Interleukin-2 based immunotherapy in patients with metastatic renal cell carcinoma

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INTRODUCTION

Renal cell carcinoma (RCC) is the most common malignancy (85%) of the kidney. The remaining are mainly transitional cell carcinomas originating from the renal pelvis. Wilms' tumor (nephroblastoma) is only seen in children. In the following, emphasis is restricted to RCC.

EPIDEMIOLOGY

RCC accounts for 2 percent of all cancer. Worldwide, 208,000 new cases and 102,000 deaths result each year from renal cancer [1]. The incidence varies among countries. The rates are highest in Western and Eastern Europe, North America, Australia and Scandinavia, intermediate in Southern Europe and Japan, and low elsewhere in Asia, South America, Africa and the Pacific [2]. In the United States, the incidence has increased by 43% since 1973, and similarly, increased incidence rates have been observed in nearly all regions. By contrast, in Denmark and Sweden the incidence rates have remained almost unchanged [3]. The absolute numbers of new kidney cases and kidney cancer deaths in Denmark during the last 30 years are seen from Figure 1.

Risk factors for developing RCC include cigarette smoking – the proportion of RCCs that could be attributed to cigarette smoking is up to 30% – hypertension, obesity and end-stage renal disease [3-5]. The median age at diagnosis is 65. The men-female ratio is 2.5:1.

PATHOLOGY, GENETICS AND TUMOR BIOLOGY

International agreement was reached in 1997 on the histologic classification of RCC on the first international multidisciplinary workshop held by World Health Organization (WHO) in collaboration with the Union Internationale Contre le Cancer (UICC) and American Joint Committee on Cancer (AJCC) [6, 7]. Thus, RCC are now separated into four different cellular types: clear, papillary, chromophobe and collecting duct (Table 1). The most common histological type of RCC is clear cell carcinoma (75% of cases). These tumors arise from the proximal tubule in the renal cortex (Table 1). This tumor type is typically sporadic, unilateral and unifocal. A large proportion of these tumors have mutations or hypermethylation at chromosome 3p, which contains the von Hippel-Lindau tumor suppressor gene. Papillary tumors occur in 15% of cases, and are the next most common histological type. Cytogenetically, these tumors frequently have trisomy of several chromosomes, as well as loss of the Y chromosome (Table 1). There are two subtypes of papillary RCC, type 1 (small cells) and type 2 (large cells with abundant eosinophilic cytoplasm) [8].

Inherited RCC is rare, only up to 2% of RCC cases cluster in families [9]. Within the last decade, four genes leading to inherited forms of RCC have been identified [10]: the von Hippel-Lindau (VHL) gene leading to clear cell RCC [11] was identified on chromosome 3 in 1993; the c-Met gene leading to type 1 papillary RCC [12] was identified on chromosome 7 in 1997; the Fumarate hydratase (FH) gene leading to Type 2 papillary RCC [13] was identified in 2002; and the Birt-Hogg-Dubé (BHD) gene leading to chromophobe RCC [14] was identified in 2002.

During the past decade, the biology associated with the VHL gene product (pVHL) has been elucidated in inherited and sporadic RCC [8, 15]. The von Hippel-Lindau tumor suppressor gene (VHL), which resides on chromosome 3p25, is mutated or silenced in 100% of inherited and 50-85% of sporadic clear cell RCC. Mutation or methylation of VHL leads to a pseudohypoxic state in which the pVHL complex does not form and/or cannot degrade hypoxia-inducible factors (HIF-1α, HIF-2α and HIF-3α). Thereby, HIF overaccumulates resulting in increased transcription of a variety of genes, including vascular endothelial growth factor (VEGF), platelet-derived growth factor B (PDGF-B), transforming growth factor-α (TGF-α), Glut 1 glucose transporter, carbonic anhydrase IX and erythropoietin (EPO) [8, 16]. Thus, VHL appears to be a critical gatekeeper for the development of clear cell RCC and for the subsequent tumor proliferation, angiogenesis, mitogenesis, erythropoiesis, glucose metabolism, and pH control [15, 16].

The grading of RCC began in 1932, reflecting the differentiation of the tumor cells as defined microscopically by increased nuclear size, irregularity, and nuclear prominance [17]. Since then, several histopathological grading systems have been proposed, but currently that proposed by Furhman et al [18] is the most widely used in the North America and that proposed by WHO is most widely used in Europe [17].

CLINICAL PRESENTATION

In the onset of RCC, there are only few early warning signs. The classical triad of hematuria, flank pain and abdominal mass is found in less than 10% [4]. Among patients who are symptomatic, more than 50% have hematuria, approximately 40% have pain, 40% have an abdominal mass, and 10% have symptoms arising from metastatic sites. Patients also frequently have nonspecific signs and symptoms.
symptoms such as weight loss, fever, malaise, hypercalcemia, anemia or erythrocytosis. Metastatic disease is present in 30% of patients at initial diagnosis. Among these patients, approximately 75% have lung metastases, 36% have lymph node and/or soft tissue metastases, 20% have bone metastases, 18% have liver metastases, and 8% each have skin and CNS metastases [4,19]. Moreover, metastatic disease will develop in 30-50% of patients relapsing after initial curative-intended nephrectomy. As a result, approximately 50% of RCC patients will suffer from metastatic disease [19].

### Staging

Historically, several staging systems have been used in parallel. The system proposed by Robson et al in 1963, and updated in 1969, was most commonly used in clinical practice [20]. However, the first international multidisciplinary workshop held in 1997 by World Health Organization in collaboration with the Union Internationale Contre le Cancer (UICC) and American Joint Committee on Cancer (AJCC) [21], led to the general acceptance of the TNM classification system as one single internationally staging system for RCC (Table 2). In 2002, an update was published, subdividing stage T1 into T1a and T1b on the basis of a 4.0 cm cut-off level.

### Prognosis

The 5-year survival probability for RCC is approximately 95% for stage I patients, 88% for stage II patients, 59% for stage III patients, and 20% for stage IV patients [22]. For the subset of stage IV patients with untreated metastatic (M1) disease, the prognosis is poor as the median survival is only 8 months [23], the overall 3-year survival is less than 10% [24, 25], and the 5-year survival is less than 2% [26]. This dismal prognosis has not changed significantly in the past 30 years [27].

### Treatment of Localized Disease

The principal treatment for non-metastatic RCC is radical nephrectomy, which includes excision of the kidney with all of the Gerota's fascia, removal of the ipsilateral adrenal gland and regional lymphadenectomy [28]. An eventual intracaval tumor thrombus should be resected [29]. The actual benefit derived from adrenalectomy and lymphadenectomy has been debated and most centers now reserve adrenalectomy for patients with large upper-pole lesions and lymphadenectomy for patients with abnormal-appearing abdominal lymph nodes on computed tomography or clinically during surgery [30, 31-34]. Laparoscopic and partial nephrectomy [35] and radiofrequency ablation [36] are presently being evaluated and incorporated into clinical practice. There is no indication for adjuvant therapy, after resection for localized RCC, outside clinical trials. Studies of radiation therapy to the renal bed combined with nephrectomy – adjuvant as well as neo-adjuvant – have shown no survival benefit, but, in contrast, a trend for a harmful effect with the expense of significant toxicity (Table 3).

