transmitted disease (STD) and has been linked to cervical, vulvar and vaginal cancer [23]. In 1979, Cooper *et al.* suggested anal cancer to be a STD after an observation in four homosexual men [24]. Later, the presence of HPV in both anal intraepithelial neoplasia (AIN) and in anal carcinomas has been demonstrated using various techniques [25-27]. Certain HPV subtypes, e.g. HPV 16, 18 and 33, have been associated with a high risk of neoplastic transformation. In case-control studies using highly sensitive polymerase chain reaction high-risk HPV was detected in 89-100 per cent of patients but not in control specimens of normal anal epithelium or rectal carcinoma [12, 28].

The HPV viral genome is divided into an early (E) and a late (L) region. The two proteins E6 and E7 are encoded in the early region and it is through the actions of these two proteins that oncogenesis is believed to occur. The E7 protein binds to retinoblastoma tumour suppressor gene products resulting in a series of events compromising cell cycle control.

The E6 protein binds to p53, which leads to degradation of p53. An important action of the P53 gene is to “seek and destroy” cells with genetic errors by causing cell cycle arrest, a phase either allowing for DNA repair or leading to apoptosis. The expression of E6 and E7 in proliferating cells also disturbs chromosome duplication and segregation during mitosis, whereby severe chromosomal instability is induced. Thus, in the HPV infected cell, with impaired cell cycle control and diminished genome control, genetic errors may accumulate eventually leading to immortalisation and malignant transformation. This model, with a gradual transformation, is consistent with the clinical picture of longstanding HPV infection preceding malignancy [29, 30].

Clinical Presentation

Symptoms at presentation may include bleeding, pain, presence of a mass, pruritus and anal discharge [31]. In addition, change in stool calibre, constipation and faecal incontinence may be present. Patient delay is common and a substantial number of patients suffer symptoms for more than 24 months prior to diagnosis [32]. In different patient series the rate of advanced tumours (T3-4) at presentation ranges between 40 and 45 per cent [33-35].

Lymph node metastases are a common feature, reported in 13-21 per cent [33-36]. Moreover, in patients presenting with T3-4 lesions lymph node metastases are seen in 25-30 per cent of patients at presentation [33, 35]. Distant metastases at the time of diagnosis are infrequent [36], but when they occur lung and liver are the predominating sites [37].

The work-up of suspicious lesions must include rectal examination and biopsy. Tumour extent is assessed using digital examination and rigid proctoscopy. Examination under anaesthesia should be used liberally. Endoanal ultrasonography (EAUS) for staging of anal carcinomas was introduced in the 1980s [38].

EAUS carries the advantage of not only evaluating the tumour, but also the presence of pararectal nodes and can be combined with ultrasound guided fine-needle aspiration [39, 40]. However, recently the value of EAUS in follow-up of anal cancer has been questioned [41]. Although the value for local staging of computerised tomography (CT) and MRI has been studied in rectal adenocarcinoma, the literature regarding epidermoid anal cancer is limited. Imaging studies regarding detection of pelvic lymph nodes has also been studied in cervical cancer, and appears to be equally efficient with CT and MRI, however, strict criteria for discriminating between positive and negative nodes are lacking [42]. In a study including 11 patients with recurrent anal cancer, MRI accurately assessed tumour extent prior to salvage surgery [43]. Various imaging modalities, including MRI, CT, ultrasonography and plain chest X-ray, are available for detection of distant metastases.

Staging

The World Health Organization Classification of Tumours includes precancerous lesions of the anus and the term AIN is preferred [44]. However, reproducibility studies have shown

considerable interobserver variation in the three-graded AIN scale [45]. The term anal squamous intraepithelial lesion (ASIL) with only a two-graded scale (low grade – LSIL and high-grade – HSIL) has been proposed. Squamous cell dysplasia of the anal margin is often referred to as Bowen disease.

The preferred term for epidermoid anal cancer is squamous cell carcinoma of the anal canal and anal margin. Previously, anal squamous cell carcinoma was subgrouped into large-cell keratinising, large-cell non-keratinising and basaloid (cloacogenic) carcinomas. Since reproducibility is poor [46], it is now recommended that subgrouping should be abandoned with the exception of two rare subtypes (squamous cell carcinoma with mucinous microcysts and small cell anaplastic carcinoma) [44].

Epidermoid anal cancer staging is by the International Union Against Cancer (UICC) Tumour, Node, Metastasis (TNM) system [47]. There are slight differences in the staging of tumours of the anal canal and the anal margin (table 1).

Table 1.
TNM Classification of epidermoid anal cancer according to UICC.

	Anal canal	Anal margin
Primary tumour (T)		
T 1	≤ 2 cm	≤ 2 cm
T 2	> 2 cm ≤ 5 cm	> 2 cm ≤ 5 cm
T 3	> 5 cm	> 5 cm
T 4	Tumour invades adjacent organ(s)	Tumour invades deep extradermal structures
Lymph node metastases (N)		
N 0	No lymph node metastasis	No lymph node metastasis
N 1	Perirectal	Regional lymph node metastasis
N 2	Unilateral internal iliac or inguinal	-
N 3	Perirectal and inguinal or bilateral iliac and/or inguinal	-
Distant metastases (M)		
M 0	No distant metastasis	No distant metastasis
M 1	Distant metastasis	Distant metastasis
Stage Grouping		
Stage I	T1 N0 M0	T1 N0 M0
Stage II	T2-3 N0 M0	T2-3 N0 M0
Stage III		T1-4 N1 M0
A	T1-3 N1 M0 T4 N0 M0	T4 N0 M0 -
B	T1-4 N2-3 M0 T4 N1 M0	-
Stage IV	T1-4 N0-3 M1	T1-4 N0-1 M1

UICC: International Union Against Cancer.

BACKGROUND

Treatment

Combined modality therapy

Historically epidermoid anal cancer was treated surgically. Treatment results following abdominoperineal resection (APR) were unimpressive, with 5-year survival rates of 40-70 per cent [48, 49]. However, with the reports on combined chemo-radiotherapy (CRT) in the 1970s, a change of paradigm came about. Nigro and colleagues first reported on three patients treated with 5-fluorouracil/mitomycin C and radiotherapy (RT) in 1974 [50]. Following CRT, two patients underwent APR with no tumour in the resected specimen and the third patient refused surgery, but showed no signs of recurrence after one year. Subsequently, data on a greatly extended number of patients were reported, leading to APR as primary therapy being abandoned, reserving it for residual or recurrent disease [51].

Following the wide acceptance of the combined modality approach outlined by Nigro *et al.*, several non-randomised studies of treatment results have reported 5-year survival rates of 50-76 per cent using various protocols regarding RT and chemotherapy [33, 35, 52-55]. Although no studies have compared surgical therapy to non-surgical therapy in a randomised manner, Goldman *et al.* studied two population-based cohorts with different therapeutic approaches, treated between 1978 and 1984, including both patients treated with primary surgery and patients treated primarily with CRT [56]. It was found that patients treated with non-surgical primary therapy had a favourable outcome compared to patients treated with primary surgery, irrespective of T-stage.

Randomised trials

During the 1990s, three randomised studies were published [57-59]. The United Kingdom

Coordinating Committee on Cancer Research (UKCCCR) and the European Organisation for Research and Treatment of Cancer (EORTC) studies were designed to compare CRT with RT alone. In the UKCCCR trial 585 patients were randomised to either RT alone (45 Gy) or 5-fluorouracil/mitomycin C and RT (45 Gy). Tumour response was assessed 6 weeks after completion of initial RT. Good responders, i.e. ≥ 50 per cent reduction of tumour size or complete response (CR), were given boost RT (15 Gy), whereas poor responders were considered for radical surgery. After a median follow-up of 42 months, the local failure rate was significantly reduced in the CRT arm (36 *vs.* 59 per cent). No statistically significant difference in overall survival at 3 years was found, however, the cause-specific survival was significantly improved in the CRT arm (72 *vs.* 61 per cent). Early treatment morbidity was significantly increased in the CRT arm (including 6 deaths), whereas no statistically significant difference in late treatment related morbidity was found [57]. The EORTC trial, which included 110 patients, had a similar design. CR was obtained to a significantly larger extent in the CRT arm compared to the RT alone arm (80 *vs.* 54 per cent). The difference in CR rate remained visible throughout the follow-up (median 42 months), resulting in a significantly improved local control rate in the CRT arm. Also, in the CRT arm, colostomy free survival was significantly improved (72 *vs.* 40 per cent). No statistically significant differences were seen in toxicity [58].

