Title: Detection of Circulating Tumor Cells in the Peripheral Blood of Solid Tumor Patients

Author: András Ladányi, M.D.

Mentors: Zsolt Tulassay, M.D., Ph.D.,
Béla Molnár, M.D., Ph.D.

Place of preparation: Semmelweis University,
School of Ph.D. Studies,
Clinical Sciences

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Reviewers:
Zsuzsanna Schaff, M.D., Ph.D.,
Margit Abonyi, M.D., Ph.D.,
János Banai, M.D., Ph.D.,
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1 Introduction

1.1 Health relevance and cancer facts

Each year, almost 7 million people die from cancer, and close to 11 million new cases are diagnosed worldwide. According to the World Health Organization (WHO), 12.5% of all deaths every year are caused by cancer [1]. That's more than the total percentage of people who die from AIDS, tuberculosis, and malaria put together [2]. The number of new cases annually is estimated to rise from 10.9 million in 2002 to more than 16 million by 2020, if current trends continue. It is estimated that there are 24.6 million people alive who have received a diagnosis of cancer in the last five years [3].

In Europe there were over 1.7 million deaths from cancer in 2004 and nearly 2.9 million new cases were diagnosed. Cancer is the second main cause of death in Europe, after circulatory diseases. According to Eurostat Health Statistics 2002 report the age-standardized European population incidence rate in the European Union (EU) in 1997 for all cancers combined (excluding non-melanocytic skin cancers) was of 345.1 new cases per 100,000 for the two sexes together, or 419.0 per 100,000 for men and 296.8 per 100,000 for women. At the incidence rates prevailing in 1997, it would be expected that one in three men and one in four women would be directly affected by cancer in the first 75 years of life.

According to the data of the National Cancer Registry of Hungary there are approximately 75,000 new cancer cases diagnosed each year and about 33,000 people die of the disease [4]. In 1995 the incidence of cancer per 100,000 population in Hungary was 566.6 and 357.2 in men and women, respectively [5]. In Hungary mortality caused by cancer is very high; cancer is responsible for approximately 25% of all deaths in the country [6]. In the EU cancer mortality in the year 2000 varied by a factor of more than 2 between the highest rate of 258.5 per 100,000 men in Hungary and the lowest rate of 122.0 per 100,000 men in Sweden [7]. Specifically, the mortality of lung cancer, the most fatal cancer in Hungary, is almost twice as high in Hungary as in the EU. (83.1/100,000 vs. 43.7/100,000, respectively) [8].

By various European and international studies Hungary is leading cancer mortality in men and ranks second place in women worldwide [9]. Although, many factors might contribute to the unfavorable cancer statistics of Hungary, according to most investigators the widespread habit of smoking and alcohol consumption, poor dietary patterns, inadequate screening, dishonest patient-doctor relationship and unsatisfactory care of cancer patients are the key responsible factors [10]. At the time of the preparation of this thesis there are about 170,000 people in Hungary receiving treatment for cancer and there are about 300,000 people currently living with the disease.

1.2 Tissue distribution and mortality of the most common solid epithelial malignancies

Solid tumors constitute about 85-90% of all malignant growths. Among solid tumors malignant neoplasms of epithelial cell origin, or as commonly called carcinomas, are the most common cancers and constitute the focus of the current thesis.

Overall, cancers of the lung, breast, bowel, stomach and prostate account for almost half of all cancers diagnosed worldwide [11]. However, the types of cancer being diagnosed vary enormously across the world. According to 2002 estimates the most common type of cancer worldwide is lung cancer with approximately 1.4 million new diagnoses each year, it accounts for 12% of all new cancer cases [12][*]. Breast (11%), followed by colorectal cancers (9%) are the second and third most common cancer diagnoses. Globally the most common causes of cancer deaths are lung (18%), stomach (10%) and liver cancers (9%).

According to the American Cancer Society (ACS) the leading sites of new cancer cases in the US in men include prostate (33%), lung (13%) and colon & rectum (10%) cancer [13]. While, in women the top three sites include breast (32%), lung (12%) and colon & rectum (11%)[†]. Lung cancer is the most common fatal cancer in men (31%), followed

[*] Excludes skin cancers.
[†] Excludes basal and squamous cell skin cancers and in situ carcinoma, except for urinary bladder.

by prostate cancer (10%), and colon & rectum cancer (10%). In women, lung (27%), breast (15%), and colon & rectum (10%) are the leading sites of cancer death.

In the EU in both sexes combined lung cancer was the commonest form of cancer diagnosed (13.2%) and of cancer death (20%). Although, colorectal cancer is almost equally common (13%), it represented a smaller proportion of deaths (11.9%). In men, lung cancer was the commonest form of cancer, followed by prostate cancer (15.5%). It is worth noting that prostate cancer was the commonest form of cancer diagnosed in men in 2004 in the EU. A strong decrease in prostate cancer mortality has been seen in Europe overall since 1992. Among women, breast cancer was by far the most common, representing 27.4% of all female cases and it was also the biggest killer with 17.4% of the total deaths. One cancer in eight in Europe was breast cancer and this form of cancer represented 7.6% of all cancer deaths. Breast cancer mortality has been declining over the last decade in the EU but not in central and eastern European countries [14].

According to the 2004 data of the National Cancer Registry of Hungary the most common newly diagnosed cancers in men are lung, colorectal and prostate representing 17.5%, 12.5% and 10.7% of the new cancer cases, respectively.* In women breast, colorectal and lung cancer was contributing to the number of new cancers cases with 20.1%, 11% and 9.1%, respectively. According to the 2003 data of the Hungarian Central Statistical office in both sexes combined lung cancer was the most common fatal cancer, responsible for 24.5% of all cancer deaths. In cancer mortality lung cancer is followed by colorectal (15.2%) and breast cancer (7%). When the two genders are analyzed separately the most common fatal cancers in men were lung (31%), colorectal (14.8%) and oral cavity cancer (7.7%). Lung cancer rates have been rising in Hungarian women and became the main cause of cancer death since 2002.

Globally, Poland and Hungary have the highest standardized rates for male and Denmark, Iceland, UK and Hungary have the highest standardized rates for female lung cancer in the world [15]. In 2003 lung cancer represented 16% of all cancer deaths. Like

* Excludes skin cancers

in men, colorectal cancer is also the second most fatal cancer, followed closely by breast cancer, accounting for 15.71% and 15.7% of cancer deaths in women, respectively.

1.3 Survival rates of cancer and the most common solid epithelial malignancies

Relative survival is the preferred method for analyzing the survival of cancer patients in population-based studies. Relative survival compares the observed survival for a group of cancer patients with the survival of members of the general population who have the same characteristics, such as, age, gender, and province of residence, as the cancer patients. Relative survival of cancer is often expressed as relative 5-year survival rates.
The 5-year survival rate is defined as the percentage of people alive at least five years after diagnosis of cancer. The 5-year relative survival rate calculation includes cancer patients who are living 5 year after diagnosis, whether disease-free, in remission or under treatment with evidence of cancer and excludes people who die of other causes. The 5-year relative survival rates are useful indicators of diagnostic and therapeutic efficacy, but since cancer can affect survival beyond 5 years after diagnosis they do not represent the proportion of people who are cured permanently from the disease. Furthermore, it should be noted that differences in survival across geographic areas and across time may arise from variations in diagnostic techniques, in the use of early detection strategies, the availability and effectiveness of treatments, and differences in the cancers that occur in a specific population.

However, with covering approximately 26 percent of the total US population probably the most comprehensive cancer survival statistics around the world are prepared by the Surveillance, Epidemiology and End Results (SEER) Program of the National Cancer Institute (NCI). Based on the SEER Cancer Statistics Review's data of the most recent year for which data are available the relative 5-year survival rate of cancer for all sites combined is 65% [16].Comparing to the 50% value of the relative 5-year survival rate of the first SEER reporting period from 30 years ago (1975-1979), this can definitely be considered as an improvement in the management of cancer patients. This is due, in part, to both earlier detection and advances in treatment. However, if we consider that during the same period, the age-adjusted mortality rate from all other causes of death

declined by 38%, it emphasizes the need for further refinements of prevention, diagnosis and treatment of cancer patients.

Many European countries publish cancer survival information but differences in data and methodology make comparisons problematic. EUROCARE (European Concerted Action on survival and Care of Cancer Patients), a collaboration of European cancer registries started in 1990, aims to standardize data collection, management and presentation throughout Europe. Three sets of results have been published by EUROCARE and according to the data of the most recent study, in which the survival of 1,815.584 adult cancer patients diagnosed from 1990 to 1994 in 22 European countries were analyzed, the five-year relative survival rates for all cancers combined in the EU is 40% in men and 52% in women [17][*]. The age-standardized 5-year relative survival of these 1990–1994 cases was a remarkable 10% higher than found by EUROCARE-2 study for cases diagnosed in the period 1985–1989, reflecting the same tendency of improvement that can be observed in the US [18].

Survival for all cancers reflects the case mix by cancer site, which differs from country to country and in this respect is a somewhat crude measure. A more correct comparison of relative survival for all malignant neoplasms would require adjusting relative survival by cancer site and stage at diagnosis.

In ***Table 1*** the relative 5-year survival rates of the top 4 most common cancers in the US are summarized by stage at diagnosis. In ***Table 2*** the relative 5-year survival rates of the top four most common cancers in Europe are summarized. Unfortunately, there is not any Europe-wide stage distribution data available for these cancers, but high resolution EUROCARE data is available for a few countries and will be presented later in *Chapter 1.5*.

[*] Survival data were collected on all malignant cancers as defined by codes 140–172 and 174–208 of the ninth revision of the International Classification of Diseases (ICD-9) and also for cases of non-malignant transitional cell neoplasms of the bladder. Nonmelanoma skin cancers and in-situ carcinomas were excluded.

In relation to lung cancer the age-standardized 5-year relative survival for lung cancer varied more than two-fold across Europe. Studies addressing the question of the reasons behind this considerable variation in survival found that differences in stage at diagnosis between some European countries seem to be the major factor [19]. As for lung cancer disparities in colorectal cancer survival between European regions exist. Studies that collected data on treatment and stage at diagnosis suggested that variation in stage at presentation is the most likely cause of survival differences; however, in some areas, the quality of care may also play a role [20, 21]. Particularly for colorectal cancer there are wide variations in diagnostic and surgical practices throughout Europe. More recently, testing for Prostate-Specific Antigen (PSA) has further increased the incidence rate of prostate cancer. The slow geographic and temporal spread of transurethral resection and PSA testing, which result in increases in the detection of early good-prognosis prostate cancers, is blamed for the conspicuous between-country variation in Europe in age-standardized 5-year relative survival for prostate cancer [22, 23]. The prognosis for breast cancer is relatively good, with 5-year relative survival exceeding 75% in most countries of Western-Europe. Since the early 1990s, many European areas have implemented mass screening for breast cancer and this is having effects on incidence, mortality and survival [24]. However, breast cancer survival is particularly low in Eastern-European countries with 5-year relative survival between 60 and 67%. The marked difference across Europe in female breast cancer survival, noted by the previous EUROCARE studies, was also found for patients diagnosed in the period 1990–1994 (EUROCARE-3) [25].

As stated above, the interpretation of inter-country survival differences is confounded by many factors, e.g. disease definition, screening or early diagnostic activity, the extent of cancer registries, completeness of case collection; and quality of follow-up for vital status, etc. However, it is commonly believed by leading Hungarian health professionals that cancer patients in Hungary have one of the worst outcomes in Europe [26, 27]. According to the vice director of the National Oncology Institute, Hungarian cancer statistics are much worse than could be justified given the country's level of development and the age profile of its population [6].

The main difficulty with interpreting Hungarian survival statistics is that there are not any officially audited data for cancer survival. Moreover, Hungary did not participate in any of the EUORCARE studies. Thus in this thesis we use other Central and Eastern-European countries' EUROCARE-3 data with similar cancer incidence, mortality and health care system as an example for comparison. For all cancers affecting female patients five-year survival rates were 43.4% in the Czech Republic and 35.3% in Poland compared to 52% across Europe and 65% in the USA. A specific example in women is for breast cancer, in which Poland and the Czech Republic were achieving a 63% survival rate compare to 77% overall in Europe. The survival gap in men's is also observable. The European 5-year weighted relative survival for all cancers was 40% in men, while in the Czech Republic, in Estonia and in Poland it was 29.3%, 25.7% and 21.9%, respectively.

The only data source available at the moment from Hungary is from a report, entitled "A pan-European comparison regarding patient access to cancer drugs", conducted by the Karolinska Institute and the Stockholm School of Economics in 2000 [28]. Nineteen countries, representing almost 75% of Europe's population, were included in this report. The 1- and 5-year survival rates in Hungary and in selected European countries are presented in ***Figure 1***. This report highlighted that cancer patients only in Poland had worse outcome than in Hungary.

When looking for reasons behind the dismal prognosis of cancer in Hungary experts say that many cancer patients are not getting adequate treatment for their disease. Below are opinions of leading health professionals about the status of Hungarian oncology practice in 2005.

According to

Dr. Miklós Szûcs,	Chairman of the cancer consultative body the Radiation and Oncology College of Experts

- 15-20% of cancer patients get care from hospitals which lack the necessary expertise and equipment [29].
- Cancer patients are often given an inappropriate combination of drugs or undergo radiation treatment for a shorter period than is required.

Dr. Arthurné Vasvári, President of the Hungarian Anti-Cancer League

- Cancer treatment is often profit-oriented because some smaller hospitals have less experts, no modern equipment and, above all, don't have the means to buy the best medication.

Dr. György Bodoky, Head of the Hungarian Society of Clinical Oncology and Head of the Oncology Department of Szent Laszlo Hospital, Budapest

- Screening, early detection and access to medications are the major pitfalls of cancer survival in Hungary [30].

Dr. László Thurzó, Head of the Oncology Clinic of Szeged

- Only 30–35% of the patients was referred to follow-up care and was therefore treated adequately. The others disappeared from the system [27].

Dr. Magdolna Dank, Head of the Department of the Radiological and Oncology Clinic

- The efficacy of a clinic, department or doctor is rarely monitored.
- There is a lack of specialists in medical oncology, radiation oncology, radiology and pathology [27].

Drs. Nils Wilking and Bengt Jönsson, Karolinska Institute and Stockholm School of Economics

- Despite the proven benefits of new innovative treatments options, patients across Europe do not have equality of access to these cancer drugs and the speed at

which patients can benefit from them depends to a great extent upon the country in which they live. The adoption and availability of new cancer drugs in the UK, Czech Republic, Norway, Poland and Hungary lag behind [31].