### Randomized Trials Evaluating the Role of Radiotherapy Combined with Radical Nephrectomy

<table>
<thead>
<tr>
<th>Author</th>
<th>Timing</th>
<th>Dose</th>
<th>Number pts</th>
<th>5-y survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van der Werf-Messing [37]</td>
<td>Preoperative</td>
<td>30 Gy</td>
<td>64</td>
<td>28% (NS)</td>
</tr>
<tr>
<td>Juusela [38]</td>
<td>Preoperative</td>
<td>33 Gy</td>
<td>38</td>
<td>47% (NS)</td>
</tr>
<tr>
<td>Finney [39]</td>
<td>Adjuvant</td>
<td>55 Gy</td>
<td>52</td>
<td>36%</td>
</tr>
<tr>
<td>Kjaer [40]</td>
<td>Adjuvant</td>
<td>50 Gy</td>
<td>32</td>
<td>50%</td>
</tr>
</tbody>
</table>

NS, non significant.
TREATMENT OF METASTATIC RCC - SURGERY

In 1933, Barnay and Churchill [47] reported the first case in which a patient underwent both nephrectomy and excision of a single pulmonary metastasis. This patient survived 23 years without recurrence. There is now general agreement that a primary kidney tumor and a simultaneous solitary metastasis should be resected with curative intent. Kovilus et al [48] from Department of Surgery, Memorial Sloan-Kettering Cancer Center, addressed the issue of whether an aggressive surgical approach should be taken in patients with recurrent disease. They reported a 54% 5-year survival rate for patients who had a curative intended resection of a solitary metastasis and a 29% 5-year survival rate for patients who had curative intended resections of multiple metastases [48]. The 5-year survival rates of patients who underwent complete resection of second and third recurrences were 46% and 44%, respectively. Thus, in case of metastatic disease, surgery should always be considered. However, a case of a solitary metastasis in RCC is rare. Most often, bulky multiple metastatic disease is seen, thereby making curative intended resection impossible.

The role of radical nephrectomy in patients with non-resectable metastatic disease is controversial. Spontaneous regression of metastatic lesions after nephrectomy have been observed in about 1% of cases [49, 50]. However, the practice of nephrectomy in mRCC, in the hope of a spontaneous regression, is not recommended [27]. The hypothesis that debulking nephrectomy prior to immunotherapy may reduce a large immunosuppressive tumor burden has promise several retrospective non-randomized trials evaluating this issue. Beldegrun et al [51] from UCLA, Los Angeles, reported that among patients who received IL-2 based immunotherapy with their primary tumor in place (n=36), the 2-year survival rate was 4%, compared with a 2-year survival rate of 44% for patients who underwent nephrectomy prior to IL-2 (n=235) [51]. However, surgery may preclude the administration of systemic therapy in a large proportion of the patients (22%-77%) because of morbidity and/or rapid tumor progression [52-54]. The survival benefit may thus be caused by selection of patients with favourable prognostic features rather than a beneficial effect of nephrectomy per se.

Two recent randomized studies [55, 56] and a combined analysis [57] have evaluated the role of debulking nephrectomy plus interferon in mRCC (Table 5). The data from these trials should be interpreted with caution. Thus, the study by Flanigan [56] took 7 years, in 80 centers, to accrue 246 patients at a rate of one patient every 2 years per institution. This raises the concern of a possible selection bias. Only patients with excellent performance status 0 or 1 (i.e. patients with no or only few symptoms) were eligible. The mortality related to the surgical procedure was lower (1.4%) [57] than earlier reported (average 2.5%) [58]. Unfortunately, the combined analysis showed a survival benefit of only 5.8 months, a 1-year survival rate of approximately 50% and a 6-year survival rate of only 3%. Moreover, one of the main hypotheses – that patients may have a better response to IFN after removal of the large immunosuppressive primary tumor – was not supported (Table 5). Therefore, it has been suggested that the survival difference occurred because of decreased morbidity and mortality from local problems caused by the primary tumor [59]. Debunking nephrectomy followed by IL-2 has not been evaluated in randomized trials. In Denmark, the recommendation is to consider nephrectomy (and/or removal of any residual viable tumor) only in patients who after IL-2 based immunotherapy have had a major response [34]. Furthermore, nephrectomy may be justified in patients when the intent is to improve quality of life, such as the alleviation of symptoms i.e. pain, hematuria, erythrocytosis, uncontrolled hypertension or persistent hypercalcemia that does not respond to pharmacologic agents [27, 60].

TREATMENT OF METASTATIC RCC - CHEMOTHERAPY

RCC is resistant to chemotherapy. Chemotherapeutic agents, either alone or in combination, have little or no effect against mRCC [28]. Yagoda et al [61] reviewed the results of 4,093 patients treated in 83 trials with 74 drugs and combinations of drugs (chemotherapeutic agents and drugs with miscellaneous activity), published between 1983 and 1993. An objective response rate of 6%, including a CR-rate of 1%, was reported. Similarly, Motzer and Russo reviewed 33 chemotherapy agents studied in 51 phase II trials, published between 1990 and 1998, comprising a total of 1,347 patients [60]. Response rates were low and moreover, responses were generally short lasting. The mechanisms of drug resistance may probably be related to the fact that 80% of cancer cells have expression of the multiple drug resistance gene, which protects against apoptosis. Furthermore, the mechanisms of drug resistance may also involve the expression of anti-apoptotic genes, which can protect against programmed cell death. The mechanisms of drug resistance may also involve the expression of anti-apoptotic genes, which can protect against programmed cell death.

### Table 4. Randomised trials evaluating the role of adjuvant systemic therapy after radical nephrectomy.

<table>
<thead>
<tr>
<th>Author</th>
<th>Treatment</th>
<th>Stage</th>
<th>Number pts</th>
<th>5-year disease free survival</th>
<th>5-year overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pizzocaro [42]</td>
<td>MPA</td>
<td>M0</td>
<td>58</td>
<td>67% (NS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Observation</td>
<td></td>
<td>62</td>
<td>73%</td>
<td></td>
</tr>
<tr>
<td>Pazzocaro [43]</td>
<td>IFN-α</td>
<td>II-III</td>
<td>123</td>
<td>57% (NS)</td>
<td>66% (NS)</td>
</tr>
<tr>
<td></td>
<td>Observation</td>
<td></td>
<td>124</td>
<td>67%</td>
<td>67%</td>
</tr>
<tr>
<td>Messing [44]</td>
<td>IFN-α</td>
<td>III-IV</td>
<td>140</td>
<td>37% (NS)</td>
<td>51% (p=0.09)</td>
</tr>
<tr>
<td></td>
<td>Observation</td>
<td></td>
<td>143</td>
<td>41%</td>
<td>62%</td>
</tr>
<tr>
<td>Clark [45]</td>
<td>IL-2</td>
<td>III-IV</td>
<td>33</td>
<td>32% (NS)*</td>
<td>80% (NS)*</td>
</tr>
<tr>
<td></td>
<td>Observation + M1 (NED)</td>
<td></td>
<td>36</td>
<td>45%</td>
<td>86%</td>
</tr>
<tr>
<td>Atzpodien [46]</td>
<td>IL-2/IFN/5-FU</td>
<td>III-IV</td>
<td>135</td>
<td>42% (NS)</td>
<td>58% (p=0.028)</td>
</tr>
<tr>
<td></td>
<td>Observation + M1 (NED)</td>
<td></td>
<td>68</td>
<td>49%</td>
<td>76%</td>
</tr>
<tr>
<td>Jocham [41]</td>
<td>Vaccination</td>
<td>I-II</td>
<td>177</td>
<td>77% (p=0.02)</td>
<td>not reported</td>
</tr>
<tr>
<td></td>
<td>Observation</td>
<td></td>
<td>202</td>
<td>68%</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MPA, medroxyprogesterone acetate; NED, no evidence of disease after nephrectomy + resection of a solitary metastasis; NS, non significant; *3-years data.