Another issue was addressed in the Radiation Therapy Oncology Group (RTOG) trial. To evaluate the role of mitomycin C, a total of 310 patients were randomised to either 5-fluorouracil and RT (45 Gy) or 5-fluorouracil/mitomycin C and RT (45 Gy). After completion of initial RT treatment, response was assessed with full-thickness

biopsies and patients with positive biopsies received boost RT (9 Gy) and additional chemotherapy. Patients with residual tumour following boost therapy were considered for APR. A non-significant difference in response rate after initial RT, in favour of patients receiving mitomycin C, was seen (92 *vs.* 86 per cent). Disease free and colostomy free survival were significantly improved in the mitomycin C arm (73 *vs.* 51 per cent and 71 *vs.* 59 per cent, respectively). Early treatment toxicity (including two deaths) was significantly increased in the mitomycin C arm (23 *vs.* 7 per cent) [59].

Although no overall survival gains were reported in these randomised trials, CRT has been established as standard of care in epidermoid anal cancer, given the advantages in local control, colostomy free survival and tumour specific survival. On the other hand, in smaller (T1-2) tumours excellent results with 5-year survival rates exceeding 90 per cent have been reported from non-randomised series using RT alone [33, 60, 61].

Cisplatin

Early treatment toxicity among patients in a mitomycin C arm of the randomised studies was increased and attempts have been made to replace mitomycin C with another agent. The role of cisplatin in the treatment of anal cancer, given concomitantly or as neoadjuvant therapy, has been investigated in several studies [62-65]. In a French study 80 patients with locally advanced (T>40 mm and/or N+) anal cancers received two cycles of neoadjuvant 5-fluorouracil/cisplatin and two cycles concomitantly with RT (45 Gy) [64]. After a 4-8 week period, good responders received additional RT (15-20 Gy). A CR rate of 94 per cent (including APR in four patients) and an overall 3-year survival of 86 per cent were achieved. Only three patients experienced in-field treatment toxicity and no treatment related mortality was reported. In addition, a retrospective analysis of 92 patients treated with RT (55 Gy) and concomitant 5-fluorouracil/cisplatin reported an overall

5-year survival of 85 per cent and a local control rate of 83 per cent [65]. No fatal acute toxicities were reported.

Ongoing trials

Currently, a randomised study aiming at 600 patients is underway in the UK [66]. The role of cisplatin, and the role of adjuvant chemotherapy after CRT are being investigated. Patients are randomised to one of four treatment arms: 5-fluorouracil/mitomycin and RT or 5-fluorouracil/cisplatin and RT, with or without adjuvant 5-fluorouracil/cisplatin. Estimated date for closure is July 2006. Another randomised trial, where one arm with 5-fluorouracil/mitomycin C and RT is compared with an arm consisting of 5-fluorouracil/cisplatin and RT, was recently closed as the study had met its patient accrual target [67].

Thus, although the therapeutic approach has dramatically changed over the past 30 years, with improved results in terms of survival and preservation of intestinal continuity, the optimal treatment regimens for locally advanced anal carcinomas as well as smaller lesions remain to be elucidated. In Europe, split course high-dose RT with (neo)adjuvant chemotherapy is drawing attention while North American authors often advocate lower RT doses delivered without a treatment gap, and with concomitant chemotherapy. In some centres, brachytherapy is preferred to external beam RT for the boost [68, 69], but there are no randomised comparisons regarding efficacy or toxicity.

Special patient groups

With respect to treatment of epidermoid anal cancer, certain patient groups require special attention. Patients who have previously been irradiated in the pelvic area may not tolerate additional RT and should thus be considered for primary surgery. Also, in elderly and frail patients dose and prescription adjustments of the CRT regimen may be necessary [70, 71]. In HIV-positive patients, the clinical impression of impaired treatment tolerance and decreased

treatment response is supported by the literature [72, 73], although no differences were found in one smaller study [74]. Low CD4 lymphocyte counts (<200 cells/ μ l) appear to affect treatment tolerance negatively [72, 75]. One group of patients in whom a therapeutic approach other than combined modality therapy should be considered are those with small lesions (T1N0 tumours \leq 1 cm) of the anal margin [76]. Local excision in selected cases can produce excellent results [49, 77].

Salvage APR

Despite progress in non-surgical therapy for epidermoid anal cancer, APR remains an important tool in the therapeutic arsenal. In different series, 16-27 per cent of patients undergo APR at some point during their course of treatment [35, 54, 64]. In a recent report including 254 anal cancer patients, all treated with curative intent, the rate of APR was 30 per cent [78]. Several reports on results after APR following failed CRT are available [78-85] (table 2). Heterogeneity in preoperative treatment, patient material

and follow-up render comparison of results difficult. However, some general conclusions can be drawn; long-term survival rates of 40-60 per cent are achievable, but post-operative complication rates of 50-70 per cent should be expected. In particular, perineal wound break-down and protracted healing times are associated with the procedure. To alleviate these problems, reconstructive measures using omentoplasty and musculocutaneous flaps have been proposed [82, 83, 85-89]. For instance, in a Danish study, including 14 patients with APR following CRT or RT alone for anal cancer, a 100 per cent primary healing rate following primary rectus abdominis musculocutaneous flap repair was reported [87].

Prognosis

Prior to the era of combined modality therapy, studies on prognostic factors in epidermoid anal cancer treated by primary surgery were undertaken. For example, Boman *et al.* found in a series of 118 anal cancer patients treated primarily with APR a significant relationship between depth of tumour invasion and local

Table 2.
Published series of APR following failed CRT in epidermoid anal cancer.

Author	Year	RT dose	N:o of APR	Follow-up (months)	5-year survival
Ellenhorn [79]	1994	30-60 Gy	38	47	44 %
Pocard [80]	1998	40-65 Gy	21	40	33 %
Allal [81]	1999	-	23	22	45 %
Smith [82]	2001	>45 Gy	22	30	23 %
van der Wal [83]	2001	34-60 Gy	17	53	47 %
Akbari [84]	2004	30-61 Gy	57	24	33 %
Ghouthi [85]	2005	-	36	67	69 %
Renehan [78]	2005	35-60	73	45	52 %

APR: Abdominoperineal resection. CRT: Chemo-radiotherapy.

recurrence, and survival [49]. Prognostic information could also be gained from nodal status. These findings were consistent with those of other authors [90].

With the introduction of combined modality therapy, prognosis is largely dependent on the sensitivity of the tumour *visavis* RT and chemotherapy. Researchers have investigated epidermoid anal cancer tumours with respect to these issues, but the prognostic impact of clinical and pathological parameters have also continued to draw attention.

Clinicopathological parameters

Stage

T-stage, or tumour size, has repeatedly been shown to carry prognostic information. In a study of 270 patients with anal cancer, treated predominantly with RT alone, tumour size showed a statistically significant relationship with survival [33]. Determinate 5-year survival among patients with tumours ≤ 4 cm was approximately 90 per cent compared with 60 per cent for patients with tumours ≥ 5 cm. Moreover, T stage also had an impact on local control rates. Peiffert *et al.* reported results consistent with these in a series of 118 patients [91]. In neither of these two reports did age, gender or histologic type have an impact on prognosis. More recently, Myerson *et al.* reported tumour extent (T1/T2N0 *vs.* T3N0 *vs.* T4 or N+) to be the only independent prognostic parameter in a multivariate analyses on local control and freedom from relapse in a study of 88 patients [55], and Deniaud-Alexandre *et al.* reported T-stage to be of prognostic value for disease free survival in a univariate analysis including 305 patients [35].

Conflicting data regarding the importance of nodal status have been reported. In the randomised EORTC trial, the survival and local control rates were both significantly lower in node positive patients [58]. In the two aforementioned French studies, one reported nodal involvement to be a significant prognostic parameter in a multivariate analysis on tumour specific survival [91],

whereas the other reported nodal status to be non-significant [33]. Allal *et al.* found that the presence of positive lymph nodes was associated with a significant increase in local failure (36 *vs.* 19 per cent) but in a multivariate analysis, where therapeutic approaches were taken into account, no significant relationship could be demonstrated regarding any of the investigated clinicopathological parameters [92].

Stage, in particular T-stage, has thus been shown to be of prognostic importance. This appears to be valid also if staging is performed using EAUS [93, 94]. In a French multicenter study of 146 epidermoid anal cancer patients, the prognostic impact of EAUS was superior to clinical UICC staging on both survival and local control [94].

Histopathology

The reproducibility of histological subtyping of epidermoid anal cancer (i.e. non-keratinising squamous, basaloid *et cetera*) is poor [46]. Hence, studies on potential prognostic impact of histologic type should be interpreted with great caution. A few studies have investigated the prognostic importance of differentiation reaching conflicting results [95, 96]. One should bear in mind that strict criteria for grading, and reproducibility studies are lacking.

Treatment time

The prognostic impact of overall treatment time was investigated in a retrospective study of 90 anal cancer patients [97]. Patients were treated with CRT consisting of split course RT to a dose of 60 Gy and concomitant 5-fluorouracil/mitomycin C. The duration of the gap (above or below median) between the two RT courses had a statistically significant impact on locoregional control. In patients with a shorter gap, locoregional control was 85 per cent compared to 61 per cent in patients with a long gap. Gap duration was an independent prognostic variable in a multivariate analysis. Also in a larger patient series, shorter overall treatment time was significantly associated

with an increased CR rate (84 *vs.* 74 per cent) in the univariate, but not in the multivariate analysis [35]. A non-randomised study of 43 anal cancer patients receiving CRT with a planned gap of only two weeks reported a CR rate of 91 per cent with acceptable toxicity [98].