1.4 The social and economic burden of cancer

The WHO has defined "burden of disease" as the significance of a particular disease for society beyond the immediate cost of treatment [32]. The most commonly used measure of the burden of cancer is Disability-Adjusted Life Years' (DALYs). This is an integrated measure of mortality and disability developed by the WHO and the World Bank [33, 34]. This indicator is the aggregation of Years of Life Lost (YLL) and Years Lived with Disability (YLD) at the population level and thus truly reflects 'the burden of disease'. In other words, one DALY can be thought of as one lost year of 'healthy' life and the burden of disease as a measurement of the gap between actual health status and an ideal situation where everyone lives into old age free of disease and disability.

In 2000, cancer accounted for close to 20 million DALYs lost in the EU. As a proportion of all DALYs lost in 2000, cancer was fourth only to cardiovascular disease, mental illnesses and injuries. In terms of overall disease burden, cancer represents 11.5% of all DALYs lost. In ***Figure 2*** the top four causes of burden of disease in the whole WHO European Region are presented.

As can be expected from the unfavorable cancer statistics of Hungary it is causing a huge burden on our society. According to the 2005 WHO European health report, which divide the WHO European Region into three such subregions[*], cancer is responsible for more than 5.3 million DALYs lost in Eur-C countries [35]. In Hungary just lung cancer alone constitutes more than four percent of all DALYs and ranks 6th place in the order of lost DALY causes (***Table 3***). Another piece of information that well represents the weight of this problem is that in the past 25 years the number of people registered

[*] Based on child and adult mortality rates the WHO European region is divided into three subregions, called Eur-A, -B and -C. Hungary is grouped into the Eur-C region together with countries like Belarus, Estonia, Kazakhstan, Latvia, Lithuania, the Republic of Moldova, the Russian Federation and Ukraine.

disabled because of cancer has doubled in Hungary, despite the fact that the criteria for being registered disabled have been tightened significantly [36].

The financial costs of cancer can be divided into direct and indirect costs. Direct costs are the resources used for prevention, treatment, etc. Indirect costs are resources lost due to an inability to work and are relevant for diseases that strike in the early years before normal retirement. Indirect costs include costs of lost production due to short-term absence from work, permanent disability and death before 65 years of age. Despite the fact that most cancers occur in older persons, indirect costs of cancer are still 2-3 times greater than the direct costs and constitute a major part of total costs for all diseases.

In the US the National Institute of Health (NIH) estimate that the overall costs for cancer in 2004 at $189.8 billion: $69.4 billion for direct medical costs; 16.9$ billion for indirect morbidity costs; and $103.5 billion for indirect mortality costs. This is just under 5 percent of total US spending for medical treatment [37].

The direct healthcare cost for cancer in the 19 European countries covered by OECD is estimated at €54 billion or €120 per inhabitant in 2002/2003. The total direct costs for cancer in Hungary and in selected European countries, the per capita and share of total healthcare expenditures are shown in ***Table 4***. There are no reliable data on the indirect costs of cancer in EU, but it is estimated that it accounts for 70-85% of the total costs [38].

Comparing total and per capita health care expenditures between Central and Western-European countries it can be deduced that there is still a wide gap in resources. In light of this, survival outcomes in Hungary, that was about two thirds of those in Western-Europe, are in fact indicative of a remarkable achievement in very difficult circumstances. However, improvement on the socio-economic burden can only be excepted if all three fronts of cancer managements are addressed.

- Better general health – primary prevention and lack of co-morbid conditions that allow more aggressive treatments

- Diagnosis – earlier diagnosis and thus better stage distribution
- Treatment – earlier and more adequate treatment in access to all cancer patients

As the health sector is driven by scientific and technological progress it has to be noted that the overall costs of treating cancer increased by 75% over the past 10 years. In the near future it is expected that cancer costs may increase at a faster rate than overall medical expenditures. As the population ages, the absolute number of people treated for cancer will increase faster than the overall population, and cancer cases will increase relative to other disease categories-even if cancer incidence rates remain constant or decrease somewhat. Costs also are likely to increase at the individual level as new, more advanced, and more expensive treatments are adopted as standards of care.

1.5 Stage distribution and survival of epithelial cancers

ACS says that 'If you can't prevent cancer, the next best thing you can do is to detect it early.' For many types of cancer the stage at diagnosis has a massive impact on how likely it is that a patient can be successfully treated. Detecting cancers at an early, more treatable stage is a major goal of cancer control efforts, and knowledge of the stage of disease at the time of diagnosis is essential for determining the choice of therapy and in assessing prognosis.

In the 1940s, a generalized staging classification of localized, regional, and distant disease was developed to show long-term trends of cancer outcome. Since then this staging scheme has been used in many descriptive and statistical analyses of tumor registry data [39]. The definitions of stages are described below.

Localized: An invasive malignant neoplasm confined entirely to the organ of origin with no lymph node involvement.

Regional: A malignant neoplasm that (1) has extended beyond the limits of the organ of origin directly into surrounding organs or tissues; or (2) involves regional lymph nodes by way of the lymphatic system; or (3) has both regional extension and involvement of regional lymph nodes.

Distant: A malignant neoplasm that has spread to parts of the body remote from the primary tumor either by direct extension or by discontinuous metastasis (e.g., implantation seeding) to distant organs, tissues, or via the lymphatic system to distant lymph nodes.

Based on this staging scheme the 1975-2001 SEER data of common cancers in the US are shown in ***Figure 3*** [40]. The corresponding 5-year relative survival rates of these tumors were presented in ***Table 1***. This data show that stage has a profound effect on outcome. Based on this one could speculate that earlier diagnosis may reduce cancer morbidity, since treatment for earlier-stage cancers is often less aggressive than that for more advanced cancers, and mortality, since earlier stage cancers are generally more responsive to treatment. In fact, the 5-year relative survival rate for people with cancers for which the ACS has specific early detection recommendations (breast, colon, rectum, cervix, prostate, testis, oral cavity, and skin) is about 82%. By ACS estimates if all Americans had early detection testing according to their recommendations, the 5-year relative survival rate for people with these cancers would increase to about 95% [41].

As we have shown earlier in *Chapter 1.3* many cancers in Europe have inferior outcome than in the US. This difference has also largely been attributed to the less favorable stage distribution of European cancers. A large study published by *Sant et al.* analyzed survival rates among women diagnosed between 1990 and 1992 using clinical data from population-based case series from the SEER program (13,172 women) and the EUROCARE project (4,478 women) [42]. This study examined the impact of disease stage, age, surgery, and the number of axillary lymph nodes (LNs) evaluated after lymphadenectomy on survival. The analysis concluded that breast carcinoma was diagnosed at a later stage in Europe than in the US. Early-stage tumors (T1N0M0) comprised 29% of all cases in the European data and 41% in the US data, whereas large LN-negative tumors (T2–3N0M0) were slightly more frequent in the SEER data than in the EUROCARE data set (19.2 and 18.6%, respectively). Both locally advanced (T4M0), LN-positive (T1–3N+M0) and metastatic tumors (M1) were more common among European patients than US patients (6.8%, 31% and 6.2% in Europe vs. 5.6%,

24% and 5.9% in the US, respectively). Lymphadenectomy was equally common in both series (81–82%), but was more extensive in the US than in Europe. For example, approximately half of the U.S. women (51%) had ≥15 LNs evaluated, compared with less than one-third of European women (28%). When stage specific survival was compared it was found that survival was higher in the US for each stage category, except for women whose disease stage at diagnosis was unknown. Survival was similar for early-stage tumors (100% in the US series and 97% in the European series) but the difference increased with advancing stage and was maximal for locally advanced tumors (T4M0), 71% in US vs. 55% in Europe.

At first glance this might suggest that breast cancer treatment in the US is more effective than in Europe. However, as mentioned above, the average number of axillary nodes examined was higher in the US series, indicating a more thorough search for occult metastasis. As comparable data were not available Socioeconomic Status (SES) and racial differences were ignored in this study. Nevertheless, proper adjustment for surgery, stage, and the adequacy of staging investigations accounted for much of the difference in risk of death between the two population-based case series. On multivariate analysis the researchers found that the disease stage at diagnosis accounted for most of the survival difference seen between the US and European groups. '…these results suggest that more resources should be invested to achieve earlier diagnosis of breast carcinoma in Europe, especially for elderly women...' the investigators concluded.

Stage is also the most important prognostic factor in explaining international differences in colorectal cancer survival. *Cicolallo et al.* examined the extent to which disease stage, staging procedures, and treatment explain cancer survival between colorectal cases diagnosed in 1990 in European and US populations [43]. A total of 2,492 European and 11,191 US colorectal adenocarcinoma patients registered by 10 European (EUROCARE) and nine US cancer registries (SEER) have been compared. The percent of Dukes A, B, C and D stages at diagnosis was 24, 30, 23 and 18 in the US versus 14, 34, 21 and 21 in Europe. Although it was found that more patients underwent surgical resection in the US than in Europe (92% vs. 85%, respectively) and there were more

patients for whom 12 or more LNs were examined by the pathologist in the US than in Europe (28% vs. 13%, respectively) analysis of the results suggested that the survival advantage for colorectal cancer patients in the US, compared with those in Europe, can be explained by the earlier diagnosis in the US.

Carcinoma of the prostate is predominantly a tumor of older men where the median age at diagnosis is 72 years in the US and 75 years in the EU [44]. Thus prostate cancer patients may die of other illnesses without ever having suffered significant disability from their cancer. Nevertheless, survival of patients with prostatic carcinoma is also related to the extent of the tumor. According to the most recent data, 67% of men diagnosed with prostate cancer survive 10 years and 52% survive 15 years [45]. When the cancer is confined to the prostate gland, median survival in excess of 5 years can be anticipated. Patients with locally advanced cancer are not usually curable, and a substantial fraction will eventually die of their tumor, though median survival may be as long as 5 years. If prostate cancer has spread to distant organs, current therapy will not cure it. Median survival is usually 1 to 3 years, and most such patients will die of prostate cancer. With the advent of the use of PSA for screening purposes in 1989-1990 the incidence of organ-confined disease at diagnosis has increased dramatically and at the same time the rate of distant-stage disease has dropped ***(Figure 4)***. Recent data from several studies suggest that mortality rates are also lower as a result of PSA screening [46, 47]. The beneficial effect of screening in reducing disease-specific mortality is likely due to the fact that treatment rather than observation may enhance disease-specific survival. Currently, US data have shown a decrease in mortality of 1% per year since 1990, which coincides with the advent of PSA screening. In the absence of randomized trials, controversy exists in regard to the value of screening of prostate cancer by PSA testing as screening methods are also associated with high false-positive rates and may identify some tumors that will not threaten the patient's health [48, 49]. The issue is further complicated by the morbidity associated with work-up and treatment of such tumors. Other theories have been proposed to account for the decrease in prostate cancer mortality, and these include changing treatment practices and artifacts in mortality rates secondary to the changing incidence [50, 51]. Two large randomized

prospective trials are underway to determine the true benefit of screening for prostate cancer using PSA testing [52].

Lung cancer is histologically categorized into two major classes, Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). NSCLC constitute 75% of all lung cancers whereas the remaining 25% are SCLCs. Due to the different biology of the two classes we ought to analyze them separately here.

Although NSCLC is a heterogeneous aggregate of different histological subclasses (the most common are: squamous carcinoma, adenocarcinoma, and large cell carcinoma) they are often classified together because approaches to diagnosis, staging, prognosis, and treatment are similar. The latest SEER data analysis indicates that 15% of patients diagnosed from 1989 to 1996 with NSCLC in the United States were reported to have "localized" disease compared with 23% with "regional" disease, 48% with "distant" disease, and 14% with ‘unstaged’ disease [53]. The survival rates for regional disease are between 40-65% [54]. Patients with locally advanced stage disease have a 5-year survival rate of 10% to 17% overall [55]. Although important therapeutic advances have been made during the past 2 decades, the median survival time is around 10-12 months with a 1-year survival rate of 30% to 35% in most studies. The 5-year survival rate of this group of patients is not any better than 1%. In summary, results of treatment of patients with NSCLC are poor except for the most localized cancers and unfortunately, cure to patients with a diagnosis of NSCLC cannot be promised at any stage.

At the time of diagnosis, approximately 30% of patients with SCLC will have tumor confined to the hemi-thorax of origin, the mediastinum, or the supraclavicular LNs. These patients are designated as having Limited stage Disease (LD) and have a median survival of about 16 to 24 months, and a 5-year survival of 10% to 25% with the application of a multimodality treatment approach [56]. The majority of patients with a histological diagnosis of SCLC already have Extensive stage Disease (ED). Without chemotherapy, the average survival of patients with ED-SCLC is only 8-10 weeks. Median survival time with standard chemotherapy regimens is 8-11 months [57].

Almost all patients relapse with ED-SCLC and less than 5% are alive at 2 years. The 5 year survival rates fall between 1% to 5% of people diagnosed with ED-SCLC.

Although all of the above indicate that early detection of cancer would be highly desirable, it has to be noted that the interpretation of apparent inter-country and stage specific survival differences may be artefactual. Survival comparisons are likely to be confounded by the stage migration phenomenon. This is due to the unequal availability of new diagnostic techniques used to determine stage [58]. It is evident that disease stage depends on the range and thoroughness of diagnostic procedures, particularly those able to reveal occult metastases. Therefore, stage-adjusted survival comparisons should take account of the diagnostic examinations used to determine the stage of disease.

2 Background

2.1 The metastatic cascade of solid epithelial tumors

Cancer is a multi-step process and occurs as a result of the loss of control of cell division, leading to the initial tumor formation, which is often followed by metastatic spread. Although cancer is being diagnosed at increasingly earlier stages, most patients continue to succumb to metastatic disease. Metastatic disease remains the most common cause of cancer-related death in patients with solid tumors and the extent of metastasis is the major prognostic indicator of survival for almost all of the solid tumors.

The first recorded use of the term metastasis to describe the process of tumor cell dissemination originates from Recamier in 1829 [59]. As long ago as 1889, Paget recognized that the distribution of secondary tumor colonies was not random, which prompted him to propose the 'seed and soil' hypothesis of metastasis [60]. The hypothesis stated that tumor cells (the seed) have a preferential affinity for certain organs (the soil). Although our understanding of the molecular genetic and biological events that contribute to tumor cell dissemination has increased considerably over the last decade there are still many unknown factors in the complex series of interactions between the cancer cell and its surroundings that leads to metastasis formation [61]. Sadly, this advance in understanding has not been matched by dramatic improvements in eradication of solid tumor metastases.