### Table 5. Randomized trials evaluating the role of debulking nephrectomy.

<table>
<thead>
<tr>
<th>Author</th>
<th>Treatment</th>
<th>Number pts</th>
<th>Response rate</th>
<th>Med survival (months)</th>
<th>1-year survival advantage</th>
<th>Survival advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mickisch</td>
<td>Nx + IFN</td>
<td>42</td>
<td>19%</td>
<td>17 (p=0.03)</td>
<td>-</td>
<td>10 months</td>
</tr>
<tr>
<td></td>
<td>IFN</td>
<td>43</td>
<td>12%</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flanigan</td>
<td>Nx + IFN</td>
<td>120</td>
<td>3.3%</td>
<td>11.1 (p=0.05)</td>
<td>49.7% (p=0.012)</td>
<td>3 months</td>
</tr>
<tr>
<td></td>
<td>IFN</td>
<td>121</td>
<td>3.6%</td>
<td>8.1</td>
<td>36.8%</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>Nx + IFN</td>
<td>161</td>
<td>6.9%</td>
<td>13.6 (p=0.002)</td>
<td>51.5% (p=0.001)</td>
<td>5.8 months</td>
</tr>
<tr>
<td>analysis</td>
<td>IFN</td>
<td>163</td>
<td>5.7%</td>
<td>7.8</td>
<td>37.1%</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Nx, nephrectomy; IFN, Interferon-alpha.
drug resistance gene that encodes P-glycoprotein [62]. Thus, no chemotherapy agent has produced response rates in proportions that justify its use in mRCC [60] and no agent should be considered standard of care in mRCC [63].

TREATMENT OF METASTATIC RCC - HORMONAL THERAPY

No hormone therapy has produced a response rate that justifies its use in mRCC, despite renal tumor cells express receptors for estrogen and progesterone. However, for many years there has been a tradition of using medroxyprogesterone acetate (MPA) and tamoxifen for the palliation of mRCC.

TREATMENT OF METASTATIC RCC - CYTOKINE THERAPY

The treatment of mRCC by immunologic manipulation has long been regarded as a promising approach. The basis for this view has originated from observations that metastases may regress spontaneously following nephrectomy [49, 50, 64-67], late relapses after nephrectomy and prolonged stabilization of metastatic disease in the absence of systemic treatment [65]. Evidence for an immune response against RCC is provided by the observations that tumor-infiltrating lymphocytes can be detected in RCC tissues [68]. Overall, spontaneous regression occurs in 1% of patients [4]. These observations have led to considerable interest in research into immunologic therapies, as this phenomenon is considered immune mediated.

Some early attempts to induce a non-specific immune response to cancer involved "Coley's toxin," a crude extract of streptococcus [69] as well as regimens of crude tumor cell preparations, bacillus Calmette-Guérin, and Corynebacterium parvum. This form of immunotherapy has generally proven ineffective and has mostly been abandoned [23].

The interferons were discovered in 1957 [70], and the first experience in the treatment of patients with mRCC was published in 1983 [71]. Interferons are glycoproteins produced by mammalian cells in response to viral infections or other inducers. Three major classes of interferons have been characterized, interferon-alpha (IFN-α), interferon-beta (IFN-β), and interferon-gamma (IFN-γ). IFNs can be produced in large quantities using recombinant DNA techniques. The interferons have a direct antiproliferative effect on renal tumor cells [72], stimulate host mononuclear immune cells, and enhance the expression of major-histocompatibility-complex molecules. IFN-α was the first active antitumor agent with reproducible activity in patients with mRCC. In total, there have been four randomized trials that compared a control of non-interferon therapy with IFN-α alone or in combination with a non-IFN-α therapy in mRCC [73-76]. The results of the two most important trials are displayed in Table 6. A Cochrane review of 42 randomized trials involving 4216 patients has confirmed the value of IFN-α in mRCC [77]. However, IFN-α as monotherapy provides only a modest/minimal survival benefit of median 2.6 months compared with controls and a reduction in 1-year mortality by 27% [77]. Only 3% of patients were alive at 5 or more years following IFN-monotherapy [78].

Interleukin-2 (IL-2) was described by Morgan et al [79] in 1976. IL-2 is a 15-kDa glycoprotein, naturally secreted by activated T lymphocytes, mostly CD4+ cells. Recombinant IL-2 has been available since 1985. The molecular structure of IL-2 appears similar to that of granulocyte-macrophage colony-stimulating factor and IL-4 [80]. IL-2 mediates its biological effect by binding to the IL-2 receptor [19]. Thus, IL-2 has no direct impact on the tumor cells [81] but mediates antitumor activity through the modulation of the host's immune response [82].

In 1985, Rosenberg et al at the National Cancer Institute reported the first observations in human on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer [83]. This study established that immunological manipulations by the administration of IL-2 to patients with metastatic cancer may mediate a dramatic tumor regression. In 1995, Fyfe et al published the mature results obtained in 255 patients with mRCC treated with high-dose (HD) intravenous (iv) bolus single-agent IL-2 in seven clinical studies conducted at 21 institutions [84]. In this patient population, 15% had an objective response (CR: 7%; PR: 8%). Responses occurred in all sites of disease, including bone, intact primary tumors, and visceral metastases, and in patients with large tumor burdens or bulky individual lesions. The 5-year survival rate was 18% and the 10-year survival rate was 10% [85]. These patients are probably cured. These results led to the USA Food and Drug Administration (FDA) approval for the treatment of mRCC in 1992. Thus, IL-2 became the first biological agent to gain such approval for this disease. It should be noted that the Danish Medical Agency approved IL-2 for the treatment of mRCC in 1989. However, toxicity related to HD bolus iv IL-2 is significant. Fyfe et al [84] reported fever, hypotension, oliguria with marked fluid retention, pulmonary edema, mental confusion, hepatic dysfunction and capillary leak syndrome. Totally, 4% of patients died of adverse events judged to be possibly or probably treatment-related and 15% of patients required admission to an intensive care unit.

With the intention to diminish the severe side effects, clinical trials evaluating continuous iv (civ) IL-2 [86] or subcutaneous (sc) IL-2 [87] were initiated. Low doses of IL-2 can be given by sc injection to outpatients, and have an acceptable risk of toxic effects for less physically fit patients [87]. At least 116 phase I-III trials of IL-2 based im-

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Table 6. Randomized phase III trials evaluating different regimes of cytokines in mRCC.