Biochemical markers

Research *in vitro* has shown that the radiosensitivity of human squamous cell carcinoma cells is largely dependent on inherent properties of the cell [99]. Should assessment of reliable markers reflecting these properties be possible, the prognostication of response to a CRT regimen would be greatly facilitated. With this in mind, a number of potentially prognostic biochemical markers have been investigated in conjunction with epidermoid anal cancer [5].

Aneuploidy

Aneuploidy represents gross losses or gains in chromosomal content and is a common feature in human carcinomas [100]. Recent evidence suggests aneuploidy to be a cause rather than a consequence of malignant transformation. The prognostic influence of aneuploidy in epidermoid anal cancer has been investigated in two reports using different techniques, reaching contradicting results [95, 96].

P53

The tumour suppressor gene P53 has been referred to as the “guardian of the genome” [101]. P53 is regarded as one of the most important tumour suppressor genes with a number of key functions such as cell cycle control, DNA damage repair and initiation of apoptosis. The P53 gene is activated by DNA damage, for instance from ionising radiation, leading to increased expression of the p53 protein. This protein binds to particular DNA sequences leading to activation of several processes. The most well understood processes are cell cycle inhibition and initiation of apoptosis. Activated p53 stimulates expression of p21, which interacts with cell cycle control mechanisms leading to

cell cycle arrest. Several mediators activated by p53 may induce apoptosis. In addition, activated p53 may influence genetic stability *via* DNA repair gene systems and inhibit angiogenesis [102]. Mutations of the P53 gene are frequently seen in human cancers. Non-mutated p53 with normal functional capability is commonly referred to as “wild-type” p53. Most mutations, but not all, lead to loss of normal function. The loss of P53 tumour suppressor activity is believed to play a major role in tumour biology [103].

Most researchers believe P53 status to have an influence on the radiosensitivity of tumours; however, the mechanisms are not fully elucidated [104]. The hypothesis that P53 mutation induces radioresistance by reducing tumour cell apoptosis after DNA damage by radiotherapy is, however, commonly accepted. The method by which p53 mutational status was studied is of importance when evaluating results from clinical studies on the possible impact of p53 expression on prognosis. In the absence of DNA damage, wild-type p53 basal expression is low. Mutated p53 on the other hand, generally, has an extended half-life. Thus, by using immunohistochemistry (IHC), increased expression of p53 will occur with mutated p53. There are several sources for erroneous interpretation such as, for example, some mutations do not increase p53 protein half-life, in some instances wild-type p53 is stabilised without a mutation, and a negative stain may mask other issues (e.g. p53 degradation by HPV E6 protein). It has been proposed that IHC should be combined with DNA sequencing for confirmation of mutational status [104].

In epidermoid anal cancer, the prognostic impact of p53 has been investigated in a number of studies using IHC [105-109]. A p53 expression rate between 34 and 78 per cent was reported in these studies. Only two studies found a prognostic impact of p53, showing a significant relationship between poor disease free survival and p53 over-expression [107, 109].

Cell proliferation

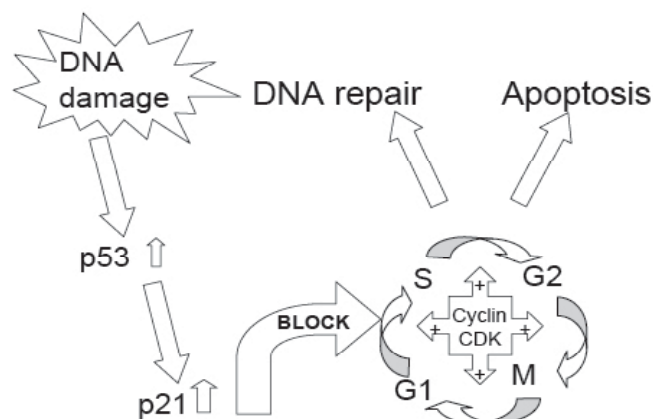
Proliferating cells are propagating through the cell cycle, which is sequentially regulated by a complex cell cycle control system [110]. The progress through the cell cycle is mainly driven by members of the protein family Cyclins, interacting with various cyclin dependent kinases (CDK). Among the Cyclins, Cyclin A may be of particular interest as it is involved in two steps of the cell cycle, both in the entry of G_1 cells into S phase and in the G_2 -M transition [111, 112]. Cyclin A has also been suggested to reflect proliferation superior to other markers, at least in colorectal adenocarcinomas [113]. CDK inhibitors negatively regulate the cell cycle [114]. p21 is an important CDK inhibitor that is a downstream effector following DNA damage induced p53 activation. p21 induced inhibition generally leads to a cell cycle arrest at the G_1 -S transition point and it appears to be the only inhibitor capable of interacting with all Cyclin-CDK complexes [115]. However, p21 can also be induced in a p53 independent manner and may also be involved in additional intracellular events. Figure 3 shows a greatly simplified model of the interaction between p53, p21 and Cyclin-CDK complexes in normal cells.

The relationship between cell cycle control and radiosensitivity may involve several

mechanisms. Cell cycle arrest points and DNA repair are most likely involved as is other factors [116]. For instance, from studies dating back to the 1960s, it is well known that differential survival of irradiated cells is dependent of phase in the cell cycle [117]. Cells are most sensitive to ionising radiation in G_2 and mitosis, less sensitive in G_1 and least during S-phase. In addition, evidence suggests an inverse relationship between proliferation and hypoxia [118, 119]. Tissue hypoxia, being a well-known factor inducing radioresistance [120], could thus be indicated by a low proliferation index.

Ki-67 (MIB-1) is a protein expressed throughout the cell cycle and is commonly used as a marker of proliferation in IHC studies. In anal cancer, only one small study including 29 patients reported prognostic impact of Ki-67 [34], whereas other studies failed to show a statistically significant relationship [121-123]. However, in the study by Allal *et al.*, there was a trend toward improved survival in the group with a high proliferation index [121]. Also using IHC, Allal *et al.* investigated the prognostic role of Cyclin D1 and Cyclin E in 98 anal carcinomas but found no correlation with local control or disease free survival [123]. In a study on 26 patients, a completely different method

Figure 3.



Simplified model of interaction between p53, p21 and Cyclin-CDK complexes.

of assessing proliferation (labelling with bromodeoxyuridine and using flow cytometry) was used, but no reference to prognostic impact was made [124]. Studies on the prognostic potential of Cyclin A in epidermoid anal cancer have not previously been reported.

In an IHC study of 94 anal cancer patients, Holm *et al.* found abrogated expression of the cell cycle inhibitor p21 to be significantly associated with overall survival [125]. However, when using a 5 per cent expression rate as cut off, no statistically significant relationship was found. Another study on p21 reported no association with local control or disease free survival [123].

The immediate interpretation of these results regarding prognostic impact of p53, p21 and Ki-67 is not obvious. Indications of improved survival with tumours high in proliferative activity can be gathered from some Ki-67 studies [34, 121]. On the other hand, the suggestion that reduced expression of the cell cycle inhibitor p21 correlates with poor prognosis [125] is contradictory to the Ki-67 results. An increased p53 expression, supposedly reflecting mutated non-functional p53, being associated with poor prognosis would be in agreement with the p21 results reported by Holm *et al.* [125]. It could be concluded that the full spectrum of intracellular actions by p53 and p21, and the implications of their action in connection with chemotherapy and RT are not fully understood. In addition, the number of studies from which conclusions can be drawn is limited. In squamous cell head and neck cancers treated primarily with radiotherapy, a number of studies have indicated overexpression of p53 and low proliferation rate to be associated with radioresistance [126].

Invasion

The capability of tumours to invade adjacent structures is one of the hallmarks of cancer [127]. Factors involving cell-cell adhesion (e.g. cadherins), cell-matrix linkage (e.g. integrins) and matrix-degrading proteases (which may

be produced by cancer cells or surrounding stromal cells) are believed to play key roles. Laminins are a family of matrix proteins, mainly localised in the basal lamina. Laminins interact with other matrix molecules and are involved in cell adhesion and migration [128]. Laminin-5 is structurally composed of three subunits (α -, β - and γ -chain), and it has been suggested that specific cleavage, by a protease in the extracellular matrix, of the γ 2-chain from the iso-form laminin-5 stimulates cell migration and invasion [129]. Expression of the γ 2-chain has been found preferentially in the cytoplasm of tumour cells adjacent to the invading front of epithelial tumours [130, 131]. In addition, there are several reports describing laminin-5 γ 2-chain expression within the cytoplasm of budding tumour cells [130, 132-134]. Tumour budding, i.e. single cancer cells or clusters of up to five cancer cells just ahead of the invasive front of a carcinoma, is a representation of the dissociation of the invasive front and is related to the invasive capability of a tumour [135].