However, the metastatic phenotype, that includes the ability to migrate from the primary tumor, survive in blood or lymphatic circulation, invade distant tissues and establish distant metastatic nodules (***Figure 5***) has been described in fine details in the past years [62]. The major steps of this process are also summarized below:

1. Penetration of lymphatics and vasculature
2. Escape from immune surveillance; survival in circulation
3. Arrest in capillary beds of distant organs

4. Penetration of lymphatic or blood vessel wall; extravasation into tissue
5. Growth of metastatic deposit in the new location
6. Tumor vascularization

This process consists of a long series of sequential interrelated steps, each of which can be rate limiting since a failure or insufficiency at any of the steps aborts the process [63]. The outcome of the process is dependent both on the intrinsic properties of the tumor cells and the responses of the host.

The major barrier to the treatment of metastasis is the biological heterogeneity of cancer cells in the primary and secondary neoplasms [64]. This heterogeneity is exhibited in a wide range of genetic, biochemical, immunological and biological characteristics, such as cell surface receptors, enzymes, karyotypes, cell morphologies, growth properties, sensitivities to various agents and ability to invade and produce metastasis [65].

2.2 Models and theories of metastasis formation

More sophisticated screening procedures have made it possible to detect smaller tumors and, consequently, have led to a significant expansion in the biopsy and excision of these lesions. This strategy for cancer control is still based on the classic Halstedian hypothesis that tumors arise locally and spread regionally and that metastases are a dissemination of a tumor, which originates in a primary site [66]. If that hypothesis of metastasis is correct, then the enormously increased ability to detect tumors at an earlier stage should certainly lead to a major decrease in mortality. In fact, what has happened is that the incidence of early-stage tumors appears to have increased whereas, despite improved treatment modalities and supportive care, age-adjusted mortality rates have not appeared to decrease. Thus the Halstedian hypothesis is inconsistent with clinical observations and novel models and theories of metastasis that better represent the *in vivo* situation are desperately needed.

The metastatic properties of tumor cells were extensively investigated in the late 1970s and early 1980s by means of ‘experimental metastasis’ assays. By studying the metastatic behavior of cultured B16 melanoma cells that were injected intravenously

into mice, *Fidler et al.* showed that cells derived from outgrowths of these cells (metastases) have a higher metastatic potential than those derived from the original cell line [67]. *In vitro* clones from the parent B16 culture varied greatly in their ability to produce lung metastases after intravenous injection into mice [68]. These observations led to a metastasis model, which proposed that most primary tumor cells have a low metastatic potential, and that during later stages of tumorigenesis rare cells acquire metastatic capacity through additional somatic mutations.

However, multiple passages of this heterogeneous melanoma cell line, both in animals and in cell culture, provide sufficient opportunity for variant genotypic cell types to arise [69]. Therefore, metastasis might be more accurately studied using spontaneous metastasis models, and cells that were not carried in culture. In such models the formation of 'natural' metastases from the primary tumors of the mice is investigated. Indeed, tumor cells derived from these metastases did not have greater metastatic capacity than those isolated from the corresponding primary tumor [70-72]. This was also true for the spontaneous metastases that arose from B16 tumors, these cells only had increased metastatic potential when placed in the experimental context developed by *Fidler et al*. [68]. The data from the spontaneous metastasis assays indicate that metastases are a random representation of disseminated tumor cells, all of which have the ability to form a metastasis. Additionally, *Weiss et al.* showed that equal-sized fragments isolated from large or small tumors showed no difference in their metastatic potential in mice, indicating that there is no apparent relationship between metastatic potential and tumor size [72].

Different hypotheses attempted to reconcile the discrepancies in the experimental findings concerning the putative selective nature of the metastatic phenotype. The 'dynamic heterogeneity' model proposes that metastatic subpopulations are generated at high rates in a primary tumor, but that these variants are relatively unstable, resulting in a dynamic equilibrium between generation and loss of metastatic variants [73, 74]. The 'clonal dominance' theory of metastasis, however, proposes that once a metastatic sub-clone emerges within a primary tumor, the progeny of this sub-clone overgrow and dominate the tumor mass itself [75, 76].

Tumor buds, projecting from neoplastic glands of colorectal cancer, were first described by *Gabber et al.* [77]. Budding or sprouting was referring to the presence of discontinuous small aggregates or single tumor cells at the invasive border of the tumor. Ultrastructurally, these tumors cell aggregates or single cells did not elaborate junctional complexes, often had incomplete desmosomes, a basement membrane was missing or rudimentary, and a brush border was absent or, at most, incomplete. The conclusion drawn by the authors was that at the invasive border differentiated adenocarcinomas focally acquire the phenotype of undifferentiated adenocarcinomas. In further studies in colorectal and other cancers de-differentiation at the invasive margin has been recognized as a strong and robust adverse prognostic factor closely related to metastatic potential [78]. Biologically, tumor budding resembles the phenomenon called Epithelial-Mesenchymal Transition (EMT) [79]. The link between this phenomenon and the formation of metastasis is discussed further in *Chapter 2.11.1.2.1.2.*

Findings from DNA-microarray studies have revived the discussion about the metastatic process. As described above, the ability of gene-expression profiles of human primary carcinomas to predict the metastatic potential indicates that the ability to metastasize is an early and inherent property of solid tumors. A study by Ramaswamy and colleagues shows that different types of human primary adenocarcinoma harbor the same gene-expression signature that is associated with metastasis [80]. These results indicate that cancer is both a local, and a systemic disease. Furthermore, the data challenge the idea that variant cells arise that give rise to metastases during the late stages of tumor progression. Thus, viewing disseminated tumor cells as rare and late events during primary tumor progression is seriously called into question by recent expression profiling studies, in which a more ubiquitous ‘‘metastatic phenotype’’ that can be assessed by gene expression analysis was proposed [81].

In contrast to the microarray studies on primary breast carcinomas, the analysis of human disseminated breast cancer cells led to a model proposing that metastatic disease evolves independently from the primary tumor [82]. This theory clearly challenges the paradigm that tumor progression to metastasis occurs through clonal genomic evolution.

The genomic alterations of disseminated tumor cells in the Bone Marrow (BM) of patients without clinical evidence of metastases generally did not resemble those of the primary tumors, in contrast to tumor cells of patients with manifest metastases.

An alternative, attractive model of metastasis is based on the finding that tumors might contain 'cancer stem cells', rare cells with indefinite proliferative potential, that drive the formation and growth of tumors [83, 84]. Experimentally, only a minority of human breast cancer cells, which were derived from fresh human tumors and grown in mammary fat pads of immunocompromised mice, were found to have the ability to form new tumors in the study of *Al-Hajj et al.* [85]. In this study cell surface markers that can distinguish between tumorigenic and non-tumorigenic cell populations were identified. The tumorigenic cell population shares with normal stem cells the ability to proliferate extensively, and to give rise to diverse cell types with reduced developmental or proliferative potential. These findings provided a previously uncharacterized model of solid tumor biology in which a defined subset of cells drives tumorigenesis, as well as generating tumor cell heterogeneity. Consistent with a cancer stem cell model is that cancer cells in the BM of patients may arise from the spread of either tumorigenic or non-tumorigenic cancer cells, and only when tumorigenic cells metastasize will frank tumors that are clinically significant develop. The prospective identification of this smaller but well-characterized tumorigenic population of cancer cells should allow for the identification of molecules expressed in these cells that could serve as targets to eliminate this critical population of cells, leading to more effective cancer therapies. Although the concept of cancer stem cells is very appealing, it must be mentioned that there remain many uncertainties, both theoretical and technical, about the interpretation of these results. Others, challenging the concept of solid tumor stem cells argue that it is very possible that in cancer cells degrees of stemness exists. and that these are variably expressed depending on the environment to which the cells are exposed [86].

2.3 Tumor cell detection in extra-tumorous compartments

The presence of very limited number of tumor cells in extra-tumorous compartments is generally termed micrometastatic disease, although the definition of micrometastasis

may vary. It may designate isolated tumor cells or a tumor-cell cluster in the absence of clinical or radiological metastatic lesions. Historically, size criteria were used to determine the presence of micrometastases within an organ, as for example in a LN. However, the original definitions of the term micrometastases included not only size criteria, but also histological architecture (***Table 4***). For this reason some pathologists want to reserve this term for small aggregates of cells within a proper tissue environment. As BM lack tissue architecture their preferred name for tumor cell contamination of this compartment is Disseminated Tumor Cells (DTCs). Although, these two terms are used interchangeably in the literature the authors believe that DTC is a more appropriate designation of these metastatic cells in the BM and therefore will continue to use this term. Whether these settled cells are precursors of clinically relevant metastases or just transiently shed cells with limited lifespan has, however, still to be investigated and will be discussed later.

Over the past several years novel technologies permitted the recovery of tumor cells directly from the Peripheral Blood (PB) of cancer patients. For the definition of such cells most authors, including ourselves, use the term Circulating Tumor Cells (CTCs). Due to smaller studies and shorter clinical follow-up only limited information is available about the prognostic impact of CTC detection in the PB of cancer patients. To date the clinical value of DTCs in BM is much clearer than that of CTCs in the PB. However, PB could be an ideal source for the detection of disseminated tumor cells for the following main reasons:

1. Simple sampling procedure

BM is only accessible by invasive intervention and thus repeated and frequent BM aspirations may not be easily accepted by patients. The acceptance of DTC detection in BM might also be slowed down by the fact that clinicians are not used to take BM sampling in patients with solid tumors, although this procedure has been safely used in patients with hematological disorders for decades. Therefore, due to the expected low patient and physician compliance, longitudinal detection necessary in disease- or therapy -monitoring is inappropriate in this source. In contrary, blood can be sampled throughout the course of the disease and provides the advantage that every clinician is

familiar with blood tests, which can be quickly done. As the collection and repeated sampling of the PB is a minimally invasive procedure the acceptability of blood-borne tumor cell detection would constitute an alternative to BM surveillance for longitudinal investigations. As the permanent access to CTCs enables follow up studies to be done for prognostication of disease outcome and monitoring therapeutic efficacy many groups currently investigate whether BM analysis can be replaced by analysis of PB. Furthermore, CTCs also provide a unique access to samples of a patient's cancer for performing several biological analyses, including gene copy number determination, gene and protein expression, etc. The ability to serially quantitate and interrogate CTCs opens new frontier of managing and investigating the cancerous disease.

2. Constitute a direct target for therapy

In solid tumors neo-vascularization is essential for growth of a neoplasm beyond a few cubic millimeters. After connection to the vasculature tumor cells can appear continuously in circulation [87]. All solid tumors analyzed so far disseminate cancer cells into the blood which is prerequisite for the formation of distant metastases. These disseminated tumor cells are the potential source of future metastatic relapse but they also constitute a readily accessible target for systemic therapy. While DTCs in the BM may rest in a dormant state (see *Chapter 2.5*) for decades the CTC pool my constitute cells with active proliferative potential. Targeting these cells in the PB before their transformation into a more therapy resistant dormant state may better eradicate the chances of a future metastatic outgrowth.

2.3.1 Comparison of tumor cell detection in the BM and PB

Preliminary data suggest that findings on CTCs and DTCs in PB and BM, respectively, do not provide congruent results [88, 89]. The reason for the discrepancy between PB and BM sampling may be attributed to the followings:

1. The frequency of CTCs appears to be lower in the PB.

BM is believed to be acting as a particularly effective filter organ for CTCs. Due to the efficient clearance of shed tumor cells from the blood it is generally believed that a greater sensitivity is required for assays attempting to detect CTCs from the PB. This intrinsic drawback, i.e. low frequency of CTCs in the PB, challenges current approaches

of CTC detection. Owing to technical issues regarding the rarity of these disseminated cells and their tendency to cluster, thereby increasing the potential for false-negative results due to the inhomogeneity of circulating blood, detection of blood-borne cancer cells may be hampered by the unavoidable statistical error of all laboratory methods [90, 91]. Due to these technical challenges only a few studies have been published that found a good concordance between detection of tumor cells in PB and BM [92-94].

2. Tumors shed cells in the circulation intermittently

Disseminated tumor cells detected in blood are merely related to the tumor load and result from transient shedding of these cells from the tumor *in situ* but are not directly linked to metastatic potential. For tumor cells, blood is only a temporary compartment and the half-life of CTCs in the blood might be short with majority of CTCs cleared from the bloodstream within 24hrs. In addition to the immunological and physical stress factors CTCs might be eliminated via systemic therapy. Thus a single detection from the PB does not necessarily reflect metastatic potential and most of the CTCs will never evolve to macroscopic metastases. This is in line with the observation that not all patients with metastatic disease showed CTCs, probably because CTCs might not be released permanently into the circulation, and a fraction of CTCs might be eliminated by systemic therapy. However, in an earlier study on a limited number of patients with epithelial tumors, a good concordance was observed between duplicate measurements of CTCs in blood [95]. Interestingly though, there are a few studies that examined repeated blood samples during the course of adjuvant therapy, and observed the occurrence of CTCs in patients being initially negative for CTCs, suggesting that CTCs might also be released from distant organs into the circulation [96].

3. Manipulation of tumors may release cells into the circulation

Many studies observed that surgical manipulation of the tumor launches tumor cells into the bloodstream [97-101]. However, it is as yet unclear whether this intra-operative tumor cell shedding represents a significant event in the development of metastatic disease. The principle of early lymphovascular ligation before manipulation of a tumor during surgical resection has been termed the 'no-touch isolation' technique. This was proposed by *Barnes* as a way of reducing the incidence of liver metastases by

diminishing the intra-operative dissemination of colorectal cancer cells [102]. Some argue that despite this precaution CTCs may disseminate intra-operatively and subsequently gain the capacity of forming detectable metastases [103]. While others believe that these intra-operatively disseminated cells that are just mechanically introduced into the circulation are eliminated completely [104]. Of special interest is to determine the timepoint of CTC sampling that is the most relevant in regard to prognosis. A recent study found that the clearance of CTCs within 24 hours of colorectal cancer resections was greatest in the subgroup of patients with the best prognosis [105]. Along the same lines, in patients with lung cancer, an increase of CTC after complete resection was a risk factor for early relapse [106].

4. The biology of DTCs and CTCs may be different

BM has so far played the most prominent role as an indicator organ of occult tumor cell dissemination. Tumor cells found in this compartment are likely to have actively invaded the parenchyma, and bone represents a clinically relevant site for distant metastasis in tumors like breast, lung and prostate cancers. While CTCs recovered from the PB still need to survive the shear stress and trapping in the capillary bed, escape the host's immune defense mechanisms, and subsequently evade into a secondary organ with appropriate microenvironment in order to develop metastasis. Thus cells in the BM in the form of DTCs have advanced further in the metastatic cascade than CTCs recovered from the PB.