<table>
<thead>
<tr>
<th>Author</th>
<th>Treatment</th>
<th>Number pts</th>
<th>Response rate</th>
<th>Median surv (mos)</th>
<th>Time to PD (mos)</th>
<th>1-year survival</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gleave [50]</td>
<td>IFN-gamma</td>
<td>90</td>
<td>4.4%</td>
<td>12.2</td>
<td>1.9</td>
<td>50%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>91</td>
<td>6.6%</td>
<td>15.7</td>
<td>1.9</td>
<td>50%</td>
<td>-</td>
</tr>
<tr>
<td>MRC [75]</td>
<td>IFN-alpha</td>
<td>167</td>
<td>14%*</td>
<td>8.5</td>
<td>4.0*</td>
<td>43%*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MPA</td>
<td>168</td>
<td>2%</td>
<td>6.0</td>
<td>3.0</td>
<td>31%</td>
<td>-</td>
</tr>
<tr>
<td>Pyrhönen [76]</td>
<td>IFN-alpha + VLB</td>
<td>79</td>
<td>16.5%*</td>
<td>16.9**</td>
<td>3.2*</td>
<td>58%*</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>VLB</td>
<td>81</td>
<td>2.5%</td>
<td>9.5</td>
<td>2.2</td>
<td>38%</td>
<td>0%</td>
</tr>
<tr>
<td>Negrier [88, 91]</td>
<td>IL-2 iv +IFN-alpha</td>
<td>140</td>
<td>18.6%*</td>
<td>17</td>
<td>20%* §</td>
<td>-</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>IL-2 iv</td>
<td>138</td>
<td>6.5</td>
<td>12</td>
<td>15%</td>
<td>12%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IFN-alpha</td>
<td>147</td>
<td>7.5%*</td>
<td>13</td>
<td>12%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yang [89]</td>
<td>IL-2, high dose iv</td>
<td>96</td>
<td>21%*</td>
<td>17</td>
<td>More durable</td>
<td>65%</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>IL-2, low dose iv</td>
<td>93</td>
<td>11%</td>
<td>17</td>
<td>CR's in high-dose group</td>
<td>65%</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td>IL-2, low dose sc</td>
<td>94</td>
<td>10%</td>
<td>17</td>
<td>-</td>
<td>65%</td>
<td>10%</td>
</tr>
<tr>
<td>McDermott [90]</td>
<td>IL-2, highdose iv</td>
<td>96</td>
<td>23%*</td>
<td>17.5</td>
<td>3.1</td>
<td>-</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>IL-2, sc + IFN-alpha</td>
<td>96</td>
<td>9.9</td>
<td>13.0</td>
<td>3.1</td>
<td>18%</td>
<td>-</td>
</tr>
<tr>
<td>Negrier [92]</td>
<td>IL-2 sc + IFN-alpha</td>
<td>122</td>
<td>10.9%</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IL-2 sc</td>
<td>125</td>
<td>4.1%</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IFN-alpha</td>
<td>122</td>
<td>4.4%</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MPA</td>
<td>123</td>
<td>2.5%</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: *, significant difference; MPA, medroxyprogesterone acetate; VLB, vinblastin; §, data is 1-year progression-free survival; mos, months.
mRCC. "targeted therapies"ing new biological immunotherapy have been completed (reviewed in [82]). IL-2 has been used in different dose schedules applying various administration routes, as either monotherapy or in combination with other cytokines, chemotherapy, endocrine treatment and adoptive cellular immunotherapy. Although a large number of randomized trials have been performed with different treatment strategies, it still remains uncertain whether the dose or combination of IL-2 with other agents substantially influence treatment outcome [82].

The most important randomized phase III trials of IL-2 based immunotherapy are outlined in Table 6. The combination of IL-2 and IFN-α resulted in a significantly higher response rate and an improved 1-year progression free survival, but did not increase overall survival [88].

Low-dose regimens have resulted in roughly equivalent response rates compared with high-dose regimens, but concerns have been raised as to whether the responses in the low-dose groups were as durable as those in the high-dose groups. Two randomized phase III trials, addressing this question, have been published recently [89, 90] (Table 6). Both studies reported a statistically significant increased response rate and a trend of more durable complete responses in favor of high-dose bolus iv IL-2 compared with low-dose sc IL-2. This difference did not, however, translate into an overall survival difference [89, 90] (Table 6).

A survival benefit of IL-2 has not been shown in randomized studies with a non-immunotherapy control [92, 93]. As seen from Table 6, all randomized trials have been small. No IL-2 treated group has exceeded 140 patients. This represents a too low statistical power to detect the long-term survival benefit actually seen in a small group of IL-2 treated patients. A large number of long-term follow-up reports have consistently been published demonstrating durable responses and long-term survival in a minority of patients (approximately 10%) treated with IL-2 [85, 91, 94, 95]. Thus, the true value of IL-2 lies in the probability that this drug can be curative for a small group of patients with metastatic disease [89]. The important issue is then to identify this subgroup of patients.

The initiation of the present thesis in 1999 represented the introduction in Denmark of IL-2 based immunotherapy as home-based therapy, in contrast to previous in-hospital treatment regimens [86, 96]. Due to the outpatient nature of the protocols, patients received instruction, guidance and monitoring during the first days of IFN-α/IL-2/histamine injections before self-administration in the patients home. Thereafter, only 2-3 ambulatory visits per month were scheduled within the following 3-9 months. A telephone "hot line" and skilled staff at the department of oncology in day-and-night preparedness is a prerequisite for the feasibility of this potentially toxic treatment - in patients with metastatic disease - on an outpatient setting.

**TREATMENT OF METASTATIC RCC - OTHER THERAPIES**

A variety of other biologic approaches to therapy have been investigated. No convincing benefit has been shown by using lymphokine-activated killer (LAK) cells [97], tumor-infiltrating lymphocytes (TIL) [98], dendritic cell vaccines [99], radioimmunotherapy with 131I-labeled monoclonal antibody to G250 [100] and thalidomide [101, 102]. The value of adding cis-retinoid has been conflicting [103, 104]. Allogeneic stem cell transplant is promising, but deserves further study [105, 106].

However, within the last 2-3 years, based on increasing understanding of the biology associated with the von Hippel-Lindau (VHL) tumor suppressor gene inactivation, a large number (>20) of new drugs, "targeted biological therapies", have been assessed in mRCC and several of these have shown promising results [107-112]. The most important trials are shown in Table 7. All trials were investigating these new drugs as second-line after cytokine therapy. To date (March 2006), three studies have been regularly published [113-115]. At ASCO 2004, response rates of 40% were reported for the first time in mRCC [115]. At ASCO 2005 (Table 7), several studies confirmed these high response rates. Whether these will translate into improved survival rates is awaited. New trials have been initiated evaluating these new drugs as first line therapy, in combination with IL-2 and/or IFN-α and as adjuvant therapy.

**IMMUNOLOGIC MECHANISMS OF ANTITUMOR ACTIVITY**

20 years have passed since the first administration of IL-2 to cancer patients. During these years, reproducible dramatic durable tumor regressions have been noted after systemic administration of IL-2 in patients with mRCC. However, the exact mechanisms mediating tumor regression have not been identified. Whereas IL-2 has no direct impact on tumor cells, which can grow unimpeded in vitro [81], it has been assumed that the impact of IL-2 on tumors in vivo derives from its ability to expand and activate lymphocytes with anti-tumor activity [81]. In fact, IL-2 has effects on several immune cells including T-cells [116], natural killer (NK) cells [117, 118], B-cells [119], monocytes/macrophages [120] and neutrophils [121] by binding to subunits of the IL-2 receptor on these cells. Three different IL-2 receptor complexes exist consisting of various subunits: (i) the α-chain (CD25); (ii) the β-chain (CD122); and (iii) the γ-chain (CD132) [122]. In addition, other immune cells are activated by cytokines produced by activated T-cells and NK cells, such as interferon-γ, granulocyte-macrophage colony stimulating factor, and tumor necrosis factor-α. The biologic effects of IL-2 has been reviewed [19] however, with no clear consistent finding [123, 124]. Thus, there is an essential need to explain which immune cell subset(s) is implicated in mediating tumor regression and to identify consistent markers of an immune response that correlate with clinical responses and overall survival.

Tumor immunology is a field with more questions than answers, i.e., is the glass half full [125] or half empty? A fundamental question is whether the immune system sees tumors as foreign or self [126].