In anal cancer, only a few studies have investigated factors related to invasion and the possible prognostic significance. In a study of 235 anal cancers treated with primary surgery, morphological evaluation of the invasive margin revealed that “infiltrating” growth pattern was a marker of poor prognosis in a univariate analysis, but not in a multivariate analysis [95]. The expression of pan-cadherin was investigated in 22 epidermoid anal carcinomas, and expression was decreased in 70 per cent of anal canal tumours, but not in any of the five tumours of the anal margin [136]. No reference to prognosis was made. Laminin-5 γ 2-chain expression, or tumour budding, has not been investigated in epidermoid anal cancer previously.

Angiogenesis

A functional tumour vasculature is essential for tumour growth and requires angiogenesis [137]. Angiogenesis may be linked to radiosensitivity by tumour hypoxia, but there is also evidence that new antiangiogenic

agents may potentiate the responsiveness of tumours to RT [138]. Counting microvessel density, angiogenesis in tumour tissue can be assessed using IHC with various antibodies (e.g. factor VIII, VEGF, CD34) [139]. CD31, a platelet endothelial adhesion molecule, is a marker of angiogenesis in different tumours including squamous cell carcinomas. Studies on angiogenesis in epidermoid anal cancer are few, however, one investigation on 24 patients found a correlation between microvessel density and depth of invasion using IHC for CD31 [140].

Prognostic factors and surgery

In a study where data from the UKCCCR trial [57] were re-analysed, it was found that among 133 patients treated for local failures following CRT or RT alone with APR, 44 per cent developed further pelvic disease and 50 per cent died of anal cancer [141]. Histopathological data was available in 105 patients and showed depth of invasion to be of prognostic importance. When the tumour was confined to the sphincter muscles or the muscularis propria, tumour specific survival and local control rates were much better compared to when tumours invaded perirectal fat, or had invaded adjacent organs (71 *vs.* 31 *vs.* 25 per cent and 16 *vs.* 52 *vs.* 75 per cent, respectively). In addition, the importance of a clear lateral margin was studied. When the lateral margin was ≤ 1 mm, tumour specific survival was 25 per cent and local recurrence after APR occurred in 60 per cent, whereas

a clear lateral margin (>1 mm) resulted in a 67 per cent survival and a 25 per cent local recurrence rate. However, in this study, patients who underwent APR following poor response to the first RT course were evaluated together with patients on whom APR was performed due to residual or recurrent tumour after full dose RT [141].

After a CRT or RT alone regimen, the clinical differentiation between treatment induced changes and residual tumour may be very difficult. Radiation induced scarring and fibrosis is generally present and suspicious lesions should be verified with a biopsy. To distinguish between residual (or persistent) tumour and recurrences, many authors have introduced a cut off at six months after termination of RT [79, 82- 84]. Several authors have found patients with residual disease having a worse prognosis than those with a recurrence [78, 82, 84, 85], but the opposite result was reported in one study [80] and no difference was seen between groups in the study by van der Wal *et al.* [83].

In a recent report on 254 anal cancer patients treated with CRT or RT alone, 73 patients underwent APR due to local disease failure (63 patients following boost RT to 50-55 Gy). Extensive analysis of factors influencing outcome of surgery revealed only persistent tumour, and positive resection margin to be statistically significant [78].

AIMS

The overall aims were to extend present knowledge on treatment, including surgery, and to explore different potential prognostic markers for the benefit of epidermoid anal cancer patients with regard to survival, preservation of intestinal continuity and treatment related morbidity.

Specific aims

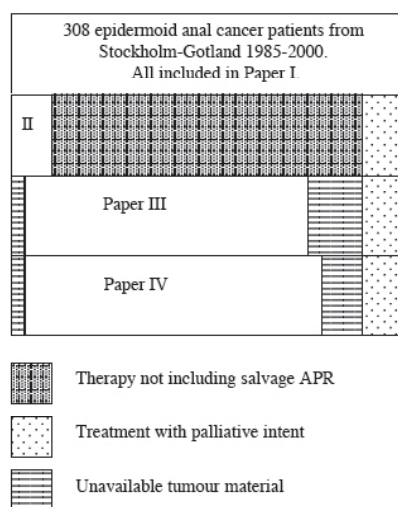
- ⊙ To review implementation of guidelines and overall results in a population-based cohort of epidermoid anal cancer patients.
- ⊙ To assess the prognostic impact of clinicopathological variables in epidermoid anal cancer.
- ⊙ To review the results of salvage APR with regard to surgical and oncological endpoints.
- ⊙ To assess the prognostic impact of clinicopathological variables following salvage APR.
- ⊙ To explore the prognostic and predictive potential of tumour budding in epidermoid anal cancer.
- ⊙ To explore the prognostic and predictive potential of p53, p21, Cyclin A and CD31 in epidermoid anal cancer.

METHODOLOGY

A consecutive, population-based cohort of 308 epidermoid anal cancer patients from the Stockholm-Gotland Health Care Region, diagnosed during the years 1985-2000, constituted the basis for this thesis. The patients were prospectively registered in a database. The database has continuously been crosschecked with the Cancer Registry for inclusion, and with the Population Registry for deaths. Parameters of interest for the studies lacking in the database were retrospectively gathered from patient records. All studies were approved by the Regional Ethics Committee.

All 308 patients were included in paper I. In paper II, 35 patients undergoing salvage APR were included. In papers III and IV, the available number of pre-treatment biopsies decided numbers to include. In paper III tumour material from 209 patients were possible to evaluate and in paper IV, 215 patients (fig. 4).

Figure 4.



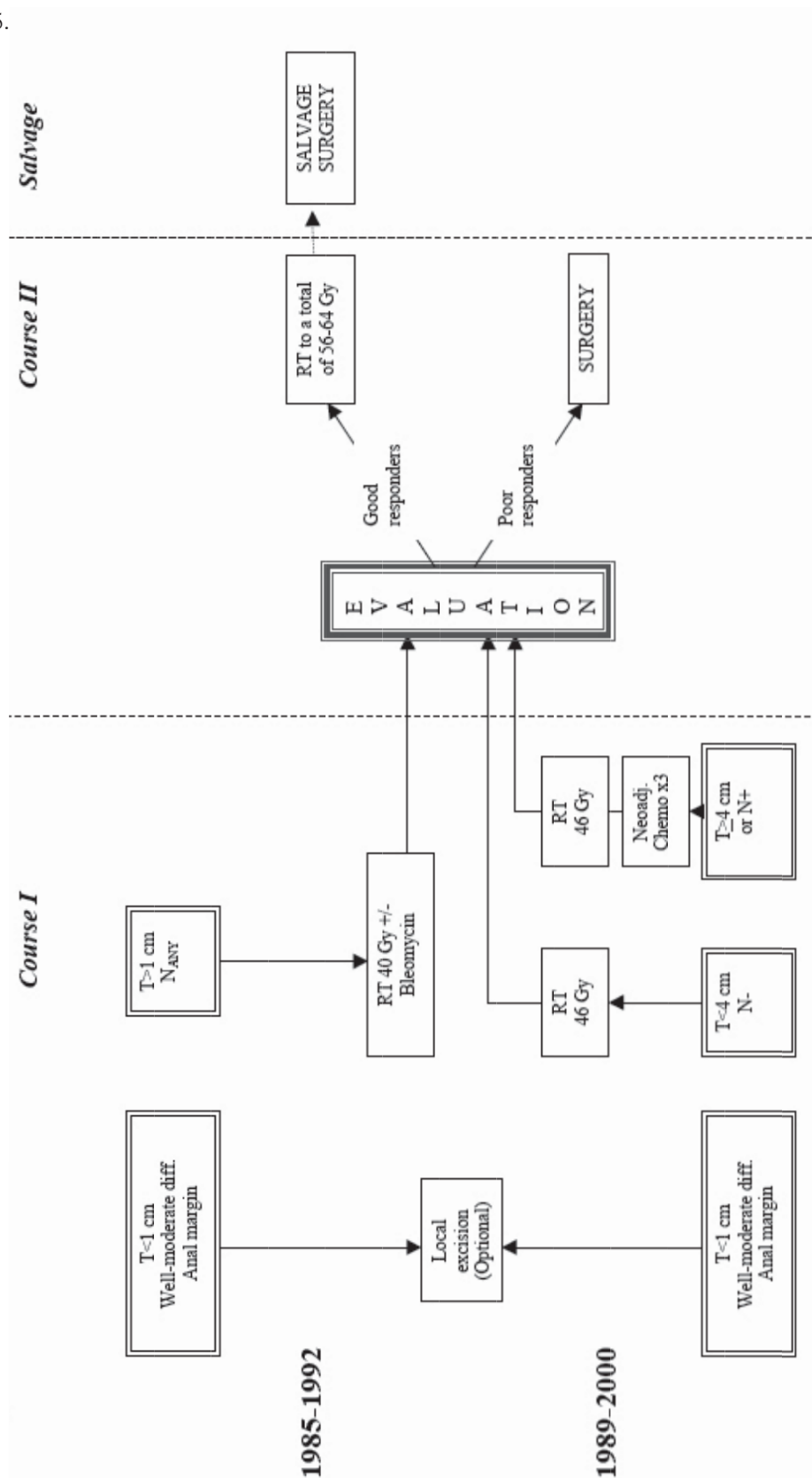
During the entire study period, defined guidelines, or protocols, prescribing recommended therapeutic approach were available. External beam RT was used exclusively, and no major changes in fractionation were made. During the study period, the chemotherapeutic approaches changed. In the early part of the series, CRT included concomitant bleomycin routinely, although patients with contraindications to this agent received RT alone. Starting in 1989, neoadjuvant platinum-based chemotherapy (carbo- or cisplatin) followed by two courses of RT constituted the CRT regimen. This “neoadjuvant CRT” was prescribed to patients with locally advanced tumours, whereas patients with smaller lesions were administered RT alone. An overview is presented in figure 5.