On the other hand, DTCs are also found in the BM of patients who never will relapse. This is may be because the vast majority of these cells remain in a dormant state and do not proliferate. However, the persistent detection of disseminated tumor cells in the circulation may be due to an immune escape mechanism or a higher malignant potential of a specific clone. One could therefore contemplate that CTCs which survive for a longer period of time in the bloodstream have higher prognostic relevance compared to dormant tumor cells in the BM. Nevertheless, further studies have to clarify this issue.

Taken together, the few data available, however suggest that the measurement of both CTCs in the PB and DTCs in the BM can provide important clinical information about

prognosis and therapy response. It is far too early to draw any conclusions on whether any of the compartments is superior to the other. It is important to continue to study tumor cell detection in both compartments in order to relate the data to clinical outcomes and to understand further the biology of metastasis.

2.4 The metastatic inefficiency and distribution of CTCs

The detachment of malignant cells from the primary tumor is an early step in the formation of metastases [107]. The release of tumor cells and their access to the circulation does not necessarily imply the subsequent development of metastases as only a tiny proportion of cells can progress successfully through all of the sequential stages leading to the development of life-threatening metastases. The mechanism of elimination of these detached tumor cells, often termed 'metastatic inefficiency', is poorly understood [108].

Between 1 and 4 million cancer cells are shed into the vasculature daily; fortunately, the vast majority never becomes macroscopic, physiology-affecting lesions [109]. The formation of a metastasis is a complex process, and only a very small percentage of CTCs (~0.05%) survives and initiates a metastatic focus [110]. This estimated proportion may vary largely depending on the geno/phenol -type of the individual disseminated cancer cells. At this moment it is uncertain at which steps in the process cells are lost, but it has generally been thought that most cancer cells are rapidly destroyed in the circulation [111], either by the immune system or hemodynamic forces [112-114]. It was thought the majority of CTC are removed from the circulation within 24 hours, by elimination in the first capillary bed they encounter. The lifespan of many CTCs are therefore short. Indeed, the examination of CTCs has revealed a high frequency of apoptosis [115, 116]. In experimental studies inhibition of apoptosis led to significantly more macroscopic metastases [117].

Recent findings suggest that early steps in metastasis, including hemodynamic destruction and extravasation, may contribute less to metastatic inefficiency than previously believed. In fact, once cells have been arrested by size restriction, extravasation from the circulation into the tissue seems not to be rate limiting [118].

Cells of high and low metastatic ability, and even nonmalignant cells, may extravasate equally well and with similar kinetics [119]. Quantitative assessment of cell loss suggests that the metastatic process to this point is quite efficient [120]. Novel findings suggest that the control of post-extravasation growth of individual cancer cells is a significant contributor to metastatic inefficiency [121-123]. In other studies of metastatic inefficiency, *Luzzi et al.* attributed metastatic inefficiency to the failure of dormant solitary cells to initiate growth and failure of early micrometastases to continue growth into macroscopic tumors [120]. Another proposed mechanism to explain the metastatic inefficiency is provided by the cancer stem cell model, which hypothesizes that metastases can only be established by tumor stem cells, which are few in number (see *Chapter 2.2*).

Because metastases tend to develop in specific locations, the distribution of CTCs between and within organs, and/or survival and growth at various locations cannot be random [124, 125]. The distribution pattern of metastases can be predicted in part by the pattern of regional venous drainage. Most metastases develop in the first capillary bed encountered after discharge from the primary tumor. However, tumors also metastasize to distant locations that are not predictable based on blood flow patterns. The high proportion of bone metastases in breast, prostate, and lung cancers are examples of selective homing of tumor cells to a specific organ.

Three major homing mechanisms have been proposed, the first one being the selective growth. Under this mechanism, tumor cells extravasate ubiquitously but selectively grow only in the organs that have the appropriate growth factors or extracellular matrix environment. The second mechanism is selective adhesion to sites on the endothelial lumenal surface only at the site of organ homing. The third major mechanism is selective chemotaxis of CTCs to the organ producing soluble attraction factors. All of these mechanisms have been found to play a role in experimental metastasis [126-128].

Despite the fact hat the metastatic process is highly inefficient and that the mechanism of tumor cell homing has been poorly elucidated, if tumor cell shedding is an early event in tumorigenesis, it may be possible to detect cancer cells in the bloodstream

before the primary tumor is large enough to be detected by standard screening examinations.

2.5 The fate of extravasated CTCs

In order to design better anti-metastatic treatment approaches it is important to understand what happens to cancer cells between their extravasation and the formation of overt metastases. Once at the metastatic site, cancer cells have three choices: to die, to become dormant, or to proliferate [129]. Most metastatic cell die in this hostile microenvironment or balances the rates of growth with rates of death [130]. It is suggested that during the process of metastasis cells are subjected to various apoptotic stimuli and the ability of apoptosis to modulate long-term growth of a metastatic tumor colony may be separate from the apoptotic regulation that determines early (24–48 hrs) survival [125].

Cells that survive in the metastatic site can forego dormancy altogether and begin to proliferate as soon as they arrive. Alternatively, some begin to proliferate later, after an unknown event that permits them to overcome inhibitory effects at the metastatic site. The clinical observation that recurrence of tumor can occur a prolonged time after removal of the primary tumor are not consistent with constant kinetic growth of tumor cells, so there must be a dormant state in the tumor cell population. In a remarkable recent study CTCs were detected in 36% of patients (n = 13) treated for breast cancer at least 7 years earlier and currently without evidence of disease [131]. This study found that patients who are candidates for breast cancer dormancy can have replicating tumor cells for as long as 22 years after removal of their tumor without evidence of progressive growth of the tumor cell population; hence, replication appears to be balanced by cell death in these patients. This represents an example that although clinically important, the biology of human tumor dormancy is very poorly understood.

Despite several modifications of cytotoxic chemotherapy in breast and gastrointestinal cancer, the efficacy threshold in chemotherapy protocols published to date is an approximately 30% reduction in mortality [132, 133]. The success of adjuvant

chemotherapy is assumed to stem from its ability to eradicate sensitive (i.e. proliferating) occult metastases before they become clinically apparent [134]. However, the success of standard adjuvant chemotherapy particularly that of chemotherapy aimed at proliferating cell populations is jeopardized by the observation that Cytokeratin (CK)-positive metastatic cancer cells rarely proliferate at the time of primary diagnosis. Therefore, current cytotoxic chemotherapy regimens are likely to fail to eliminate such dormant, non-proliferating tumor cells, which may explain metastatic relapse even after high-dose chemotherapy. In view of the dormant nature of these cells, therapies that are also directed against quiescent cells, such as antibody-based immunotherapy, might be complementary to chemotherapy [135].

Nonetheless, if extravasated CTCs do begin to proliferate, the rates of proliferation have an extraordinarily broad range that depends on the tumor and cell type, the individual and the metastatic site. Proliferation is certainly not continuous and may not necessarily follow the Gomperzian model of growth widely accepted for primary tumors [136]. Cells that do start to grow may form micrometastases of only a few cells before they die, suggesting that the associated metabolic activity may make them more vulnerable to destruction [137, 138]. The rate at which cells within micrometastases proliferate may also depend on location within the organ [125]. It appears then that the first few divisions of tumor cells at the secondary site constitute a rate-limiting step in the metastatic process [116].

Continued growth eventually depends on the development of a microvasculature capable of serving the metabolic needs of the tumor [139]. Initially this may be provided by existing vasculature which the tumor cells tend to grow around, but as the developing tumor grows larger, angiogenesis is needed to limit the distance between vessels. Transport from blood vessels to metabolically active tissues is limited by diffusion and tumor growth beyond the size of 1-2mm is already angiogenesis-dependent [140]. The presentation of large tumors is attributed to a successful switch to the angiogenic phenotype.

In animal models cells that have been recovered from the liver after several weeks of dormancy were successfully grown in culture. When re-injected into the mammary fat pad, they can form tumors, proving that given the right conditions they can grow even after months of dormancy [138]. Thus dormancy can also exist in the form of metastases that are in a state of suppressed angiogenesis, with proliferation balancing cell loss [130]. Any factors that tip the balance towards net proliferation or apoptosis, may result in tumor progression or regression [137]. Thus, dormancy in the form of either solitary cells or pre-angiogenic micrometastases presents a danger of recurrence of life-threatening metastases.

2.6 Shortcomings of current cancer staging parameters

Current tumor staging parameters are only able to statistically predict the risk of recurrence for populations of patients without giving indications about the possible outcome for any one individual, which, after complete surgical excision of localized disease, is primarily influenced by the presence of unidentified CTCs.

Evidence for early metastatic events exists on four different levels:

1. Locoregional and distant relapses occur after 'curative' resection of early stage disease in just about every solid tumor.
2. The existence of Carcinomas of Unknown Primary (CUP).
3. Reports of detection of microscopic metastasis in regional LNs, in the BM and PB of patients with early stage solid tumors.
4. With directly observing the metastatic process with intravital microscopy of animal models.

2.6.1 Locoregional and distant relapses occur after 'curative' resection of early stage disease

The observation that, despite complete resection of their primary tumor, patients with initial stage M0 also relapse demonstrates that seed cells of distant metastasis must have spread before surgery or even before first diagnosis.

After successful treatment up to 50% of colorectal patients develop metastasis and eventually die from the disease [141, 142]. Despite the fact that the cancer being completely resected surgically, even in the earliest stage of the disease, when the tumor has only penetrated the most superficial layer of the bowel wall (the mucosa), a few patients (1-3%) may still experience recurrence of their cancer [143]. In stage II colorectal carcinoma patients the recurrence rate (local and distant) can be as high as 25-40% after curative resection of the primary tumor. This suggests that these patients already have small amounts of cancer that have spread outside the bowel and were not removed by surgery. With standard techniques this extra-colonic spread is below the limits of detection. It remains under debate, whether all stage II patients or at least stage II patients with additional risk factors should receive adjuvant chemotherapy ([144, 145]. It is therefore of high importance to define prognostic criteria for this patient group to help identify high-risk patients for tumor relapse, who might benefit from adjuvant therapeutic regimes.

Similarly, breast cancers metastasize very early in their course. 12–37% of small (<1 cm) mammographically detected breast cancers already have metastasized at diagnosis [146, 147]. Approximately 10–15% of patients with breast cancer has an aggressive disease and develops distant metastases within 3 years after the initial detection of the primary tumor. However, the manifestation of metastases at distant sites 10 years or more after the initial diagnosis is also not unusual [148, 149]. Approximately one-third of women with breast tumors that have not spread to the LNs develop distant metastases, and about one-third of patients with breast tumors that have spread to the LNs remain free of distant metastases 10 years after local therapy [150, 151]. Although axillary LN status, as determined by histologic examination, is still considered the most accepted prognostic indicator in invasive breast cancer, negative LNs do not preclude aggressive and subsequent distant disease. Today, the traditional prognostic markers are able to confidently identify the group of approximately 30% of patients, who are most likely to have either a very favorable or a very poor outcome. For the remaining 70% of patients, of whom approximately 30% will still develop metastases, new prognostic markers are needed to help identify low-risk and high-risk groups, to pinpoint those

patients who are most likely to benefit from systemic adjuvant treatment. The identification of additional markers predictive for the behavior of individual tumors is a significant challenge in breast cancer research.

Of men who by imaging studies and clinical parameters seem to have localized prostate cancer, 15% to 40% will develop recurrent disease [152, 153]. Even when the disease is pathologically organ confined at surgery, 5% to 20% of these patients harbor foci of micrometastatic disease that will later manifest itself as recurrent disease despite definite local therapy. Widely used biochemical, histopathological, and clinical criteria such as PSA level, Gleason score, the clinical tumor stage, and molecular genetic approaches assaying loss of tumor suppressors or gain of oncogenes had only limited success with respect to prostate cancer patients' stratification and demonstrated a significant variability in predictive value among different clinical laboratories and hospitals [154]. Furthermore, best existing markers cannot reliably identify at the time of diagnosis a poor prognosis group of prostate cancer patients who ultimately would fail therapy [155]. Risk assessment schemas have been developed to help predict the clinical outcome for men with localized prostate cancer treated with definitive local therapy and are widely used to guide clinical decision-making. However, one of the significant deficiencies of these classification systems is that they have only limited utility in predicting the differences in outcomes readily observed between patients diagnosed with prostate cancers exhibiting similar clinical, histopathological, and biochemical features. Consequently, these variables are losing their discriminatory power, creating the need for novel diagnostic and prognostic markers that is capable to improve the classification accuracy of prostate cancer patients with respect to clinical outcome after therapy.

Also in NSCLC the dissemination of malignant cells to regional LNs and distant organs can occur at an early stage of primary tumor growth and is regularly underestimated by currently available clinical and pathologic staging procedures [156]. For example, approximately 40% of patients who undergo surgical resection of NSCLC without overt metastases (pT1-2, N0, M0, R0) relapse within 24 months after surgery [157]. This is also reflected in a poor 5-year survival rate and suggests that an occult tumor load is the

major reason for the high mortality rate in surgically treated lung cancer patients. Nevertheless, many patients with early stage NSCLC (stage I and II) are curable by surgical resection. In patients with locally advanced disease surgery plays an important role in order to provide local tumor control. Since surgical resection offers the highest chance for cure in NSCLC, the intention of all staging efforts must be to identify all patients, who benefit from a surgical approach [158].

At the time of diagnosis SCLC is almost invariably disseminated or at least so locally advanced that it is virtually always amenable to surgical therapy. Sixty to 65% of patients with SCLC have ED at the time of diagnosis and more than 85% of SCLC patients have mediastinal lymph-node metastases [159]. Combination chemotherapy is the cornerstone of treatment for SCLC patients, yielding high initial response rates of 65 to 85%, including 50%-60% complete responses (CRs). Despite high response rates, relapse and progression occur in the majority of SCLC patients, and median survival is <1 year for patients with extensive-stage disease. Patients with LD-SCLC receiving chemoradiotherapy sometimes are cured, but the median survival is limited to 15 to 20 months in the majority of patients, and the 2-year survival rate is 40% [160]. Ten to 30% of patients with progressive disease may respond to salvage chemotherapy regimens, but the responses are of short duration. Surrogate markers that could aid the development and evaluation of novel agents and treatment schedules are desperately needed in order to increase the disease free interval and overall survival in this very aggressive malignancy.