---

**Table 7. Trials evaluating new biological "targeted therapies" in mRCC.**

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<thead>
<tr>
<th>Author</th>
<th>Design</th>
<th>Number</th>
<th>Response</th>
<th>Median survival</th>
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<tr>
<td>Yang [113]</td>
<td>Bevacizumab, highdose</td>
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<td>10%</td>
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</tr>
<tr>
<td>Motzer [115]</td>
<td>SU11248 (2 consecutive phase II)</td>
<td>106</td>
<td>40%</td>
<td>Too early</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>AG-013736</td>
<td>52</td>
<td>46%</td>
<td>Too early</td>
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</tr>
<tr>
<td>Rini</td>
<td>ASCO abstract 4509</td>
<td>Interim</td>
<td>Low but many SD</td>
<td>Too early</td>
<td>6 (&lt;0.00001)</td>
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</tr>
<tr>
<td>Excluder</td>
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MAGE-1, the first tumor antigen recognized by cytolytic T lymphocytes [127]. However, most human antigens expressed on tumor cells are normal non-mutated differentiation antigens [128]. Antigens that are only expressed in association with cancer are rare, including RCC [129]. To date, only few tumor antigens have been identified [130], with the total of 8 in RCC: Adipophilin [131], Fibroblast growth factor 5 (FGF-5) [132], G250/CAIX [133], intestinal carboxyl esterase gene (ICE) [134], M-CSF [135], RAGE1, PRAME, and glycoprotein 75 [136], with the role of G250/CAIX most elucidated [137, 138]. However, the immunologic mechanisms of antitumor activity are not completely understood [139]. Until now, no correlation between clinical benefit and the detection of antigen-specific T-cell responses has been achieved [140]. Therefore, NK cells are particularly attractive for the treatment of cancer because, unlike T lymphocytes, their anti-tumor activity does not depend on recognition of tumor-specific antigens, which remain poorly defined for the vast majority of malignancies [118].

As most patients with metastatic disease fail to respond to immunotherapy, effective immunotherapy against cancer has been suggested to be a question of overcoming immune suppression and immune escape [141]. Escape from immune recognition may be achieved by three distinct but closely related principles (Table 8) (I). Escape by loss of tumor recognition (i.e., loss or alteration of molecules which are important for the recognition by, and activation of, the immune system) (II). Escape by loss of susceptibility (i.e., escape from the effector mechanisms of cytotoxic lymphocytes) (III). Escape by induction of immune suppression (i.e., induction of immune dysfunction) [142].

The present thesis has tested the hypothesis that effective immunotherapy against cancer is a question of overcoming immune suppression in terms of chronic inflammation and oxidative stress. In basic tumor immunology, the negative effect of NO and H2O2, produced from activated macrophages and neutrophils, on T and NK cell functions is well-established [143]. Among macrophage and neutrophil products, reactive oxygen species (ROS) may not only induce genomic instability [144] and thereby potentially contribute to malignancy [145], but also damage anti-tumor immune effector cells [143, 146-149]. Intratumoral macrophages isolated from melanoma metastases inhibit NK cell function by the release of ROS [150]. Intratumoral NK and T cells isolated from mRCC show signs of oxidative damage [151, 152]. IL-2 cannot activate NK cells in vitro in the presence of monocytes or macrophages [153]. Taken together, H2O2 secretion by activated macrophages and neutrophils is one important mechanism behind the tumor-induced immune suppression with decreased signal transduction and poor effector functions of T and NK cells observed in cancer patients [143]. Monocytes/macrophages and neutrophils therefore represent an important drug-target for cancer treatment, with the aim of reducing the number and/or function of these cells. However, the clinical impact of this oxidative stress hypothesis in relation to IL-2 based immunotherapy was unknown at the initiation of the present thesis.

Extensive laboratory work for a decennium has identified histamine dihydrochloride (HDC) as an anti-phagocyte drug-candidate [154]. Targeting NADPH-oxidase through binding to the H2-receptor on monocytes [155] and neutrophils [156], HDC specifically blocks the formation and release of hydrogen peroxide (H2O2), thereby protecting NK and T cells from oxygen radical-induced inhibition and apoptosis [148]. Thus, NK and T cells remain viable and responsive to IL-2 [153, 157, 158].

In the present thesis, we have clinically explored this potential mechanism in two phase II trials in mRCC in collaboration with oncological departments in Sweden and United Kingdom, representing the introduction of histamine in patients with mRCC. As a supplement to the clinical trials, we initiated biological studies based on serial blood and tumor samples to search for a potential histamine effect in situ.

**PATIENT SELECTION**

Efforts to improve overall results also include the identification of prognostic factors, which allow treatment to be better directed towards those patients most likely to benefit. Given the toxicity and cost of IL-2 therapy, the benefit of a model predicting response as well as long-term survival to such therapy would represent a significant advance.

**AIMS OF THE STUDIES**

Two critical questions have driven the present thesis. First, which properties of the immune system are responsible for the dramatic tumor regression seen in some patients with mRCC following IL-2 administration? And second, can histamine increase the efficacy of IL-2 based immunotherapy by ending the immune suppression induced by phagocyte generation of reactive oxygen species, i.e. does a clinical testing of histamine support the oxidative stress hypothesis formulated in a pre-clinical setting?

The aims of this thesis were as follows:

- To improve the treatment of patients with mRCC.
- To identify parameters that may help to identify patients more likely to benefit from IL-2 based immunotherapy, either at baseline or as early as possible within the treatment course.
- To increase our understanding of the immunologic mechanisms of IL-2 based immunotherapy.

**PATIENTS AND METHODS**

In the following, an overall overview of the patients, treatments, number of blood- and tumor samples and applied methods is presented. A more detailed description is given in the individual articles.

**PATIENTS**

All data of this thesis were prospectively collected. A total of 181 patients, encompassing 120 Danish patients, 20 Swedish patients and 41 patients from United Kingdom entered phase II clinical trials (n=150) or were treated outside protocols as standard therapy (n=31). No patients were lost to follow up. Only Danish patients were asked for informed consent to the supplemental blood and tumor samples (Figure 2).
ETHICS
Approval from the Ethics committees and the Medical Agencies in Denmark, Sweden and UK were obtained before start of the studies. Written informed consent was obtained from all patients before treatment initiation and before blood- and tumor biopsy sampling.

TREATMENTS
All patients received s.c. IL-2 based immunotherapy at low or intermediate dose levels. Due to the outpatient nature of the treatment, patients received instruction, guidance and monitoring during the first days of injections before self-administration in the patients home. Thereafter, only 2-3 ambulatory visits per months were scheduled within the treatment course. An overview of the protocols is given in Table 9 and a graphical overview of the schedules is given in Figure 3.

Main inclusion criteria were bi dimensionally measurable, histologically confirmed mRCC; progressive disease; age 18 to 75 years; Karnofsky performance status ≥ 70; no brain metastases. Pre-study, all patients had a brain-, chest-, abdominal- and pelvic CT scan plus a bone scan. Bone lesions on bone scans were verified by CT scans or X-ray.

CRITERIA FOR TUMOR RESPONSE
Patients were evaluated for objective response, according to standard WHO criteria [159], (a) complete response (CR), defined as total disappearance of all clinical disease; (b) partial response (PR), defined as a reduction of more than 50% in the bidimensionally product diameter; (c) stable disease (SD), defined as a reduction of less than 50% or an increase in size of less than 25%; and (d) progressive disease (PD), defined as an increase in size of more than 25% in the bidimensionally product diameter or appearance of new lesions.

BLOOD SAMPLES
At baseline, all patients had a blood sample, which included differential count, hemoglobin, platelet count, creatinine, sodium, potassium, glucose, bilirubin, alkaline phosphatase, albumin, ALAT, LDH, calcium and ion-calcium and partial thromboplatin time (APTT).

For flow cytometry and cytotoxicity assay, a total of 250 blood samples were obtained serially during treatment in paper II. An additional 28 and 165 blood samples were obtained for the analysis in paper III and VII, respectively. Thus, a total of 443 blood samples were analysed by flow cytometry and cytotoxicity assays.