Paper I:

Therapeutic modalities used and adherence to treatment protocols in all 308 patients were analysed. Median follow-up was 66 months with a range of 12-194 months. Locally advanced tumours were defined as $T \geq 4$ cm and/or $N+$ in this and the following papers. Among the 276 patients treated with curative intent, outcomes in relation to stage and used treatments were analysed. Results were presented as overall survival, death with no evidence of disease, complete response rate and recurrence rate.

Among patients with locally advanced tumours two treatment groups were distinguished. During the years 1985-1992, 51 patients with locally advanced tumours were treated with RT alone or RT with concomitant bleomycin. These patients were grouped together, based on results from an earlier publication [142]. From 1989 to 2000, 91 patients with locally advanced tumours received neoadjuvant platinum-based chemotherapy followed by RT. Comparisons were made between these two

Figure 5.



Overview of therapeutic approaches used during the study period.

groups regarding the aforementioned endpoints.

An analysis on prognostic impact on survival of clinicopathological parameters, including gender, age, and location of tumour, histology type, tumour size, and nodal status was made on all 276 patients treated with curative intent. Prognostic factors for both survival and locoregional failure, using the same variables with the addition of therapeutic approach, were analysed in patients with locally advanced tumours.

Paper II:

In the cohort consisting of 308 anal cancer patients, 39 patients suffered an isolated locoregional failure after two courses of RT to a minimum of 60 Gy. Retrospectively, operative notes and patient records were reviewed. Thirty-five of these patients underwent salvage APR. Patients who underwent APR as their sole therapy, or after an initial course of RT to less than 50 Gy, or for reasons other than locoregional failure were not included. Twenty-one patients in whom the failure was diagnosed within 6 months after termination of RT were classified as having persistent disease and 14 patients who presented with a locoregional failure after more than 6 months were classified as having a recurrent tumour.

Outcomes after salvage APR, with regard to surgical and oncological results, were analysed. Prognostic impact on survival, of clinical and pathological variables was assessed.

Papers III and IV:

In the group of 276 patients treated with curative intent, 230 formalin-fixed wax-embedded original, pre-treatment diagnostic biopsies were possible to retrieve. The quality of some blocks was inadequate, leaving 209-215 biopsies for analysis with respect to different markers.

The investigated biochemical markers were selected to reflect properties of interest

for tumour behaviour and radiosensitivity. In broad terms, tumour properties and corresponding marker in focus were: invasive capability – laminin-5, apoptosis and DNA repair – p53, cell cycle progression – Cyclin A, cell cycle inhibition – p21, and angiogenesis – CD31.

IHC was performed using standard methods. From the biopsy-containing blocks 4 µm sections were cut and subjected to the standard horseradish peroxidase avidin-biotin complex (ABC) technique. In paper III, an in-house monoclonal antibody for the γ2-chain of laminin-5 was used. Commercially available antibodies for p53 (DO-1, Santa Cruz Biotechnology Inc., Santa Cruz, USA), p21^{WAF1} (Oncogene Research Products, Boston USA), Cyclin A (Novocastra Laboratories, Newcastle, UK) and CD31 (DAKO, Glostrup, Denmark) were applied in paper IV. Counterstaining in Mayer's hematoxylin was performed prior to mounting. As negative controls, the primary antibodies were replaced by bovine serum albumin.

A senior pathologist reviewed all slides for confirmation of diagnosis. In paper III, tumour budding was defined as dissociated single cells or clusters of up to five cancer cells with cytoplasmic laminin-5 γ2-chain staining, just ahead of the invasive front. All slides were evaluated with regard to tumour budding by one investigator and 50 randomly selected slides were re-evaluated by another investigator with a 96 per cent concordance. In paper IV, two investigators using a double objective microscope evaluated all slides. Staining for p53, p21 and Cyclin A was analysed semi-quantitatively based on percentage of positive tumour cells. The following cut-offs were used to discriminate between high and low expression: p53 >5 per cent, p21 >5 per cent and Cyclin A >20 per cent. When CD31 was analysed, the entire section was viewed in low power to identify "hot spots". Then, in a high-power field (x 400), a graticule was used and all stained vessels were counted. In CD31 the median vessel count was used as cut-off between high and low expression.

Results from IHC were correlated to clinical outcomes and the prognostic value was analysed.

Statistical methods

Paper I-IV:

For comparison between proportions of nominal data, the χ^2 -test was used. In paper IV, two tailed Fisher's exact test was used for assessment of correlation between different markers. Ordinal variables were compared using the Mann-Whitney *U*-test. The Kaplan-Meier method was used to estimate overall and tumour specific survival. In paper I, failure-free survival was also estimated, using the

method of Kaplan-Meier. In paper II, survival was calculated from the time of surgery, and in papers I, III and IV, survival was calculated from date of diagnosis, until death or date of last follow-up. Survival comparisons between different groups were made by the log-rank test. The relative hazard was estimated using Cox proportional hazards regression model. In all papers, statistical significance was accepted at $p < 0.05$.

All statistical analyses were made using a statistical software package (STATISTICA, '99 edition, StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Treatment

Paper I and II

Of the 308 consecutive patients, 276 (90 per cent) were treated with curative intent. All but 12 (4 per cent) were treated in accordance with the defined treatment protocols used during the study period. Non-surgical sphincter preserving therapy was intended for 257 patients, but among these, 33 patients (13 per cent) were assessed as poor responders after the initial RT course to 40-46 Gy. As part of primary therapy, these patients underwent APR after a median RT dose of 46 Gy. Two hundred and twenty-four patients received non-surgical sphincter preserving therapy including RT to a total dose of 60 Gy or more.

Locally advanced tumours were diagnosed in 150 patients. Treatment according to guidelines was given to 142 patients. Therapy consisting of RT +/- bleomycin was delivered to 51 patients and neoadjuvant CRT to 91 patients. An overview of treatments delivered is presented in figure 6.