2.6.2 The existence of Carcinomas of Unknown Primary

Metastatic CUP accounts for as many as 3% to 5% of solid tumor patients referred to oncologists, making it the seventh most common malignancy [161, 162]. These are patients for whom a diagnosis of metastatic malignancy is made without knowledge of the site from which the tumor originated despite a thorough history and physical examination with appropriate ancillary testing. When tumors present at an advanced stage, conducting a directed diagnostic workup might identify the primary tumor site. However, in many other instances, the source of the metastatic tumor cannot be identified despite a focused search and thus 15% to 25% of cases will not yield evidence

of the primary tumor even at postmortem examination [163]. CUP therefore demonstrates that a large tumor extent apparently is not an absolute requirement for a systemic spreading to occur [82]. Thus, the clinical experience of CUP leads one to accept that also in early clinical stages of a malignant growth cells can dissociate from the tumor's parenchyma, penetrate into adjacent tissue compartments and are spread via the vascular system.

2.6.3 Detection of microscopic disease in patients with early stage solid tumors

Although studying of minute number of cancer cell dissemination to the LNs is an area of active research in this thesis we focus on the other two compartments, the BM and PB, for two major reasons. First, LN involvement is implemented in and validated for all major tumor risk classification systems. Second, once LNs are resected together with the primary cancer for tumor staging, no longitudinal monitoring of therapy is feasible. Thus the clinical relevance and utility of these different compartments could be remarkably different.

BM has played a prominent role as an indicator organ of tumor cell dissemination because it is easily accessible by aspiration, and represents a relevant site of distant metastases for many solid tumors. Bone is the clinically most relevant site of metastatic disease in patients with solid epithelial tumors and is one favored site for formation of metastases in tumors like breast, lung and prostate. However, also in other cancers where bone metastasis is less frequent, as for example in colorectal cancer, BM contamination with DTCs has been shown to be a common event.

Single CK-positive cells found in BM of carcinoma patients years before overt distant metastases occur in the skeleton or other distant organs were the first direct evidence for clinical significance of DTCs in the BM. Often, many years after resection of the primary tumor and treatment of cancer patients, cancer cells can grow out of DTCs deposits to form overt metastases. Several studies support the hypothesis that tumor cells in the BM of cancer patients can be regarded as the precursor of clinically manifest distant metastases [164, 165]. Indeed, evidence is accumulating that the presence of

occult carcinoma cells of epithelial origin in the BM is an independent risk factor in many solid tumors (***Table 6, 7, 8*** and ***9***).

Among solid tumors, the clinical relevance of DTCs has been most extensively studied in breast cancer patients (***Table 6***). BM is the single most common site of breast cancer metastasis, and up to 80% of patients with recurrent tumors will develop BM metastases at some point during evolution of their disease [166]. It is also the most frequent initial site of clinically detectable breast cancer metastases [167]. Recently, the highest level of evidence for the prognostic impact of DTCs in primary breast cancer was reached by a pooled analysis comprising more than 4,000 patients, showing poor outcome in patients with DTCs in the BM at primary therapy [168]. The analysis evaluated patient outcome over a 10-year follow-up period (median, 5.2 years) using a multivariable piecewise Cox regression model. All patients were free of distant metastases at the time of primary surgery; 90% had pT1 and pT2 tumors; 58% were node-negative; and 70% had received adjuvant therapy. The prevalence of DTCs was 30.6% and significantly associated with larger tumor size, higher grade, LN metastasis and hormone receptor negative tumors (each, $p< 0.001$). The presence of DTCs was a significant prognostic parameter for poor overall and breast cancer-specific as well as disease-free and metastasis-free survival during the entire 10- year observation period (univariate mortality ratios, 2.15, 2.44, 2.13 and 2.33, respectively; $p< 0.001$). In multivariable analysis, DTCs outperformed the traditional prognostic variables for poor survival. In univariate subgroup analysis, breast cancer-specific mortality among patients with DTCs was significantly elevated ($p< 0.001$) for those on either adjuvant endocrine (mortality ratio, 3.22) or cytotoxic therapy alone (mortality ratio, 2.32) and for patients who had pT1N0 disease and did not receive adjuvant systemic therapy (mortality ratio, 3.65). The present analysis meets the criteria for Level I evidence for the prognostic value of DTCs and the results suggest means for selecting patients in need of prolonged adjuvant therapeutic interventions.

In addition, five recent independent studies comprising a total of 2,506 breast cancer patients consistently reported that the presence of DTCs in the BM had a strong prognostic impact on patient survival using validated methodologies and adequate clinical observation periods [164, 165, 169-171]. In addition to being identified as an

independent prognostic factor, the presence of DTCs in the BM was associated with axillary LN metastasis, pathologic tumor stage, tumor grade, hormone receptor status and vessel invasion in both locally recurring and advanced stage breast cancers [169-176]. However, in contrast to the above mentioned studies several investigators found that the incidence of DTCs did not correlate with the outspread of tumor cells into LNs in breast cancer patients [177-179]. Other investigations found no association with adverse outcome or only partly confirmed the prognostic value of DTCs in the BM of breast cancer patients (***Table 6***).

Soon after initiation of the search for BM micrometastases in breast cancer, BM samples of patients with other types of carcinomas were explored for the presence of DTCs. In the absence of other factors of poor prognosis, DTCs in BM has been found to predict a decreased overall survival and disease-free survival in other cancers, like NSCLC, SCLC, prostatic cancer and colorectal cancer, signing an early systemic dissemination of malignancy (***Table 7, 8*** and ***9***).

A recent meta-analysis of 2,494 patients with all types of carcinoma found a prevalence of BM DTCs of 35% [180]. Fourteen of the 20 studies reviewed showed a correlation between BM metastases and relapse-free survival. But in only two studies was the presence of marrow metastases on multivariate analysis determined to be an independent predictor of survival. Even though that meta-analysis included a substantial number of patients, it comprised individual studies with rather divergent detection methods, thus making comparison of the studies extremely difficult.

In summary, the finding of absence and presence of prognostic influence, respectively, might be related to varying technical factors, such as the choice of detection methods, as well as the limited statistical power of single studies with small patient series.

2.6.4 Direct observance of the metastatic process in animal models

Traditional approaches to study hematogenous metastasis do not enable one to directly observe the metastatic process *in vivo*, and conclusions often have been based on reasonable inferences instead of primary observations.

An ideal way to follow cancer cells through the early stages of the metastatic process is to use high resolution Intra-Vital Video Microscopy (IVVM). IVVM permits the study of events in the metastatic process that were previously inaccessible by commonly used *in vivo* and *in vitro* assays. Although intravital microscopy can be used to provide detailed still and video images of the metastatic process, the real power of the technique lies in being able to quantify specific parameters such as the dissemination rate, survival, extravasation and metastatic growth of cancer cells.

The greatest fidelity to donor cancer tissue was achieved with Green Fluorescent Protein (GFP) models employing orthotopically implanted tumors. These tumors closely emulate the metastatic behavior of the donor in rate and seed similar target tissues. Also, the GFP-expressing tumors are sufficiently bright that they often could be viewed through simple video equipment situated externally to the animal [181]. The GFP imaging affords unprecedented continuous visual monitoring of malignant growth and spread within intact animals without the need for anesthesia, substrate injection, contrast agents, or restraint of animals required by other imaging methods [182].

In the study of *Naumov et al.* CHO-K1 cells that stably express GFP, were used to visualize and quantify the sequential steps in metastasis within mouse liver by IVVM from initial arrest of cells in the microvasculature to the growth and angiogenesis of metastases [183]. With this approach individual, non-dividing cells, as well as micro- and macro-metastases could clearly be detected and quantified in the liver. Just within 24 hours after initial arrest, most cells had completed extravasation and others were in the process of extravasating. Two days after injection small micrometastases of 2-4 cells accounted for 11.8% of the total tumor foci observed. By the fourth day multicellular micrometastases with a diameter from 20 to 380μm were readily observable. The authors have also identified a population of single undivided cells surviving in the tissue

up to two weeks after injection. These apparently dormant cells could have the potential to be activated at a later time and commence growth. This situation might resemble the clinical scenario of contamination of BM with DTCs.

Wang et al. established a colon cancer model in which trafficking of CTCs to the liver in the portal vein was studied by IVVM [184]. Within 72 hours after transplantation of tumor cells, on the ascending colon in nude mice, metastasis was visualized *ex vivo* on a single-cell basis around the portal vein by GFP imaging. These experiments not just demonstrated the critical role of the portal vein in metastasis to the liver but also indicated that the dissemination of cancer cells happens shortly after implantation.

In the study of *Koop et al.* the fate of cancer cells was studied with injection of B16F10 melanoma cells the into chorioallantoic membrane of chick embryos [118]. Tumor cell survival and extravasation was studied one day later by IVVM. The study demonstrated that greater than 80% of injected cells survived and extravasated by 24h, indicating that tumor cell destruction in the blood is not necessarily a rate-limiting step in the metastatic cascade (see *Chapter 2.1*). However, few extravasated cells began to grow, with only 1 in 40 forming micrometastases. Furthermore, few micrometastases continued to grow, with only 1 in 100 progressing to form macroscopic tumors by day 13; in fact, by then most micrometastases had disappeared. Surprisingly, 36% of injected cells remained by day 13 as solitary cancer cells, 95% of which were shown to be dormant. These results suggest that metastatic inefficiency is principally determined by two distinct aspects of cell growth after extravasation: the failure of solitary cells to initiate growth and the failure of early micrometastases to continue growth into macroscopic tumors.

2.7 The principle of CTC detection

The accuracy of clinical cancer staging and diagnostic procedures is significantly limited. Current state-of-the-art imaging techniques, such as magnetic resonance imaging and spiral computed tomography, have a detection limit of lesions with a diameter 3–5mm, corresponding to about 10^8 cancer cells [185, 186]. Hence, in clinical practice, detection of smaller residual cancer cell aggregates or metastatic sites has so far been impossible. Consequently, such small lesions have not been taken into account

for the definition of ‘high risk’ patients who require further systemic therapy. However, many studies have shown that residual cancer cells remaining after surgery at the resection margin, in local LNs, in the BM, or in the PB pose a significant risk for the patient to develop metastatic disease (see *Chapter 2.6.3*).

To establish a metastasis, tumor cells have to invade their surrounding host tissue, enter the circulatory blood stream, arrest in capillary beds of distant organs, invade the host tissue and proliferate. As small tumors of less than a few mm in diameter already receive a vascular blood supply, it is likely that cancer cells have spread throughout the body years before they are first detected [187]. The development of an assay to detect these cells before the manifestation of distant metastases might therefore be useful for patient prognosis.

In fact, a considerable body of evidence indicates that tumor cells are shed from a primary tumor mass at the earliest stages of malignant progression [188]. Some of these cells will travel via the PB to sites anatomically distant from the primary tumor and form metastases [189]. As individual cells present in low numbers, such disseminated cells can be occult to standard methods of investigation such as microscopic examination of routinely stained cyto/histology slides. However, these cells are understood to be a source of eventual lethal metastases, the major cause of treatment failure in cancer patients. Detection of these cells is a promising method for both diagnosis and clinical management of cancer patients as well as monitoring treatment. Translating this principle into a clinically viable technology is the main focus of our efforts.

In general, new markers, such as DTCs in the BM and CTCs in the PB, may serve as prognostic factors, indicating the further course of the disease, or as predictive factors, with regard to expected therapy response. Moreover, new markers themselves may serve as targets for new biological therapies. However, before new markers can be implemented in everyday patient management, they need to fulfill certain quality criteria regarding determination methodology and demonstrate clinical relevance [190]. Next, to have a plausible biological rationale for using the particular marker, its

determination method needs to be robust, standardized, and quality-assured. Its clinical impact needs to be validated by independent clinical studies and, finally, the marker must be clinically useful, that is, it must be able to support clinical decision-making independently of existing markers.

2.8 History of CTC detection

The first publication on CTCs is attributed to Ashworth, who in 1869 described a case of cancer in which cells similar to those in the tumor were found in the blood after death [191]. However, it was the experimental work of Zeidman, Fidler and Gullino in the 1950s and 1960s that laid the foundations for the demonstration of tumor cells in extra-tumorous compartments. Zeidman concluded from experiments on rabbits that tumor cells can traverse capillaries and spread to the whole organism [192].

The detection of CTC gained great attention again when Engell first reported the detection of CTCs in humans with various types of carcinoma using a cell block technique [193]. Indeed, between 1955 and 1965, several thousand cancer patients were tested for CTC by investigative teams using different cytological methods. The initial studies reported a very high positivity rate among cancer patients (up to 100%) [194]. However, these results were soon shown to be due to false-positives since circulating hematopoietic elements, especially megakaryocytes, were often confused with tumor cells. When cell preservation techniques were improved allowing a better morphological analysis, the detection of true CTCs by light microscopy was shown to have a very low sensitivity in cancer patients [194]. Routine cytological examination of blood specimens for CTC was therefore abandoned in 1965.

The issue of CTCs resurfaced later with the advent of Immunocytochemistry (ICC). Fidler was the first to measure blood-borne metastasizing tumor cells quantitatively in the PB of animals [111]. *Butler and Gullino et al*. calculated that the daily cellular turnover of a tumor is very high in relation to the tumor mass [109]. In that model they also found that a malignant tumor can induce metastases both in progression and regression. The absolute number of tumor cells in arterial and venous blood tended to correspond to the normal distribution of the blood volume. Studies presented verified

that the released tumor cells distribute so rapidly in the vascular system that blood obtained by venous puncture can be employed for detecting tumor cells in PB.

At the same time sensitive immunocytological tests were developed to detect DTCs in the BM of cancer patients. These tests were shown to identify tumor cells in the BM with much greater sensitivity than conventional techniques [195]. Indeed, these immunocytological assays were said to detect a single tumor cell seeded amongst 10,000-100,000 Mononuclear Cells (MNCs).

Despite evidence of the prognostic value of this determination in some studies, the detection of CTCs and DTCs by ICC is not routinely used in cancer staging protocols. This is mostly due to the absence of any clinical significance in some studies [196-198]. Meanwhile, there was the prospect of developing an even better method for the detection of occult tumor cells using nucleic acid analysis. This hope materialized with the advent of the highly sensitive Polymerase Chain Reaction (PCR) technique in the late 1980s that has greatly facilitated the detection of these cells. Since 1987, a variety of PCR-based techniques have been devised for the identification of CTCs and micrometastases in leukaemias, lymphomas and various types of solid malignancies.

Numerous studies have concluded that the presence of DTCs in the BM is of prognostic significance in different types of solid tumors, such as NSCLC, colorectal, esophageal, prostate and neuroendocrine carcinomas [199, 200]. The most extensive database on BM micrometastasis and prognosis exists for breast cancer, involving over 4,000 patients [201, 202]. Despite these important observations, the use of BM examination for DTCs detection is not yet part of routine clinical staging. This is predominantly because several other studies found no statistically significant relationship between DTC detection and prognosis [180, 196, 203, 204]. The most likely explanation of the discrepancy between these studies is the technical and methodological differences applied for DTC and CTC detection. Since the frequency of DTCs in the BM of cancer patients can be as low as one tumor cell in the background of 10^5-10^6 normal hematopoietic cells, the reliable detection and quantification of such rarely occurring cells challenges available analytical methods.