TUMOR SAMPLES
Core needle biopsies (18G cutting needle) were collected by standard ultrasound-guided procedures [160]. The tissue processing was standardized. Thus, all patients had baseline biopsies and repeated biopsies at day 2 in week 3 and week 8 (non-IFN containing regimen) or at day 1 in weeks 5, 12, 19, 24, 31 and 36, if possible (IFN-containing regimen). (Figure 3 and Table 10 and Table 11). The time-points were selected to coincide with routine outpatient visit, according to the immunotherapy schedule. The biopsies were obtained within the first days of the week, allowing almost identical fixation time for the tumor tissue.

Of the 120 consecutive Danish patients, written informed consent and baseline biopsies were obtained from 101 patients. Four patients did not complete one course of therapy because of toxicity and were not evaluable for objective response. Two patients had only fine needle biopsies performed. These 6 patients were excluded.
from the tumor analyses. Thus, 95 patients had baseline assessable tumor biopsies. The total number of patients having a tumor biopsy is outlined in Table 10. Eleven patients had more than one biopsy obtained at each time point, most frequently from both the primary kidney tumor and a metastatic lesion.

Carefully evaluation of the biopsies demonstrated insufficient tumor tissue and necrosis in 59 biopsies (21%) of a total of 284 biopsies obtained, giving 225 sufficient tumor biopsies. The number of patients with a sufficient tumor biopsy for immune and tumor cell evaluation is indicated in Table 11.

Two cases of deviations from Table 11 and paper V and VIII should be mentioned. In paper V, tumor FasL staining could not be assessed on week 5 in one patient treated with low-dose IL-2, IFN-α, and histamine, rendering a total of 57 patients for the analysis on treatment in paper V. This patient had, however, detectable intratumoral immune cells as well as KI-67-positive tumor cells, and therefore this patient was included in the analysis in paper II, III, and IV.

In one patient with a baseline biopsy only, the intratumoral CD57+ lymphocytes could not be assessed as the neoplastic cells also stained positively for this marker. In this patient, all other immune staining was successful and, thus, this patient was not excluded from the analyses in paper II, III, IV, and V. However, for paper VIII, only patients with a complete set of data were included in the multivariate analysis, rendering a total of 85 patients at baseline for this paper.

Tumor specimens were classified by histological subtype and Fuhrman nuclear grade by a single central pathologist.

METHODS – IMMUNOHISTOCHEMISTRY

Since the introduction in 1991 of microwave oven antigen retrieval in formalin-fixed, paraffin-embedded tissues as an enhancement method for immunohistochemical staining [161], immunohistochemistry of formalin-fixed, paraffin-embedded tissues has been increasingly used and has been thoroughly tested over years [162]. By using this technique, it is possible – in situ – to localize immune cells within the tumor stroma and to locate the protein in cell type and cell structure (nucleus, cytoplasm, membranes). The superior morphology, easy storage for reuse, as well as the feasibility of cutting numerous 2 μm sections from a small core biopsy (measuring at a maximum 1 mm in diameter and 1 centimeter in length) favors paraffin-embedding technique compared with frozen-tissue. However, the precise concatenation of methodological details has a major effect of the final results and, thus, standardization of all procedures is important [162]. Within the present thesis, we focused on (i) standardization of the length of time for the formalin fixation and paraffin embedding; (ii) the time span between cutting of sections and staining as short as possible (few weeks) to avoid protein degradation; (iii) standardization for the microwave oven heating process, e.g. constant target retrieval buffer volume, constant processing time, constant number of slides, – and the use of a microwave oven with a turntable; (iv) standardization of procedures for immunohistochemistry staining. Only well-known and commercially available antibodies were used. All immunostaining were performed in an automatic DAKO TechMate staining machine with a capacity of 120 slides per run. We used EnVision [163] as a simple, two-step visualization system of very high sensitivity to avoid problems with endogenous biotin in tissues as heat-induced epitope retrieval procedures may enhance the reactivity of endogenous biotin [162] highly expressed in especially kidney and liver tissues. Overall, as a result of the standardization efforts, day-to-day variation was reduced and variation contributed by laboratory processing was minimized, but not deleted.

METHODS – ASSESSMENTS OF INTRATUMORAL IMMUNE CELLS

Immunohistochemistry studies normally use semiquantitative scoring assessment of intratumoral immune cells. Based on the observation that immune cells not necessarily infiltrate tumor areas in a homogeneous distribution, we used stereological examination in measuring intratumoral immune cells in order to avoid sampling bias [164]. Unfortunately, this technique is associated with considerable workload and time consumption.

Stereochemistry is mostly about sampling and only little about estimation [165]. The sampling principles are simple: systematic, uniformly random sampling at all levels [165, 166]. The optical fractionator principle is the classical method [167]. Despite only a small fraction of the tumor specimen is actually needed for estimation, the method requires initially the whole tumor for unbiased serial sections. Ultimately, a fraction of the sections is sampled for the study. This final sample is cut by at least 25 μm after processing, drying and mounting, and is the subject for identifications of particles by microscopy [168]. Immunohistochemistry is not implemented in stereology because of the requirement of thick sections. Special issues concerning stereological examination in the present thesis should be mentioned:

(i) As the assessments was based on core biopsies obtained from accessible tumors, the fractionator principle was not applicable [167], and the material is, thus, biased by definition. Thus, estimation the total number of immune cells within the whole tumor was not possible. Therefore we only estimated the number of immune cells/mm² tumor.

(ii) Immunohistochemistry was a prerequisite in the present study as identification of lymphocyte subsets based on morphology alone was impossible. Thus, we were unable to use the disector method. However, as lymphocytes are small and completely uniform cells, we assumed that thin sections might be sufficient for the estimates.

(iii) We only assessed one section of each immune staining per patient. This was done because of the workload and also because of the cost of immunohistochemistry. However, assessing more than one section would have reduced the contribution of variance at this level.

(iv) Whereas patients with progressive disease (PD) had very few and easily detectable intratumoral lymphocyte subsets, responding patients had large numbers of lymphocyte subsets, which in several cases made enumerations difficult. Moreover, whereas tumors in patients with PD remained unchanged, tumors in responding patients loosened, the tumor cell density per mm² tumor diminished, and fibrotic areas developed. This observation has also been described in regressing tumors in

<p>| Table 10. Number of patients having a biopsy. |</p>
<table>
<thead>
<tr>
<th>Dose level</th>
<th>Week</th>
<th>w0</th>
<th>w5</th>
<th>w12</th>
<th>w19</th>
<th>w24</th>
<th>w31</th>
<th>w36</th>
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<td>Low dose</td>
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Timing of tumor biopsies is illustrated in Fig 3. Week 3 and week 5 biopsies were classified as "on-treatment biopsies" in paper # 5.

<p>| Table 11. Number of patients with a sufficient biopsy |</p>
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<th>Dose level</th>
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metastatic melanoma [169]. According to our counting rules, necrotic and fibrotic areas were avoided and only a cell with staining restricted to the plasma membrane, a visible nucleus and located within the counting frame was counted as positive.

The unit for intratumoral immune cell infiltration was given as number of immune cells per mm$^2$ tumor. This unit does not take into account regressive changes in responding patients. The immune – tumor cell ratio would have been a more accurate unit for the measurements. Thus, we may have underestimated the immune infiltrate in infiltrating patients.

(v) Using this technique with an unbiased counting frame, a high level of reproducibility was found, as has also been demonstrated by others [170, 171].

METHODS – CHROMIUM-RELEASE ASSAYS

In order to assess the killing potential of NK cells – which should be regarded as the most important functional capability - the $^{51}$Cr-release 4-hour assay against the NK sensitive target K562 cell line was used. This assay is the gold standard but is, however, associated with a considerable workload [172].