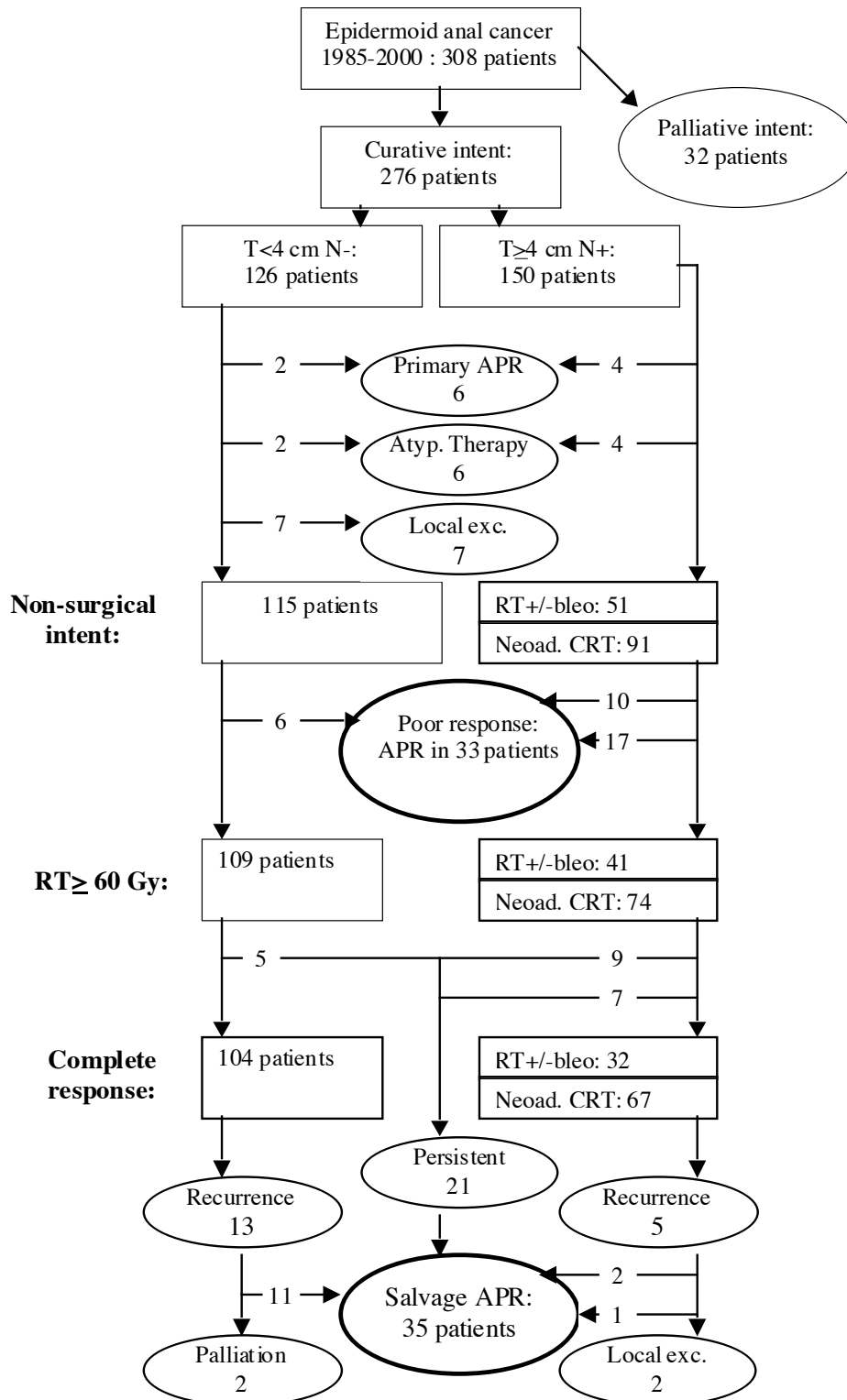
Median survival in the cohort of 308 patients was 96 months (range 1-194+ months). Among patients who had died, 49 per cent died with no evidence of disease. Overall survival rates at 5 and 10 years were 60 and 44 per cent, respectively. Median survival among patients treated with palliative intent was 6 months (range 1-70 months). In patients treated with curative intent, overall 5-year survival rate was 68 per cent and among patients who had died, 61 per cent died with no evidence of disease. Overall survival rate in relation to stage is presented in figure 7.

In the group of 126 patients with tumours <4 cm and N-, overall 5-year survival was 79 per cent. In only six of these patients, was APR performed after initial RT due to poor response, however an additional five were found to have residual tumour after

termination of RT, leading to salvage APR. Also, recurrences were found in 13 patients of whom 11 went on to have salvage APR. The results regarding survival found in this group of patients with small tumours (T1 and T2 <4cm) and negative nodes, treated basically with RT alone, are in agreement with results reported by other investigators [33, 35, 60, 61]. The notion that RT alone is adequate in these patients appears to be supported by these results; however, a 12.5 per cent recurrence rate gives cause for concern. A corresponding figure of 10 per cent was reported in two French series [33, 35]. Also, one should bear in mind that the study by Martenson *et al.* included only 18 patients [60], and in the study by Ortholan *et al.* no tumours were >1 cm [61]. In two reports where CRT including cisplatin/5-fluoruracil was used, an overall 5-year survival rate of 80-100 per cent and a local control rate of 88-100 per cent in T1-2 tumours were reported [63, 65]. These two reports included almost 200 patients collectively, and one chemotherapy-related death and two discontinuations due to toxicity were reported. Also, late toxicity resulting in severe anal dysfunction necessitating a colostomy occurred only in seven patients. Thus, it may be justified to administer CRT, in particular cisplatin-based, not only to patients with locally advanced tumours, but also to patients with smaller lesions.

An overall survival of 58 per cent was found among patients with locally advanced tumours. The patients who were treated with neoadjuvant CRT had a significantly higher survival rate compared to those who had received RT +/- bleomycin (63 *vs.* 44 per cent, $p=0.02$) (fig.8). Irrespective of treatment used, about twenty per cent of patients with locally advanced tumours had a poor response to initial RT and underwent APR as part of initial therapy. In patients having received RT +/-

Figure 6.



Overview of delivered treatment in all patients.

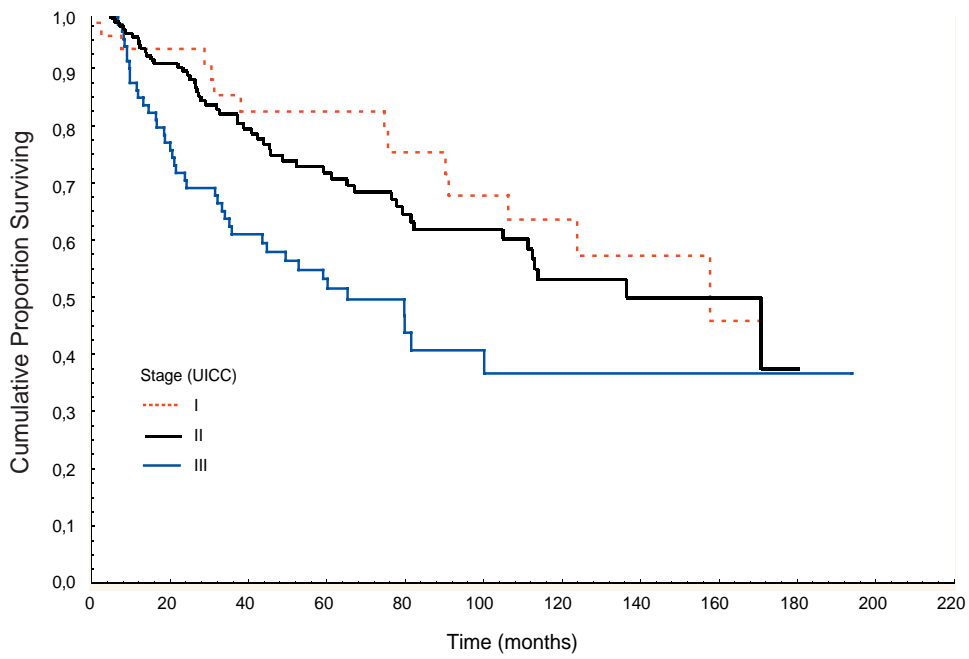


Figure 7. Overall survival in relation to stage (n=276).

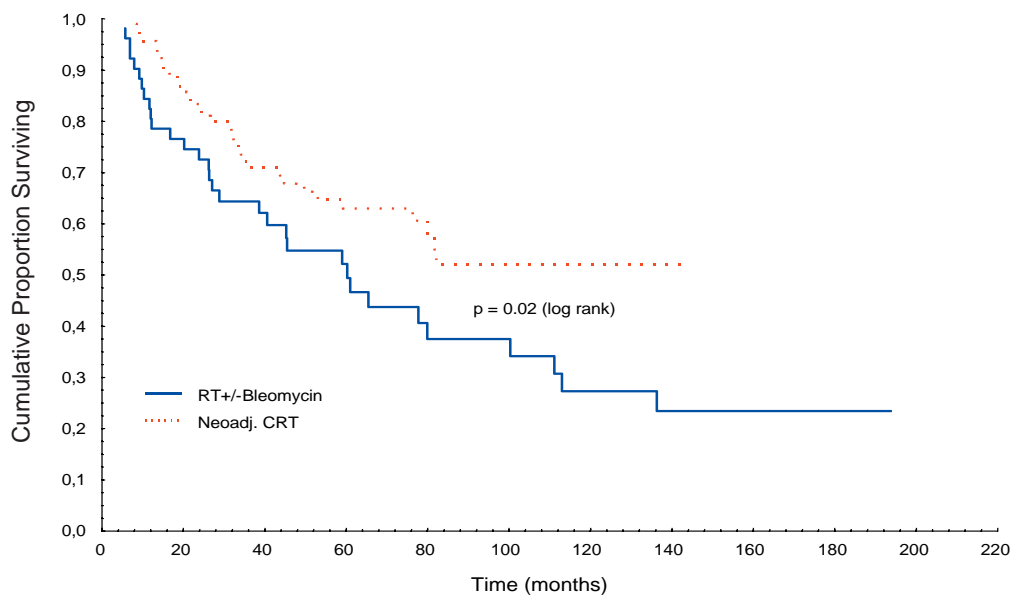


Figure 8. Overall survival in relation to therapy. Locally advanced tumours (n=142).

bleomycin 22 per cent of patients were found to have a persistent tumour after non-surgical therapy, compared to 9 per cent among those having received neoadjuvant CRT ($p=0.06$). A significantly increased CR rate was observed among patients who had received neoadjuvant CRT compared to patients from the pre-neoadjuvant era (92 *vs.* 76 per cent, $p < 0.01$). Following initial CR, five patients suffered recurrences, three of whom underwent salvage APR.