Another approach to detecting occult micrometastases is measuring CTCs in PB. It is hypothesized that CTCs in PB represent 'cells in transit' that have the potential to give rise to distant metastasis. The presence of such cells would thus indicate an unfavorable prognosis but also constitute a readily accessible target for systemic therapy. Therefore, a simple blood test for CTCs would be the most straightforward approach for risk stratification of cancer patients. However, the cells are present in ultra-low concentrations in the PB, and such rare cells are challenging to measure reliably. In fact, there is considerable disagreement in the current literature over the incidence of CTCs in PB at different stages of the disease. The data discussed below shows that a reliable measurement depends on both sample preparation protocol and detection technique, and hence research in this area must take both into account simultaneously in order to develop a reliable and clinically useful approach.

However, there is good reason to be optimistic about the value of CTC detection. A recent clinical trail involving 177 metastatic breast cancer patients showed the number of CTCs in PB after Immunomagnetic Enrichment (IME) provided prognostically useful information regarding progression-free and overall survival in a subset of patients [205, 206].

2.9 Characterization of CTCs

The occurrence of CTCs is the result of two basic competing factors: first, the ability of the cancer cells to disseminate, and secondly, the effectiveness of tumor cell elimination mechanisms. Therefore, detection of CTCs seems to be a logical approach to assess prognosis of the patients to define surrogate markers.

However, the detection of CTCs does not necessarily reflect metastatic potential. A malignant tumor is continuously shedding tumor cells and most of these cells are eliminated in the first capillary bed, being the LNs, the lungs and the liver and only a minority will be released progressively and arrested in a secondary capillary bed, the BM being the most easily assessable [114, 207]. Furthermore, tumor cells are found in

BM from patients who never will relapse and patients can survive several months with a peritoneo-venous shunt for malignant ascites without developing distant metastases, although high amounts of tumor cells are continuously shedded into the venous circulation [208].

In summary, isolated tumor cells found in BM or PB are the result of this continuous shedding from the primary tumor and most of them will never evolve to macroscopic metastases [209]. Whether a detected individual CTC is a dangerous precursor of further clinically relevant metastasis or just a transiently shed cell with a limited lifespan is a crucial question to be answered. Besides their mere detection the only way to understand the biological significance of CTCs is to perform some sort of characterization.

2.9.1 Phenotypic characterization of CTCs

The ICC-based characterization of CK-positive CTCs supports their classification as epithelial-derived neoplastic cells. In double labeling studies on CK+ cells HER-2/neu (c-erb-B2) protein expression, down-regulation of Major Histocompatibility Complex (MHC) class I as well as the expression of the urokinase Plasminogen A (uPA) receptor (a molecule that regulates proteolytic activity needed for extracellular matrix degradation), seem to be linked to specific malignant characteristics, such as dissemination, immune-escape and metastatic outgrowth [135, 210-213].

Another antigen with a presumably modulated, differential expression on CK+ cells is the Epithelial Cell Adhesion Molecule, EpCAM [213]. EpCAM was found to be frequently absent on the cell surface of CTCs as down-regulation of EpCAM expression would permit tumor cells to be released from contact-mediated controls within the primary tumor, while re-expression at the secondary site would facilitate organ-specific homing of disseminated tumor cells. Moreover another molecule that can enhance the mobility of CTCs through the production of Matrix Metalloproteinases (MMP) 1, 2 and 3 in peritumoral fibroblasts EMMPRIN, is found to be expressed on approximately 90% of CTCs in BM [214].

Interestingly, there are also indications that CK+ cells are mostly dormant tumor cells because the expression of proliferation markers such as Ki-67, p120 and Proliferating Cell Nuclear Antigen (PCNA) were rarely reported [135, 215]. Therefore, many CTCs escaping apoptosis are likely in a latent stage (dormant cell-cycle arrest). The ineffectiveness of current adjuvant therapies in eliminating CK+ cells in the BM likely to be caused by this state of dormancy as cytotoxic therapies primarily target proliferating cells [216].

2.9.2 Genotypic characterization of CTCs

There are indications that CTCs may exhibit a considerable genetic diversity, reflecting the instability and micro-heterogeneity observed in primary tumors. Muller and coworkers were the first to detect genomic changes in CK+ cells [217]. They combined immunological detection of micrometastatic cells by an antibody against CK18 with Fluorescence In Situ Hybridization (FISH). In breast cancer patients with mainly small CK+ cell clusters in the BM samples, they could detect HER-2/neu (c-erb-B2) gene amplifications, a genomic change that is frequently found in breast cancer and associated with a bad prognosis. In a more recent study *Meng et al.* analyzed isolated CTCs for the presence of HER-2/neu (c-erb-B2) amplification and compared this with the HER-2/neu (c-erb-B2) status in the primary tumor [218]. Nine of 24 patients whose primary tumor was HER-2/neu (c-erb-B2) negative acquired HER-2/neu (c-erb-B2) gene amplification in their CTCs. This suggests that CTCs acquire genomic aberrations after dissemination, independent of those arising in the primary tumor.

In prostate cancer, a gain of chromosome 7 and/or 8 was noted in at least 70% of the CK+ cells, from which it was concluded that the majority of CK+ cells from this cancer are cytogenetically aberrant. This provided the first direct evidence of the malignant nature of CK+ cells in BM. Because it had been observed that 25% of cancer patients with epithelial cells in BM do not show disease progression, the authors suggested that the patients be stratified according to genomic aberrations [215].

The availability of new protocols for the amplification of the whole genome has enabled a more detailed analysis of disseminated tumor cells [219]. Novel laser-based

microdissection techniques have made it possible to isolate and if necessary to pool CTCs and perform detailed analysis on their genetic content [220]. Using a procedure involving whole-genome amplification and subsequent Comparative Genomic Hybridization (CGH) of single immunostained cells, it has been shown that CK+ CTCs in the BM of breast cancer patients without clinical signs of overt metastases were genetically heterogeneous [221]. This unexpectedly high genetic divergence, particularly at the level of chromosomal imbalances, was strikingly reduced with the emergence of clinically evident metastasis. Therefore, the authors concluded that the selection of clonally expanding cells leading to metastasis seems to occur after dissemination has taken place. Also with using the single-cell CGH technology *Gangnus et al.* analyzed tumor cells in BM of early-stage breast tumor patients for genomic changes [222]. Authors found that the viable CTCs had a plethora of copy number changes in their genome. In fact, all examined cells in their study showed chromosomal copy number changes with a substantial intercellular heterogeneity and differences to the matching primary tumors. One possible explanation for these findings is that the CTCs may have separated from their primary tumor at an early stage and these aberrations might have been acquired at a later stage of tumor progression. This supports the concept that early disseminated cancer cells evolve independently from their primary tumor.

Based on these novel insights, the decision that the primary tumor should be the gold standard for making treatment decisions at a later date is challenged. Some authors already advocate that CTCs would represent a safe 'real-time' biopsy to detect genetic changes in general as cancer progresses [223]. *Smirnov et al.* generated gene expression profiles for CTCs using a method that compares the RNA extracted from the CTC-enriched fraction of a PB sample with the RNA extracted from the corresponding CTC-depleted fraction [224]. With this approach a novel set of genes was identified to detect and characterize CTCs in blood samples of metastatic colorectal, breast and prostate cancer patients. These insights into the global expression profile of CTCs could lead to the development of novel diagnostics tools and may have important consequences for therapeutic approaches to eradicate these cells.

2.9.3 Functional assays on CTCs

A major limitation of functional assays is that even after short-term culture, the number of tumor cells available for molecular analysis is still small (on average, between 1,000 and 10,000 cells), which limits further functional analyses of these cells. In this context, permanent cell lines developed from disseminated cells might be helpful tools. Pantel and coworkers expanded CK+ cells present in BM samples by using special culturing techniques and succeeded in establishing several cell lines that express various epithelial markers [224, 225]. Unfortunately, it was necessary to immortalize the cells by microinjection of the SV40 large tumor antigen (SV40-T). Because SV40-T damages human chromosomes and drives karyotypic instability of normal diploid cells within days, genomic data from the expanded cells should be viewed with caution [226]. However, some interesting observations came out of these studies. First, the number of *in vitro* expanded CK-positive cells was not correlated to the number of CK-positive cells in the BM aspirates, which was consistent with the former findings demonstrating that the growth kinetics of these tumor cells are variable [227]. Second, they demonstrated that the extent of *in vitro* expansion of CK-positive cells was a better prognostic indicator than the mere detection of these cells in BM samples [228].

The potential of CTCs to form solid metastases (i.e. lung) *in vivo* has recently been reported. *O'Sullivan et al.* demonstrated that CK-positive cells isolated from the rib marrow of esophagogastric cancer patients were tumorigenic in athymic nude mice [229]. The first report of the formation of xenografts from carcinoma cells taken directly from the PB of patients was published by *Pretlow et al.* In their study prostate cancer from 2 of 11 patients and colon cancer from 1 of 3 patients were found to be growing as metastases in the lungs of the nude mice after *s.c*. injection [230].

2.10 The clinical significance of a reliable CTC assay

Normal, non-dysplastic epithelial cells are not capable of migrating outside their original host organ, except in chronic inflammation after epithelial-mesenchymal transition [231]. Thus, the presence of extrinsic epithelial cells in BM or PB indicates

the malignant nature of these cells. The demonstration of extrinsic epithelial cells in these mesenchymal compartments and their further characterization possesses a potential for clinical oncology applications such as:

2.10.1 Risk assessment and early detection of cancer

A potential application of CTC detection from PB is the early diagnosis of cancer. At some ill-understood point during primary tumor growth, invasive subpopulations of the primary tumor cells gain access to the circulation. While the literature supports the concept that many tumor cells shed from a primary tumor are immediately destroyed in the circulation and most shed cells do not in fact result in metastasis, detection of such shedding may provide an early clue as to the presence of malignancy. With ancillary use of appropriate tissue and/or organ specific markers, CTC screening could be an early indicator of metastasis-capable malignancy. This aspect is of particular importance as there are no established methods to screen for many types of cancers, the most common being lung cancer. Many investigators are excited about the prospect to test the feasibility of CTC detection in patients with high risk for cancer.

2.10.2 Staging and selection of primary therapy

Cancer cells are being disseminated into the blood stream during the growth of solid tumors and their occurrence has been shown to indicate a poor prognosis. It has also been shown that the number of CTC varies during treatments and that this information might be used to evaluate treatment success or failure. In current clinical practice, treatment decisions are made according to the histological and/or molecular analysis of primary tumors. Yet, it has been described that the gene and protein expression profiles of primary tumors and their metastatic counterparts are dramatically different [82, 232]. Furthermore, after removal of the primary tumor, residual tumor cells and metastases derived thereof compose the therapeutical target cells. The emergence of new drugs directed against specific cancer related molecules will make it mandatory to stratify treatment according to properly identified molecular targets, introducing future applications for CTC characterization. Characterization of CTCs will possibly provide more information about metastatic potential, drug sensitivity and the development of a

therapy resistance than analysis of the primary tumor and will eventually help to identify subgroups of patients receiving benefit from a particular therapy. In summary, current classification systems, are not sufficiently accurate to predict the course of disease and the response to the selected therapy. The importance of a surrogate marker assay permitting immediate assessment of whether therapy has eliminated systemic disease is therefore evident.

2.10.3 Monitor effectiveness of post-surgical therapy and recurrence

The permanent access to CTCs enables longitudinal studies to monitor therapy outcome. This aspect is substantially improved by recent quantitative methods allowing the identification of changes in the quality and number of CTCs. With improved understanding it may be feasible to predict which patients with early stage disease will recur despite adjuvant therapy and if so which drug is most likely to be efficacious for recurrent and/or metastatic disease. New prognostic markers are urgently needed to identify patients who are at the highest risk for developing metastases, which might enable oncologists to begin tailoring treatment strategies to individual patients.

In summary, CTC detection, quantitation and characterization can fulfill the above mentioned goals in the following manner:

- Determine Presence - Risk assessment for cancer
- Count - Assess prognosis, direct therapy, monitor disease
- Phenotype & Genotype - Diagnose, specify therapy

While, the requirements for clinical application are that the detection of CTCs has to bring an improvement in [233]:

1. The determination of patients' prognosis independent from the basic systems (e.g. TNM).
2. The possibility to monitor therapy outcome.
3. The earlier disclosure of primary tumors as well as their metastases.

4. The possibility to identify the presence of drug targets for therapy selection by their molecular characterization.

2.11 Methods of CTC detection

Haematogenous spread of tumor cells has been recognized and is under investigation for more than a century, but technical problems of detection and identification are accompanying it's history. Several CTC detection methods have been explored and described in the literature. Each of these methods relies on either a difference in protein expression or in genetic composition between the tumor cells and the normal hematopoietic cells.

The technology is primarily based on cell-based detection, with ICC, Fluorescent-Activated Cell Sorter (FACS) or molecular detection relying on nucleic acid amplification techniques. At present, cell-based detection is constrained by the absence of a means of detection that is both efficient and robust. Consequently these approaches require additional, typically complicated and almost certainly cell-damaging methods of enrichment to achieve adequate speed and sensitivity. Although the other alternative approach, nucleic acid amplification, has the required sensitivity, it lacks the required specificity, as it does not allow for morphological confirmation of the malignant cytologic features of the cells in question and it is very problematic to quantitate. In summary, each of these existing technologies has critical limitations for CTC detection and quantification and these are discussed in detail below.

2.11.1 Cell-based methods:

2.11.1.1 Flow cytometry

Flow cytometric assay based detection involves the preparation of BM and PB cell suspensions and their subsequent immunofluorescent staining. Based on their size, cell shape, color and intensity of fluorescence CTCs are then distinguished, enumerated and in FACS machines separated, from normal hematopoietic cells.

Flow cytometry has the highest analysis rate of the scanning assays with reported rates around 10^4 cells/sec-1 [234]. The major difficulty of applying flow cytometry for CTC detection is that without applying sophisticated cell-sorting technologies, the cells constituting the 'positive' signals are lost, and thus are not available for morphologic examination. Furthermore, it is generally perceived that the system noise does not permit the reliable detection of objects below the frequency of $1:10^4$. The one reported exception requires very complicated preparation with 8 different fluorescent tags and slower scan rates [235]. Enrichment procedures can increase the concentration of CTCs to a level detectable by flow cytometers but at the cost of sensitivity loss and substantial added processing complexity [234]. For these reasons flow cytometry based detection of CTCs has been abandoned by most investigators and the clinical relevance of this method still remains to be demonstrated.