METHODS – FLOW CYTOMETRY

Flow cytometry employs instrumentation that scans single cells flowing past excitation sources in a liquid medium. The technology is unique in its ability to provide rapid, quantitative, multiparameter analyses on single cell characteristics [173]. Flow cytometry has been thoroughly tested over years. The Flow cytometer used in the present thesis was regularly calibrated. Lymphocytes were gated on the basis of forward and side scatter. At least $10^4$ cells were analysed for each sample. The percentage of lymphocyte subsets obtained by flow cytometry was converted into absolute numbers by multiplying with the simultaneously obtained differential lymphocyte count.

METHODS – STATISTICS

The statistical methods are standard for this type of data. P-values are two sided. For comparing two unpaired groups, the nonparametric Mann-Whitney U-test or Fisher’s exact test were used. For comparing two paired groups, the Wilcoxon signed rank test was used. For comparing three or more unmatched groups, the Kruskal-Wallis test was used. The cumulated survival rate was analysed by Kaplan-Meier and the log-rank test was used to analyse for survival differences among subgroups of patients. Overall survival was measured from first day of treatment until death or last follow-up evaluation. Time to progressive disease was measured from first day of treatment until disease progression. All calculations were performed using SPSS 10.0 and 11.0 statistical software.

RESULTS

An overall overview of the results that form the thesis is summarised in the following. A detailed description of the results is given in the individual articles.

1. The thesis represented the establishment in Denmark of s.c. IL-2 based immunotherapy for mRCC as an outpatient home-therapy and, moreover, documented the safety and feasibility of the treatment (Article I and VI).

2. Of 120 Danish patients with mRCC – initially estimated as non-candidates for curative surgery – receiving IL-2 based immunotherapy, a total of 12 patients (10%) achieved no evidence of disease (NED) after either immunotherapy-only (n=5), immunotherapy plus surgical resection of residual disease (n=5) or surgery plus re-treatment with immunotherapy (n=2).

3. Of 120 Danish patients receiving IL-2 based immunotherapy, the estimated 5-year survival rate was 16% (Paper VIII). This seems consistent with results previously obtained with intermediate to high doses of i.v. IL-2 based immunotherapy (Figure 4).

4. IL-2 based immunotherapy, administered subcutaneous in low- or intermediate dose levels, was able to induce objective tumor responses in a minority of patients (Paper I, III, VI and VIII).

5. The present thesis represented the introduction of histamine as an adjuvant to IL-2 based immunotherapy in mRCC. In a low-dose schedule of IL-2 and IFN-α, histamine did not appear to add efficacy (Paper I). However, in two randomised phase II studies, in which the dose of IL-2 was doubled and IFN-α was omitted, the Danish study showed a trend towards benefit in favor of IL-2/histamine, whereas the UK study was negative for all end points (Paper VI). Thus, a randomised phase III trial is warranted to clarify the potential role of adding histamine to IL-2 in mRCC.


Figure 4. Overall survival of 120 patients with mRCC receiving IL-2 based immunotherapy. Median survival 15.1 months. Estimated 5-year survival 16%. Vertical and horizontal axes, the survival probability and months of follow-up, respectively. Tick marks (†) indicate last date of follow-up.

Figure 5. A patient with baseline lung metastasis and lymph node metastases in retroperitoneum and left hilus (left lane, arrows). The patient received IL-2 and histamine and achieved complete response. The patient is alive with no recurrence after 43 months of follow-up.
7. In responding patients, cytokine therapy induced substantial changes in the blood and tumor tissue leukocyte composition, correlated with both response and survival. In contrast, in progressing patients, both the absolute number and the relative composition of leukocyte subsets in blood and tumor tissue remained unaffected by cytokine therapy (Paper II, III and V).

8. Circulating as well as intratumoral phagocytes— monocytes and neutrophils— were shown to be powerful negative prognostic factors for IL-2 based immunotherapy (Paper III, VII and VIII).

9. The thesis emphasized the value of accompanying clinical trials with blood and tumor tissue assessments. Based on the randomized trials by themselves, no effect of adding histamine to IL-2 was recognized. However, by the biological analyses, a potential effect of histamine was clearly demonstrated (Paper VII).

10. Our data suggested that IFN-α in vivo had only modest effect on tumor proliferation in patients with mRCC. Tumor Ki-67 (MIB-1) reactivity after one month of immunotherapy appeared to be a significant predictor of patient survival (Paper IV).

11. Our observations did not support the hypothesis that FasL tumour "counterattack" has an effect on the clinical outcome in metastatic renal cell carcinoma during IL-2 based immunotherapy (Paper V).

12. The thesis pointed on five clinical and three supplemental immunological independent prognostic factors of survival in patients with mRCC receiving IL-2. The three independent immunological parameters had significant discriminatory power as supplemental risk factors in prognostic models based on the clinical risk factors, identifying subgroups within the favorable clinical group with estimated 5-year survival rates of 60%, 25% and 0%, respectively (Paper VIII).

**DISCUSSION**

Detailed discussions of the different aspects of the thesis are included in the individual articles. The main and central issues are further discussed in the following.

With a worldwide mortality of 102,000 in the year 2002, RCC remains a major challenge for the oncologist. There is no effective treatment for the large majority of patients where the disease is a much more powerful determinant of outcome than any therapy [77]. Thus, RCC remains a model for the translation of laboratory research into clinical practice, as demonstrated in the present thesis— the first systematic collection of serial tumor core biopsies and blood samples during IL-2 based immunotherapy [175]. For comparison, the study by Bukowsky [175] comprised 27 biopsies (at baseline and 14 during or after therapy) from 17 patients; the study by Cohen [176] included one patient (baseline and after therapy biopsy); and the study by Rubin [177] included 12 patients with baseline nephrectomy only. Only the study by Bukowsky [175] also included blood samples, but these were obtained at different time points than the tumor biopsies. No consistent finding has been demonstrated in these studies. Thus, an effort to integrate immune cellular infiltrates with standard clinical criteria for tumor regression has largely not been done [174]. For comparison, based on 443 serial blood samples and 225 serial tumor core biopsies in the present thesis, we were able to map the orchestration of immune cells in blood and tumor at baseline and during IL-2 based immunotherapy. An understanding of IL-2 based immunotherapy as a "targeted therapy" requiring immune cells for tumor rejection emerged from these analyses. Moreover, the "image" of the immune cell composition in peripheral blood seems "delayed" compared with the "image" of the intratumoral immune cell composition. In short, analyses revealed that high numbers of baseline intratumoral CD4+, CD8+, CD56+ and CD57+ T- and NK cells and high numbers of on-treatment intratumoral CD3+, CD4+, CD8+ and CD57+ T- and NK cells were significantly correlated with objective response. In contrast, no baseline blood immune cells were correlated with objective response. High numbers of on-treatment total blood lymphocytes, CD3+, CD3+CD56+ and CD57+ lymphocyte subsets and low numbers of blood neutrophils were significantly correlated with objective response. The immune cells were also correlated with survival. These analyses revealed that no blood lymphocyte subsets were correlated with survival whereas high numbers of baseline blood neutrophils, on-treatment blood neutrophils and on-treatment blood monocytes were correlated with short survival. Of intratumoral immune cells, high numbers of baseline CD57+, baseline CD4+, and on-treatment CD3+ were significantly correlated with favorable survival whereas presence of baseline neutrophils was correlated with short survival. Thus, neutrophils and monocytes/macrophages are "bad guys" and T cells and NK-cells are "good guys" for the outcome of IL-2 based immunotherapy. All statistical differences for blood and tumor phagocytes have the same negative direction with both objective response and survival as endpoint. In contrast, all statistical differences for blood and tumor lymphocyte subsets have the same positive direction with both ob-
the clinic. The present thesis indicates a direction for future clinical immunotherapy efforts.

**GENERAL CONCLUSIONS**

- Immunotherapy should always be accompanied by laboratory questions based on blood and tumor sample collections.