Although the superior outcome among patients who received neoadjuvant CRT

appears to be clear, interpretation must be with some caution. Firstly, it was not a randomised trial. The patients who had been treated with neoadjuvant CRT were compared with historical controls. Secondly, the two groups that were compared in the study were not identical (table 3). However, results consistent with the ones reported on neoadjuvant CRT here, have been reported by others using cisplatin/5-fluorouracil and RT [63-65]. The efficacy of cisplatin-based CRT regimens in anal cancer is currently being investigated in randomised trials, and future results will add to present knowledge.

Table 3.
Comparison between treatment groups RT+/-bleomycin and neoadjuvant CRT (T \geq 4 cm or N+) (n=142).

	RT+/-bleo		Neoadj. CRT		p
	n	%	n	%	
n	51		91		
Gender (Female : Male)	37 : 14		72 : 19		n.s
Age (yrs) median (range)	74 (40-90)		68 (36-86)		$p < 0.05$
Tumour location					$p < 0.05$
Anal margin	11	22	4	4	
Anal canal	40	78	87	96	
Histology					n.s
Squamous	39	76	61	67	
Basaloid	12	24	30	33	
T-classification (UICC)					n.s
T 1	1	2	5	5	
T 2	20	39	35	38	
T 3	21	41	42	46	
T 4	9	18	9	10	
Tumour size (mm) median (range)	51 (20-100)		51 (10-100)		n.s
N-classification (UICC)					$p < 0.05$
N 0	38	75	41	45	
N 1	7	14	30	33	
N 2	6	12	19	21	
N 3	0	0	1	1	
RT-dose (Gy) median (range)	64 (40-68)		64 (26-68)		n.s
Duration of RT (days) median (range)	63 (28-123)		54 (17-172)		$p < 0.05$
APR after 1:st course of RT (n)	10	20	17	19	n.s

UICC: International Union Against Cancer. RT: Radiotherapy. CRT: Chemoradiotherapy.
APR: Abdominoperineal resection. n.s: not statistically significant.

While regimens with concomitant CRT are being widely used, neoadjuvant administration of chemotherapy was prescribed in the present series. The principal aims of neoadjuvant chemotherapy are improved survival by eliminating subclinical disseminated disease and organ preservation by reducing the number of tumour cells to be killed by radiation, but only a few reports on neoadjuvant CRT in anal cancer are available [64, 143]. The advantages of concomitant chemotherapy are shortened overall treatment time and possible synergistic effects between radio- and chemotherapy. However, the latter also represents a risk, as it may lead to increased toxicity. In clinical practice, concomitant therapy hinders the use of maximal dosage of both chemo- and radiotherapy. On the other hand, should neoadjuvant chemotherapy kill cells in the primary tumour that would easily have been killed by RT, no gain is seen, although, potentially, radiation dose can be lowered.

An important observation in the two groups (RT+/- bleomycin *vs.* neoadjuvant CRT) concerns the number of persistent tumours after full dose RT. One may speculate that the high rate (22 per cent) observed in the RT+/- bleomycin group was, at least partly, due to patients who actually had a (undetected) poor response to initial RT and, thus, should have undergone APR at an earlier stage. Should this assumption be true, it stresses the importance of accurate assessment of patients after initial RT.

In a report on a series of 106 anal cancer patients, Tanum *et al.* found a 15 per cent rate of late treatment-related symptoms, including seven per cent faecal incontinence [144]. Two reports on anal manometric measurements after (chemo)-radiation for anal cancer have shown reduction in both resting and squeeze pressure [145, 146]. When assessed with a questionnaire, quality of life was generally maintained despite this objective impairment [146]. However, in a study from Canada, investigating 50 anal cancer patients following CRT with EORTC

quality of life questionnaires and comparing results with a group of healthy volunteers, significant reduction was found in several scales [147]. Outcome with regard to anorectal function was not studied in the investigated cohort of 308 anal cancer patients, however, the formation of a colostomy without any evidence of persistent or recurrent disease occurred in four per cent. This is a crude measurement of the rate of treatment induced severe anorectal dysfunction, and indicates that results in this series are comparable to earlier reports [144].

The results reported from the Stockholm-Gotland cohort show that results parallel to those reported from clinical trials are achievable on a population level. Adherence to pre-determined guidelines, centralised management of patients within a specialised multidisciplinary team and close follow-up may be key elements in the treatment of epidermoid anal cancer patients.

APR

For different reasons, six patients were treated primarily with an APR among the 276 patients treated with curative intent. In 33 patients the response to the first course of RT was assessed as poor, and these patients underwent APR after a median RT dose of 46 Gy. Following either persistent tumour or recurrent disease after two courses of RT an additional 35 patients underwent salvage APR. Thus, the rate of APR among the 276 curatively treated patients was 27 per cent. Estimated overall 5-year survival among APR-patients is presented in table 4.

Although no post-operative mortality was observed, complications were common in the group of 35 patients undergoing salvage APR. Twenty per cent required a second laparotomy due to complications and 66 per cent had delayed healing of the perineal wound. In the 33 patients undergoing APR after the initial course of irradiation, these figures were lower at 13 and 23 per cent, respectively, indicating that additional RT increases post-operative complication rate.

Table 4.
5-year survival rates (Kaplan-Meier) in different groups
undergoing APR in epidermoid anal cancer.

	n	RT dose	5-year survival
Poor responder	33	≤ 50 Gy	41 %
Persistent tumour	21	≥ 60 Gy	33 %
Recurrent tumour	14	≥ 60 Gy	82 %

APR: Abdominoperineal resection.

Comparison between different series of APR in anal cancer in the literature would be facilitated, should terminology be more precise. Most CRT or RT alone treatment regimens involve split course irradiation, although shortened gaps have been introduced [98, 148]. The gap allows for patients to recuperate and, also, for assessment of tumour response after the initial RT. In poor responders after initial irradiation, APR should be considered as an option, and part of primary treatment. For example, in the UKCCCR trial 8 per cent of patients had a poor response after 45 Gy and 65 per cent of these patients went on to have an APR at this stage of the treatment [57]. A second course of RT (boost) reaching a potentially curative radiation dose is prescribed to patients in whom treatment response is good (i.e. CR or near CR) in most CRT regimens. The term salvage APR should be reserved for these patients, whose initial tumour response was assessed as good and, thus, received boost RT, but were then found to have a local disease failure necessitating a salvage APR. Again, using the UKCCCR trial as an example, 82 per cent received boost RT and among these patients 38 per cent suffered either residual disease or recurrent disease after 60-70 Gy and were potential candidates for salvage APR.

The separation of the entity “salvage APR” from APR as part of primary therapy is relevant as preoperative radiation dose may

increase postoperative complication rate. Also, a salvage APR procedure represents a failure in the treatment response assessment and is, at least theoretically, avoidable, whereas APR after an initial first course of RT represents true practise of the concept of combined modality therapy. Alas, there is no uniform consensus regarding this terminology, which is reflected in published series of APR in anal cancer [78-85] and in recently published treatment regimens [98].

The survival rates among patients undergoing APR at different stages of their course of therapy presented in table 4 indicate that patients with a poor response to initial RT and patients with persistent tumour following two courses of RT, both have poor prognosis. Since additional RT in poor responders appears to add only morbidity and no survival benefits, this observation underlines the importance of assessment of treatment response after initial RT. The ongoing trial with adjuvant chemotherapy to anal cancer patients may provide answers whether this group of patients should receive adjuvant chemotherapy following APR [66]. The results in table 4 may also indicate that certain tumours have a more malignant phenotype, being resistant to CRT and producing poor results also after surgery, whereas other tumours respond well to (chemo)-radiotherapy and, should a recurrence appear despite this, the response to APR is excellent.

Prognosis

Clinicopathological parameters (Paper I-II)

The prognostic impact of clinicopathological parameters on overall survival was analysed in the cohort of 276 patients treated with curative intent (table 5). In the univariate analysis, tumour size exceeding 4 cm, presence of positive lymph nodes, and age over 70 years were significant indicators of poor prognosis. In the multivariate analysis, only age was an independent prognostic variable. A more comparable patient group were those with locally advanced tumours who had received treatment according to guidelines. Apart from age, a multivariate analyses regarding overall survival could not define any independent prognostic variables. Thus, in the investigated cohort of patients no strong clinicopathological prognostic markers were found. In the clinical setting, this calls for caution when using these parameters as prognostic tools.

The hypothetical total rate of poor response to initial RT can be estimated by adding the number of patients undergoing APR after the first course of RT to the number of patients with residual tumour after two courses of irradiation. Of the 257 patients intended for non-surgical therapy, 54 patients (21 per cent) underwent APR due to either poor response to initial RT or persistent tumour. In patients with locally advanced tumours, 30 per cent were poor responders, using the above definition. The impact of different clinicopathological variables on the risk of poor response is outlined in table 6.