2.11.1.2 Immunocytochemistry

2.11.1.2.1 Technical considerations

2.11.1.2.1.1 Advantages of immunocytochemistry

Early attempts used cytological identification of tumor cells and distinguishing between normal cells and tumor cells were based solely on morphological features [193]. However, the identification of CTCs only on the basis of cytomorphological criteria is extremely difficult and impractical due to its low sensitivity. The advent of ICC in the 80s that permit the identification of CTCs on the basis of qualitative features greatly facilitated the investigations of DTCs and CTCs. Currently, ICC is considered to be the gold-standard method for reliable CTC detection.

Immunological detection of CTCs involves the incubation of cells with monoclonal, polyclonal or a cocktail of antibodies directed against epithelia-specific antigens, which are then identified by immunocytochemical or immunofluorescent method. The specific binding is usually detected by the application of secondary antibodies conjugated with enzymes and their chromogenic substrates or fluorophores. The sensitivity and specificity of cancer cell detection by immunochemical or immunofluorescent staining techniques are determined by multiple parameters: the expression level of the target

antigen in disseminated cancer cells vs. normal hematopoietic cells, the affinity of the primary antibody, and the development technique.

The major advantage of this technology is that it allows for morphological, immunological or molecular cytogenetic studies to be performed on the recovered CTCs. The simultaneous morphological analysis vastly improves the specificity of the ICC assay as characteristics of the malignant features can be recognized on the positively stained cells. Furthermore, with multi-marker characterization high-content cellular analysis can be performed on CTCs. Thus, in addition of the mere detection and quantitation of CTCs, critical information such as their metastatic potential and therapeutic sensitivity can be deciphered. Another practical advantage of ICC method is that the technology is widely available in clinical pathology laboratories and pathologists are familiar with the requirements for specimen preparation [199].

2.11.1.2.1.2 Problems related with ICC

- Robustness

Primarily, the sensitivity of the ICC assay is proportional to the cellular volume analyzed. The estimated frequency of CTCs in the BM and in the PB is in the range of 1 tumor cell in the background of 10^6-10^8 normal hematopoietic cells [236]. Studies not using enrichment typically analyze $\leq 10^6$ cells with cell-based assays as processing more cells would be highly time-consuming and impractical. Assuming a uniform distribution of cancer cells, Poisson statistics can be used to predict the probability of false-negative detection at different sample sizes (***Table 10***). However, even assuming that the cancer cell prevalence is at the higher end of its range (10^{-6}) the risk of a false-negative detection is unacceptably high (36.78%) with this sample size. Thus detection with these cytological methods is mostly limited by the relatively small number of analyzed cells [237]. The recent introduction of Automated Digital Microscopy (ADM) systems is trying to overcome the time-consuming manual microscopic analysis (see *Chapter 4.3.1*).

- Difficult read-out

The most critical variables in the ICC methods are the choice of the primary antibody and the scoring criteria of tumor cells. Recent attempt to standardize the subjective readout of immunostained cells has been undertaken by the International Society of Cell Therapy and Graft Engineering and summarizes criteria for the classification of cells as tumor or non-tumor [238]. Sub-categories in their classification scheme include tumor cell, uninterpretable cell, probable hematopoietic cell and hematopoietic cell. Because the threshold for positivity often is set to >=1 positive cell/sample, the conclusion of a positive sample has to be carefully identified.

- Cellular-loss

The pre-analytical steps of an ICC-assay involve some sort of preparation of the BM or PB in order to bring the cells of interest to a substrate that is suitable for analysis. These can consist of buffy-coat preparation, recovery of MNCs on Density Gradient (DG), red blood cell lysis, cytospin preparation, direct cell adhesion, and the series of vigorous washings steps during staining. Each of these steps that precede the microscopic examination involves a substantial risk of cell loss. It is estimated that the overall cell loss of an ICC procedure can be as high as 60% [239, 240]. In addition, whether there is any preferential loss of CTCs during these processes have not been thoroughly investigated and is currently unknown.

- Down-regulation of target antigens

Just like in primary tumors and their metastasis ICC analysis supports that there is a considerable heterogeneity of antigen expression in CTCs [241]. Expression of tumor-related antigens on CTCs can be very weak due to down-regulation of antigens which are strongly expressed in the primary tumor or in the originating tissue. For this intra- and inter-tumor heterogeneity it is necessary to use a mixture of antibodies targeting multiple common epitopes and independent antigens. Recent *in vitro* studies provided evidence that the expression of individual CK components, such as CK 8, 18 and 19, may also be down-regulated during mobilization of tumor cells [242]. This phenotype switch that is often called EMT is marked by a complex and coordinated set of

molecular changes leading to cell behavioral changes [243]. The same histologic, molecular, and transcriptional, elements that change during EMT are commonly associated with carcinoma progression, leading to the obvious possibility of EMT as a part of the metastatic process. Therefore, the down-regulation of CK components and the parallel aberrant expression of vimentin in epithelial cancer cells might be related to local invasiveness and metastatic potential [244].

- Cross-reactivity of antibodies

Macrophages, B cells, T cells and some other cell have a great propensity to fix antibodies via their Fc receptors in a non-specific manner [209]. To avoid this, instead of using full IgG's antibodies, F(ab)2, or Fab-fragments that do not contain the Fc-region, may be used. In addition, by exploiting the preferential avidity of Fc receptor for human> mouse> rabbit> swine> goat immunoglubulins, one may use a blocking of the receptors with a reagent which will not interfere with the secondary reagents and with which the secondary antibodies can be absorbed.

Even with using Monoclonal Antibodies (MoAbs) that were previously believed to be highly specific and validated to a certain epitope cross-reactivity reaction can and do occur. One example for such cross-reaction is the MoAb 2E11 that was commonly used for detection of DTCs in the BM of cancer patients. In a recent study a strong cross-reactivity reaction was shown with this antibody to epitopes displayed by hematopoietic cells [245]. However, with modification of the staining protocol specific staining of epithelial cells could be achieved. Nevertheless, the authors of this study concluded that as long as there is no antibody available which is highly specific for epithelial cells, detection of micro-disseminated tumor cells in BM by antigen-antibody reaction should be verified morphological criteria.

- Phagocytized cellular debris

Although, it is not a real cross-reactivity reaction fragments of CTC or normal epithelial cells phagocytized into hematological cells can also be stained un-specifically [128]. For example, PSA-positive cells in the PB could be monocytes that have been pinocytosed free PSA in serum or phagocytosized PSA-positive tumor cells [246].

Again, morphological inspection of the positively stained cells by a trained cyto-pathologist may help to eliminate such events.

- Cross-reactivity of reagents

Common immunocytology indicator enzymes like horseradish peroxidase or alkaline phosphatase can give rise to false-positive cells because of endogenous enzymes [247]. In the study of *Borgen et al.* it was found that some plasma cells can very strongly react with the alkaline phosphatase-anti-alkaline phosphatase complex [248]. Although, endogenous enzyme activity may be blocked by treating the tissue with various blocking agents, (as for example peroxidase activity may be blocked with phenylhydrazine, azide plus nascent hydrogen peroxide, or periodic acid, while endogenous alkaline phosphatase activity may be blocked by adding levamisole) a complete blockade is often unfeasible [249]. Morphological evaluation and adequate controls are therefore important to ensure the diagnostic specificity of the assay.

- Presence of non-malignant epithelial cells in the BM and PB

Even in subjects without malignancy very low numbers of normal epithelial cells were shown to be present in the BM and in the PB. Although, the presence of normal epithelial cells in these compartments are usually temporary and can be related to benign epithelial proliferative diseases and/or tissue trauma, without repeated sampling and morphological/phenotypical characterization they may be confused with CTCs [250, 251].

2.11.1.2.2 Target selection

2.11.1.2.2.1 Intermediate Filaments, Cytokeratins

Intermediate Filaments (IFs) form the third fibrous element of the cytoskeleton in almost all vertebrate cells. The diameter of the IFs varies between and 7-11nm and their diameter are ‘intermediate’ between microfilaments (6nm) and microtubules (25nm). Gel electrophoresis of whole cells and of cytoskeleton preparations shows that IF proteins are major cytoplasmic components. Within the adult anima, biochemical, immunologic, and chemical evidence suggests the existence of five distinct subgroups of IF proteins.

These are:

1. the CKs characteristic of true epithelia
2. neurofilaments characteristic of many but not all neurons
3. glial fibrillary acidic protein filaments found in various glial cells
4. desmin filaments characteristic of most myogenic cells
5. vimentin filaments typical of various non-epithelial cells, in particular those of mesenchymal origin.

Although probably many functions of the intermediate type filaments are still unknown, IFs provide tissue and differentiation specific markers which are remarkably stable even under conditions where other markers are lost, e.g. during malignant transformation or cultivation *in vitro* [252, 253]. As they are major constituents of epithelial cells and their specificity of expression is retained in malignant tumors, they have been used extensively in diagnostic tumor pathology [254]. The intermediate-size filament-type characteristic of the cell of origin seems to be retained not only in the primary tumors but also in their metastases.

CKs, the intermediate-size filament of the cytoskeleton of epithelial cells, show an astonishing complexity. There seem to be more than 20 distinct components, differing in their molecular weights and isoelectric points. Grouping of CKs into different subclasses can be performed by either gel electrophoresis or by antibody based approach. Either conventional polyclonal sera or MoAbs are suitable to target specific subsets of CKs. By now many MoAbs against CKs have been described [255, 256]. Different types of epithelia are characterized by typical sets of individual members of the CK family [257]. The CK expression profile, which reflects both epithelial type and differentiation status, makes them suitable as histological markers of differentiation [258].

CKs have been used extensively as markers of CTC detection [259]. Normal cells of the PB and BM are of mesenchymal origin, thus they do not express CK proteins making the disseminated epithelial cells readily distinguishable [260]. By use of MoAbs which

recognize epitopes specific for individual CKs one should be able to distinguish and analyze the different epithelial lineages. At present, ICC using CKs as epithelial marker proteins is the most common method for CTC detection.

Although testing for other antigens, such as the Epithelial Membrane Antigen (EMA) or mucin-1, has been tried but current data suggest that antibodies directed against these polymorph epithelial mucins label 2%–10% of the mesenchymal mononucleated cells of healthy volunteers [261]. Many studies have therefore used MoAbs against CKs as these proteins are stably and abundantly expressed in a majority of epithelial tumors and in a majority of the cells in these tumors. Furthermore, sufficient methodological validation of the detection antibodies has only been reported for anti-CK antibodies [135]. Although illegitimate CK mRNA expression by hematopoietic cells might be detected by sensitive PCR technologies, this problem is not relevant to ICC approaches because CKs are very rarely detected in mesenchymal cells [262-264].

2.11.2 Molecular methods

In the last few years, molecular detection techniques have been used extensively to identify CTCs. Theoretically, the amplification of tumor-specific abnormalities present in the DNA or mRNA by PCR could provide a very sensitive and specific means of CTC detection. In fact, this method was shown to be very useful in minimal residual disease detection in hematological malignancies [265]. Two different groups of molecular markers were reported for the identification and characterization of CTCs (***Table 11***) these comprise:

1. Genetic alterations.
2. Tissue specific molecular markers including epithelial and organ specific mRNA transcripts whose expression is retained during carcinogenesis. Gene transcripts specific for the tissue of cancer origin are indicative for disseminated cells when the gene is normally not expressed at the site of investigation. The utility of current molecular tests for the detection of CTCs are limited by lack of tissue-specific markers in many solid tumors.

3. Cancer cell specific molecular markers composed of genomic alterations and cancer cell specific mRNA transcripts.

However, in solid tumors the main limitations in the determination of genomic alterations like translocations, point mutations, allelic losses and amplifications are that:

a) Only a few tumor-types show a consistent genomic alteration; and
b) High sensitivity detection of mutations can only be achieved in cases where these occur in a few specific codons of a gene or when the mutation is already known (e.g. after sequencing of primary tumor material [233].

2.11.2.1 PCR

The PCR method involves the extraction of DNA from cell preparations and then the enzymatic amplification of specific DNA sequences using oligonucleotide primers that flank and therefore define the region of interest in the target DNA. In CTC detection this means the PCR amplification of tumor-specific abnormalities present in the DNA.

2.11.2.1.1 Technical considerations

PCR has been used to detect free DNA within plasma. However, PCR has also had difficulty with poor specificity [266]. This is due in part to the longer half-life of DNA in plasma when compared to mRNA. As a result it is unclear whether the free DNA that is amplified from plasma is from CTCs in the circulation or if the DNA is being shed from primary tumors, metastatic tumors or from normal tissue [267].

2.11.2.1.2 Target selection

Concerns over assay specificity with PCR may be overcome by the identification of tumor specific DNA modifications, such as with methylation-specific PCR primers [268]. However, age-dependent hypermethylation is another source of potential problems. As for example age-dependent hypermethylation in promoter regions of up to 50% of the genes hypermethylated in colorectal cancer has been found in normally aging colonic mucosa [269].

Loss of Heterozygosity (LOH) is another mechanism that frequently leads to the inactivation of tumor suppressor genes in cancer cells. For the detection of LOH in cancer cells, polymorphic tandem repeats (microsatellites) in non-coding regions are amplified by PCR. A comparative analysis of both the non-neoplastic cells and a tumor sample from the same patient is necessary to reveal the loss of one allele in cancer cells. DNA mismatch repair deficiency is frequently observed in hereditary carcinomas of patients with HNPCC syndrome, as well as in about 15% of sporadic cancers, such as gastrointestinal carcinomas, bladder cancer, lung cancer, head and neck cancer, and endometrial cancer [270]. Unfortunately, LOH and microsatellite alterations are not restricted to malignant cells, but they also occur (less frequently) in premalignant lesions, or in nonmalignant tissue due to chronic inflammation or chronic exposure to carcinogens. Hence, as criteria of malignancy, both LOH and microsatellite instability are of limited diagnostic value. Moreover, with a detection limit of 1 cancer cell in up to 500-1,000 nonmalignant cells, analysis of these alterations provide a relatively low sensitivity for the detection of malignant cells in clinical samples.