Histamine dihydrochloride as an adjunct to IL-2 is an example of translational research from basic biology to clinical trials. Following the first observation in 1982 by Seaman et al [146], a large number of in vitro and in vivo observations from independent laboratories have supported the observation of oxidative suppression of NK and T cells by phagocytes (i.e. monocytes, macrophages and neutrophils) [147, 150-152, 182]. Therefore, in basic tumor immunology – in the preclinical setting – the negative effect of NO and H₂O₂ produced from activated macrophages and neutrophils on NK and T cell functions is well-established [143]. The observations by Kristoffer Hellstrand et al that histamine protects NK and T cells against oxygen radical-induced dysfunction and apoptosis by specifically blocking the formation and release of hydrogen peroxide (H₂O₂) from phagocytes, and moreover, maintains the activation of NK and T cells by IL-2 and/or IFN-α, are compelling [148, 153, 155, 157, 158, 183-186]. Therefore, the pre-clinical basis for introducing histamine in combination with IL-2 and/or IFN-α in clinical trials was obvious. Despite that, several clinical trials of histamine in combination with IL-2 and/or IFN-α were unsuccessful in acute myelogenous leukaemia [187], chronic hepatitis C [188], multiple myeloma [189], metastatic melanoma [190-192] and mRCC [193]. However, our biological analyses in mRCC (paper VII) support the oxidative stress hypothesis [143, 154, 194-196] and a potential effect of histamine was clearly demonstrated. This emphasizes the value of accompanying clinical trials with blood and tumor tissue assessments. Indeed, our assessment of the oxidative stress hypothesis in blood and tumor tissue is the first to establish a biological rationale in humans for the use of histamine in conjunction with IL-2. Thus, targeting H₂O₂ by histamine seems to enhance the antitumor activity of IL-2 in situ in a subgroup of patients with low monocytes/neutrophils or high NK cells. The explanation for the lack of a histamine effect when IFN-α was co-administered is unclear.

Since its introduction to the clinic in 1985 [83], IL-2 remains the only established immunotherapy approved by the US Food and Drug Administration for the treatment of mRCC. However, despite 20 years of clinical trials, no combination therapy has proved better than IL-2 treatment alone in terms of long-term survival [88, 90]. Thus, cancer immunotherapy is most dependent on the application of advances in knowledge of basic science and for its translation to the clinic. The present thesis indicates a direction for future clinical immunotherapy efforts.

**FUTURE ASPECTS**

The results obtained in this thesis have to be confirmed in independent and larger studies. First of all, the validation of our prognostic model – based on clinical factors supplemented with immunological factors – is of high priority as these features may help to select patients more likely to benefit from IL-2 based immunotherapy. A validation study is currently underway examining 120 patients treated at the Department of Oncology in Aarhus, between September 2002 and September 2004 with low-dose IL-2 and IFN-α (same schedule as in the present thesis). Moreover, an international kidney cancer working group has been established to identify independent, validated predictors of survival and ultimately to develop a single validated prognostic model, based on >4000 mRCC patients.
receiving IL-2 based immunotherapy [197]. The possibility of validation of our three independent immunological features – blood neutrophils, tumor neutrophils and tumor CD57+ NK cells – in this >4000 patient material will be pursued.

Secondly, the translation of our results from metastatic disease to primary RCC will be an important future aspect. We will evaluate our hypothesis – that IL-2 based immunotherapy is a “targeted therapy” requiring immune effector cells for tumorlysis – in an adjuvant setting. Thus, adjuvant IL-2 based immunotherapy should only be offered to patients with >50 CD57+ NK cells/mm² tumor tissue, no intratumoral neutrophils, and blood neutrophils <6.0 × 10⁹/L. This should be evaluated in a randomised adjuvant trial.

Third, future developing and assessing drugs that block the generation of oxygen radicals as an adjunct to IL-2 is a viable therapeutic opportunity in renal cell cancer. These drugs – including histamine – should be evaluated in randomised trials appropriately stratified for monocytes and neutrophils in blood and tumor.

Fourth, the improved understanding of the biology associated with renal cancer have generated a large number (>20) of new drugs, “targeted biological therapies”, currently assessed in mRCC. Whether these new drugs – either alone, as first line therapy or in combination with IL-2 and/or IFNα – will improve survival rates will be the cutting edge questions for clinical trials in the foreseeable future. A lesson learned from the present thesis is that it would be beneficial to require (serial) blood and tumor tissue collections in parallel with these clinical studies.

**SUMMARY**

The present thesis consists of 8 published articles focusing on interleukin-2 based immunotherapy in metastatic renal cell carcinoma (mRCC). This disease represents a significant challenge, as the tumor is resistant to current chemotherapy, hormonal therapy and radiation therapy. However, IL-2 based immunotherapy may induce dramatic durable tumor regression by manipulating the immune system, however, only in a minority of patients.

Two critical questions have driven the present thesis. First, which properties of the immune system are responsible for the dramatic tumor regression seen in some patients with mRCC following IL-2 administration? And second, can histamine increase the efficacy of IL-2 based immunotherapy by ending the immune suppression induced by phagocyte-generation of reactive oxygen species?

120 Danish patients, 41 UK patients and 20 Swedish patients were treated with low- or intermediate dose IL-2 based immunotherapy in an outpatient setting. As monitoring of the Danish patients, 443 serial blood samples and 225 serial tumor core biopsies were obtained.

The regimen of outpatient low-dose subcutaneous IL-2 and IFN-α in mRCC is safe and active. In the Danish patients, an estimated 5-year survival rate of 16% was observed.

From the blood and tumor analysis, an understanding emerged that IL-2 based immunotherapy is a “targeted therapy” requiring intratumoral immune cells (CD4+, CD8+, CD56+, CD57+ T- and NK cells) for treatment effect. In contrast, monocytes and neutrophils were harmful for the outcome of IL-2 based immunotherapy. In progressing patients, the leukocyte subsets in blood and tumor tissue remained unaffected by cytokine therapy.

The fate of a patient with mRCC prior to IL-2 and IFN-α based immunotherapy cannot be determined by measuring baseline tumor features of FasL expression or Ki-67 (MIB-1) proliferation marker.

We established a biological rationale for the potential use of histamine in conjunction with IL-2 in mRCC. A large confirmatory randomised phase III trial of IL-2 with and without histamine in mRCC appropriately stratified for monocytes and neutrophils in blood and tumor tissue is warranted.

In a multivariate analysis, 5 clinical features (PS, bone metastases, lymph node metastases, low hemoglobin and high LDH) plus 3 supplemental immunological factors (intratumoral CD57+ NK cells <50 cells/mm², intratumoral neutrophils >0 and blood neutrophils >6.0) were independent prognostic factors of short survival in patients with mRCC receiving IL-2, identifying subgroups with estimated 5-year survival rates of 60%, 25% and 0%, respectively. These features may help to select patients more likely to benefit from IL-2 based immunotherapy.

**ABBREVIATIONS**

CR: Complete response  
RCC: Renal cell carcinoma  
mRCC: Metastatic renal cell carcinoma  
HDC: Histamine dihydrochloride  
HLA: Human leukocyte antigen  
H₂O₂: Hydrogen peroxide  
IFN-α: Interferon-alpha  
IL-2: Interleukin-2  
LDH: Lactate dehydrogenase  
NK: Natural killer cells  
NO: Nitrogen oxide  
PR: Partial response  
PS: Performance status  
ROS: Reactive oxygen species  
SD: Stable disease  
PD: Progressive disease  
VHL: von Hippel Lindau

**THIS DOCTORAL THESIS IS BASED ON THE FOLLOWING ORIGINAL PAPERS**


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