Even though the multivariate analyses failed to provide any valuable prognostic markers, it appears that stage is of importance for treatment response and survival. Figure 7 provides a graphical illustration of this impression and in table 6 the importance of T-stage in relation treatment response is emphasized.

Table 5.
Cox proportional hazard regression on survival on all patients treated with curative intent (n=276).

	Number of patients	Number of events	Univariate		Multivariate	
			Relative hazard	95 % CI	Relative hazard	95 % CI
Gender: Male : Female	58 : 218	24 : 83	1.25	0.79 – 1.97	1.29	0.81 – 2.06
Age: ≥ 70 : < 70 years	132 : 144	70 : 37	2.71	1.81 – 4.04	2.86	1.88 – 4.34
Location:						
Anal canal : Anal margin	236 : 40	91 : 16	1.03	0.60 – 1.75	0.86	0.50 – 1.49
Histology:						
Squamous : Basaloid	200 : 76	79 : 28	0.92	0.60 – 1.42	0.72	0.46 – 1.12
Tumour size:						
≥ 40 : < 40 mm	108 : 168	51 : 56	1.74	1.19 – 2.56	1.38	0.91 – 2.10
Nodal stage: N- : N+	211 : 65	76 : 31	1.59	1.04 – 2.42	1.50	0.95 – 2.38

CI: Confidence interval.

Table 6.

	Poor response (%)	p
All treated with non-surgical intent (n=257)		
Female / Male	20 <i>vs.</i> 25	0.36
Age (yrs) <70 / ≥ 70	17 <i>vs.</i> 26	0.07
Anal canal / margin	23 <i>vs.</i> 10	0.10
T1-2 / T3-4	14 <i>vs.</i> 36	< 0.001
N- / N+	20 <i>vs.</i> 25	0.33
Locally adv. no / yes	10 <i>vs.</i> 30	<0.001
Locally advanced tumours (n=142)		
RT+/-bleo / Neoadj. CRT	37 <i>vs.</i> 26	<0.18

Hypothetical rate of poor responders in relation to clinico-pathological parameters.

Despite the limited number of patients included in the study on results following salvage APR, a statistically significant difference in 5-year overall survival rates was found between patients with persistent and recurrent tumours (33 *vs.* 82 percent, $p < 0.05$ log rank). Independent prognostic significance of recurrent *vs.* persistent tumours could possibly be shown, were there larger patient series available for analysis.

Tumour budding (Paper III)

The analyses of 209 biopsies showed that the presence of tumour budding was associated with improved prognosis (overall 5-year survival 74 *vs.* 64 per cent, $p < 0.05$). Other outcomes, such as tumour specific survival rates, recurrence rate, local failures, and distant metastases were all in favour of patients whose tumours showed tumour budding without being statistically significant. Tumour budding was an independent prognostic factor in a multivariate analysis including T-stage, N-stage and histological type.

The observed difference in survival in favour of patients whose tumour exhibited budding was significant in a statistical sense. However, the difference was relatively small and different therapeutic approaches were present in the series. Also, CR rates were similar whether tumour budding was present or not and

budding could not assist in the prediction of poor response to (chemo)-radiation. The presence of tumour budding in colorectal adenocarcinomas, which are treated primarily by surgery, is associated with poor prognosis [149]. One may speculate that tumour budding reflects not only aggressiveness but also factors affecting the radiosensitivity of a tumour such as, for instance, tumour hypoxia. This is the first report on the prognostic significance of tumour budding in anal cancer, and the first report involving properties of invasion in anal cancer patients treated primarily with a non-surgical approach. Future investigations may clarify the importance of budding in epidermoid anal cancer.

Intracellular markers (Paper IV)

The prognostic potential of four markers was explored in 215 anal carcinomas. Analyses of p53 and CD31 revealed no prognostic impact of these markers. Reduced expression of p21 showed a trend towards inferior survival (62 *vs.* 71 per cent, $p = 0.08$) and a significantly increased failure rate (27 *vs.* 14 per cent, $p < 0.05$).

There was a relationship between overexpression of Cyclin A and overall, and tumour specific survival (77 *vs.* 59 per cent, $p = 0.005$, and 81 *vs.* 64 per cent, $p = 0.009$, respectively). A multivariate analysis

including T-stage, N-stage and therapeutic approach showed Cyclin A expression to be an independent prognostic factor (table 7). In addition, Cyclin A overexpression was associated with significantly fewer isolated locoregional failures (12 *vs.* 24 per cent, $p < 0.05$) and a trend toward fewer failures after an initial CR (14 *vs.* 25 per cent, $p = 0.06$). However, rate of poor response to initial RT was not predicted by Cyclin A expression rate.

The results regarding p53 in this study, and in relation to earlier reports, illustrate the difficulties in assessing prognostic importance in IHC studies, as outlined earlier. The results on p21 are partially consistent with earlier reports showing reduced expression to be an indicator of poor prognosis [125]. More importantly, the finding that both the expression

of a cell cycle inhibitor (p21) and the expression of a proliferation marker (Cyclin A) appear to have an impact on prognosis strengthens the notion that tumour cell proliferation rate is of importance for response to RT.

Heterogeneity within the tumour with respect to marker expression may be of relevance for interpretation of results. The degree to which marker expression rate differs in various parts of an anal carcinoma is not known. Recently it was reported that all of 21 anal cancer biopsies strongly expressed EGFR and none expressed HER-2, leading to the assumption that heterogeneity, at least regarding these markers, is limited [150]. Moreover, as epidermoid anal cancer is treated primarily by non-surgical means, evaluation of tumour properties must rely on biopsies in clinical practice.

Table 7.

Cox proportional hazards analyses on overall and tumour specific survival

	Univariate		Multivariate	
	RH	95% CI	RH	95% CI
Overall survival				
T3-4/T1-2	2.03	1.26-3.28	2.34	1.37-3.98
N+/N-	1.43	0.88-2.32	1.55	0.89-2.70
Neoadj. CRT/RT+/-bleo	1.09	0.68-1.75	0.57	0.32-1.01
Cyclin A high/low	0.55	0.34-0.88	0.54	0.34-0.87
Tumour specific survival				
T3-4/T1-2	2.07	1.17-3.65	2.37	1.26-4.45
N+/N-	1.44	0.83-2.53	1.61	0.84-3.07
Neoadj. CRT/RT+/-bleo	1.07	0.62-1.84	0.54	0.28-1.05
Cyclin A high/low	0.52	0.30-0.90	0.51	0.29-0.88

RH: relative hazard. CRT: chemo-radiotherapy.

RT: radiotherapy. bleo: bleomycin. CI: Confidence interval.

Summary

Combined modality therapy is the primary therapeutic approach in epidermoid anal cancer. The optimal regimens regarding radiotherapy and chemotherapy in early tumours and locally advanced tumours are not known. Poor responders to (chemo)-radiation should be considered for abdominoperineal resection after initial radiotherapy. Assessment of treatment response at this stage is of importance, as additional irradiation has no survival benefits. Patients with residual tumour or recurrence after a full chemo-radiotherapy or radiotherapy alone regimen should be considered for salvage abdominoperineal

resection. Including all patients, an overall survival rate at 5 years of 60 per cent is achievable in an unselected population-based patient series.

For prognostic information, clinical and histopathological parameters should be used with caution; however, T-stage appears to be of prognostic value. Presently, no biochemical markers are available in clinical practice. Markers related to tumour cell proliferation rate merit further study. Reliable predictors of poor response to (chemo)-radiation are lacking.

CONCLUSIONS

- ⊙ The Stockholm-Gotland epidermoid anal cancer patient cohort 1985-2000 was treated highly in accordance with guidelines. Treatment results comparable to those in clinical trials were achieved in an unselected population-based patient series. In locally advanced tumours, neoadjuvant platinum based chemotherapy appeared to provide superior results compared with RT with or without bleomycin.
- ⊙ Stage, in particular T-stage, appears to be of prognostic value in anal cancer but clinicopathological variables should be used with caution.
- ⊙ Long-term survival rates exceeding 50 per cent are achievable after salvage APR in epidermoid anal cancer. Complication rates are high and appear to be associated with pre-operative radiation dose.
- ⊙ Salvage APR for recurrences yields significantly better survival rates than salvage APR for persistent disease.
- ⊙ Tumour budding detected by laminin-5 γ 2-chain IHC may be of prognostic importance in epidermoid anal cancer. Further studies are needed for confirmation and clarification of clinical implications.
- ⊙ Increased Cyclin A expression may be a marker of radiosensitivity, and may prove to be a valuable prognostic marker in epidermoid anal cancer. p21 appears to be of little value, and p53 and CD31 appear to be of no prognostic significance in epidermoid anal cancer.

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