Point mutations in some genes are consistently found in various types of malignancies. For diagnostic applications, predominantly mutations in p53 and K-ras have been investigated. The p53 gene is mutated in about 50% of all carcinomas, with hot spots in exons IV–VIII. The highest mutation rates of K-ras occur in pancreatic carcinoma (75%), thyroid carcinoma (50%), colorectal cancer (40%), and pulmonary adenocarcinoma (about 25%) [270]. Point mutations of K-ras are exclusively found in codons 12, 13, and 61. The majority of K-ras mutations is found in codon 13, and can be selectively amplified by a special primer set. The maximal sensitivity achieved with this type of mutation-specific PCR assay is in the range of $1{:}10^4$-10^5 [271]. This is because even under highly optimized conditions a complete digest of all DNA molecules with the restriction site recognition pattern can never be achieved [272]. Just like with the other alterations described above, K-ras mutations were also detected in benign and inflammatory conditions, as for example in premalignant pulmonary lesions, colon adenomas, and pancreatitis [273-275]. In summary, currently available DNA-

based PCR assays do not provide optimal sensitivity and specificity for the detection of rare cancer cells in clinical samples.

2.11.2.2 Reverse Transcriptase-PCR

Reverse Transcriptase-PCR (RT-PCR) involves the extraction of mRNA from cells and then its conversion into cDNA through the use of specific primers and the enzyme reverse transcriptase, and then the amplification of cDNA by either nested or non-nested PCR reaction. The PCR product is then visualized by various techniques.

2.11.2.2.1 Technical considerations

2.11.2.2.1.1 Advantages of RT-PCR

RT-PCR is more rapid and can be more easily automated and standardized than ICC, so it is more suitable for routine laboratory analysis. Theoretically, RT-PCR is more sensitive than ICC, because a very low transcription-level with weak or no translation can be detected. In practice it means that tissue-specific mRNA can be present despite a negative protein-based assay. As for example, PSA mRNA transcripts have been detected in poorly differentiated prostate carcinoma cells that do not express the PSA protein [276].

2.11.2.2.1.2 Problems related to RT-PCR

- Illegitimate transcription

As a single copy of tissue-specific mRNA molecule is enough to generate a signal through amplification via RT-PCR, it is a highly sensitive method of CTC detection. This sensitivity, however, is the inherent limitation in the approach as the technique is highly prone to false-positive detections. This is mostly because the target genes can be expressed at a low level in hematopoietic cells (estimated at one mRNA molecule in 100-1,000 cells) [277]. This phenomenon, called illegitimate transcription, was first described by *Chelly et al.*, who noted that the transcripts of the Duchenne muscular dystrophy gene were not only detectable in the muscle and brain cells (as expected), but also at low levels in a range of other cell types [278, 279]. The interference of illegitimate transcription has already been demonstrated in several target genes of CTC detection, such as CKs, MUC-1 and CEA [261, 280-283].

- Cell-free RNA

Several studies successfully amplified mRNA from cell free fractions such as the serum or plasma [284-287]. This result means that, contradictory to previous thinking, the detection of RNA signal from the PB is not necessarily an indication of the presence of intact tumor cells but rather could come from freely circulating nucleic acids in the PB [288]. The elucidation of the biology of RNA release, clearance, and possible functionality of clinical use is area of intensive investigation [289].

- Instability of RNA and the presence of PCR-inhibitors

Additional sources of error on the mRNA level include the instability of the RNA molecule. Most human tissues, including blood and BM have high levels of RNAses. The degradation of RNA during the various steps preceding the PCR reaction itself accounts greatly for the high variability of sensitivity of RT-PCR results [290]. Another problem could be the presence of contaminants sample like heparin, hemoglobin, melanin and porphyrins in the PB or BM. These can unfavorably alter the efficiency of the PCR reaction. Therefore, careful controls are necessary to ensure that there is amplifiable RNA in the sample.

- Up- and down -regulation of mRNA half-life by molecules present in the blood

It was demonstrated that transcription of so-called tissue-specific genes is inducible in hemopoietic tissues under certain conditions, as for example the inflammation process [291]. On the other end, various molecules in the PB and BM were shown to induce down-regulation of the target genes of CTC detection. For example PSA mRNA expression was shown to be decreased by anti-androgen therapy and in poorly differentiated prostatic carcinoma [276, 292]. The deficient expression of the marker gene in CTCs may lower the actual sensitivity of the RT-PCR assay *in vivo* as compared with experimental model systems that use tumor cell lines for sensitivity estimation [293].

- Inability to visualize tumor cells and perform functional assays

Another important drawback of molecular methods is the inability to employ morphologic criteria to confirm the presence of CTCs. Without visual inspection, clinically important information about CTCs, such as the presence of CTC clusters or fragmented cells is lost [87, 184]. Furthermore, molecular assays require the destruction of cells to extract their nuclei acid content. Thus, downstream functional assays that could theoretically yield in important information regarding the growth and metastatic potential of CTCs cannot be performed.

- Difficulty with quantification

RT-PCR positivity only reflects the presence of circulating cells containing target mRNA. It does not, however, correlates with the number of CTCs. Accurate quantification of CTCs is problematic with RT-PCR. With the exponential nature of PCR amplification combined with the small quantities of target molecule, a minimal variation in cycling conditions can significantly influence the yield of the final product. Furthermore, quantification of rare transcripts is complicated, requiring replicates of samples and carefully selected housekeeping genes and/or artificially added internal standards to normalize the level of expression [294, 295]. In addition to the technical difficulties, another problem is that the transcription rate of target genes varies between individual tumor cells, thus it is impossible to know whether the signal is coming from a few cells with high expression or from many low expressing cells. Thus, it might be difficult to interpret the RT-PCR results and distinguish between changes in tumor cell numbers or changes in expression levels. The situation becomes even more complex if the RT-PCR test is used to monitor therapy-induced changes in minimal residual disease because the therapy might affect the expression level of the marker transcript in tumor cells [202]. Some of these observed problems may be irrelevant if well-validated quantitative real-time RT-PCR methods become widely available (see *Chapter 4.2.1*).

- Prone to false-positive detection

The occurrence of false-positive results is a difficulty which is born of the immense power of the technique. Therefore RT-PCR assays generally require more careful

handling and processing and are more sensitive to contamination than conventional PCR assays or ICC-based methods. Only scrupulous attention to laboratory practice and the physical separation of nucleic acid extraction, PCR amplification and the manipulation of the amplicons will prevent this [296].

- Presence of pseudogenes

Pseudogenes are defunct relatives of known genes that have lost their protein-coding ability or are otherwise no longer expressed in the cell [297]. For some of the target genes of CTC detection, as for example for CK18 and CK19, processed pseudogenes are present in the genome [298, 299]. Because the amplification products of cDNA and processed pseudogenes are of the same size, residual contaminating genomic DNA in the RNA preparation may cause false-positive results. This problem can be solved with either selecting target genes that lack known pseudogenes sequences, as for example CK20, or with careful primer design and the systematic utilization of DNAse [300, 301]. However, for absolute certainty the sequence of the amplified products needs to be determined.

2.11.2.2.2 Target selection

RT-PCR-based amplification of transcripts present in cancer cells, but not in surrounding non-neoplastic cells, is increasingly applied to sensitively detect isolated cancer cells in clinical samples. For the detection of dissemination of solid tumor cells to be successful, distinct characteristics of cancer cells are required for diagnostics on a molecular level. However, solid tumors are notable for their extreme genetic heterogeneity and the rarity of tumor-specific genetic abnormalities. Only chimeric gene-transcripts, resulting from chromosomal translocations are truly tumor-specific, but these are very infrequently found in solid tumors. Ewing's sarcoma is an exception from this rule as the amplification of chimeric tumor-specific mRNA has been performed successfully in this type of solid tumor [302]. Other exceptions are the virus-associated cancers, such as anogenital carcinomas or cervical cancers associated with persistent infections with ‘high-risk’ Human Papilloma Virus (HPV) types. The fusion transcripts in this case are consisting of both viral and human sequences and can be

specifically detected with PCR [303]. However, for the vast majority of human cancers no truly carcinoma-specific transcripts have yet been identified.

Alterations in gene expression, including alternatively spliced isoforms, and altered ratios of gene expression are other common genetic alterations seen in cancer [304, 305]. In this respect the expression of specific variant splice products of the CD44 gene has been most extensively studied. However, further studies revealed that these alternative spliced transcripts are also expressed in the proliferating basal cells of various epithelia [306-308]. Thus again it turned out that these changes are neither specific nor sensitive for any individual cancer [309].

Most currently used target genes for RT-PCR based detection are involved in tissue differentiation (mainly epithelial genes) or in malignant transformation (***Table 11***). Tissue-specific markers can be used if they are expressed in the tissue of origin of the respective tumor cells, but not in tissues, in which the tumor cells should be looked for. The first report on these assays dated back until 1991 when *Smith et al.* used for the first time RT-PCR amplification of tyrosinase transcripts to detect circulating melanoma cells in PB samples [310]. The first comprehensive data set presenting the detection of tissue-specific gene expression by RT-PCR in the PB changed even the view of how cancer cells invade. The detection of PSA-positive cells in the PB of prostate cancer patients by RT-PCR substantiated that hematogeneous micrometastasis can occur without LN involvement [311].

Since then numerous assays have been developed for different types of cancers using a broad variety of tissue/differentiation specific transcripts. Generally, the promise of the use of these markers to detect CTCs is on their lack of detection in negative controls. However, two major issues my influence the specificity of these assays. First, by definition tissue-specific markers are expressed in tumors as well as in their normal counterparts. Thus the mechanical introduction of normal or benign cells in the circulation after invasive procedures and/or by non-malignant pathological processes may lead to false-positive detections. [280, 312-317]. Second, illegitimate transcription

(see *Chapter 2.11.2.2.1.2.)* of the target genes in non-transformed leukocytes in the PB or BM poses a tremendous amount of problems with specificity of these types of assays.

Another attractive target for CTC detection by RT-PCR amplification is the family of oncofetal antigens, whose expression is restricted to embryonic development and transcriptionally activated in the course of carcinogenesis. The most commonly used target genes include the Carcinoembryonic Antigen (CEA), the Beta chain of the Human Chorio Gonadotropin (β-HCG) and the Alfa-Fetoprotein (AFP). However, just like with tissue-specific markers many studies has found significant levels of illegitimate transcription also among healthy individuals or under irrelevant pathological conditions [298, 318-321].

A further class of tumor associated antigens otherwise exclusively expressed in embryonic tissues or in the adult testis are the various antigens of the 'cancer-testis' gene family. Currently, the 'cancer-testis' family includes more than 20 antigens in the MAGE, GAGE, BAGE family, whose characteristics, aside from the highly tissue-restricted expression profile, include heterogeneous protein expression in cancer and likely correlation with tumor progression [322]. Although, their apparently restricted expression in non-transformed tissues suggests that illegitimate transcription might be of minor importance, but the above mentioned features dramatically restrict their clinical utility as CTC markers [323].

When summarizing the studies conducted by RT-PCR using the above mentioned markers a very high inconsistency has to be considered. Clearly, illegitimate transcription of the respective markers and the appropriate RT-PCR design poses a tremendous amount of problems, which needs to be solved. These aspects render the various RT-PCR studies reported so far almost totally incomparable in meta-analysis. Close collaboration of laboratories and in particular inter-laboratory quality control is urgently required until these assays can be recommended as diagnostic tools in the daily clinical practice. Until then, despite the large number of exploratory studies, the prognostic relevance of CTC dissemination detected by RT-PCR-based assay remains unclear.

2.11.3 Enrichment methods

Producing an analyzable aliquot of nucleated cells from BM or PB samples, which are relatively low-cellularity fluids, requires some degree of concentration and/or enrichment. To increase the specificity and sensitivity of cancer cell detection several approaches for CTC enrichment have been developed. These sample processing protocols are relatively vigorous, utilizing harsh chemicals, multiple centrifuging steps, DG separation and/or IME. While extremely effective at enriching the proportion of analyzable cells (both nucleated hematopoietic cells and rare CTCs), these methods are well known to result in considerable cell loss and cell damage, with estimates as high as 60-70% cell loss.

Mainly, two different biological characteristics of cancer cells are exploited for their purification:

1. Their divergent morphology and physicochemical properties in comparison to hematopoietic cells (such as their greater size, different buoyant density, membrane permeability).
2. Their antigenicity.

Enrichment strategies that belong to the first group are often called physical separation methods, while the antigen-dependent enrichment group consists of immunological methods.

2.11.3.1 Physical separation methods

These methods utilize the typical physical and intrinsic characteristics of tumor cells like their greater size, their different buoyant density and cell membrane permeability. The efficiency of physical separation methods depends on the choice of an appropriate cut-off value for the cell density, chemical property or cell size.

a) The minimal centrifugation method relies on the different buoyant density of nucleated cells from other blood components [324]. Advantages of this method include gentleness and the non-chemical processing while disadvantages include possible incomplete sequestration of tumor cells within the buffy coat layer (i.e. cells 'lost' in discarded fluid) and the necessity of tube-tube transfer.

b) Red blood cell lysis with ammonium chloride is a well accepted method of nucleated cell separation. This method relies on the fact that membranes of anucelated cells are more susceptible to chemical reaction with certain reagents than nucleated cells, and consequently they can be preferentially lysed [325]. The major advantage of the technique is that it requires minimal centrifugation and transferring steps. The disadvantages include possible damage of tumor cell membranes and their consequent lyses due to the biological unpredictability of the fragility of malignant cells.

c) Hypotonic red blood cell lysis takes advantage of the fact that red cells undergo lysis in H_2O very quickly, while nucleated cells are damaged much more slowly. Therefore, a very brief pulse with H_2O will lyse all of the red cells and leave the white blood cells intact. Again, due the biologically unpredictability of malignant cells some optimization of the procedure may be required to insure that the cells of interest are not lysed.

d) Dextran can also be used to deplete red blood cells as it increases the viscosity of the blood and sediments erythrocytes, whereas leukocytes stay in the plasma fraction. The advantage of this method is that throughout the procedure physiological values of pH and osmolality are maintained. However, it is usually difficult to achieve a complete depletion of red blood cells with this method and erythrocyte contamination could interfere with the assay [326].

e) Density Gradient separation with nonionic synthetic polymer of sucrose was originally developed for the enrichment of MNCs from blood or BM [327]. DG cell separation is a more proactive sorting of the MNC population using fairly vigorous centrifugation in specialized media. Many CTC studies perform a DG centrifugation

with medias of various brand names (e.g. with Ficoll™, Histopaque™, OncoQuick™). One key advantage of this technique is a more complete sequestration of nucleated cells, while disadvantages include increased numbers of centrifugation steps, washes, transfers and the potential loss of tumor cells. It was found that cancer cells can also be detected in the granulocyte fraction, since cancer cell aggregates may differ significantly from single cancer cells in their buoyant density [328]. Hence, it can not be excluded that significant numbers of CTCs are lost if they are isolated by DG centrifugation. Nevertheless, it has to be acknowledged that the vast majority of our knowledge about the clinical importance of DTCs and CTCs was generated using DG centrifugation procedures.

f) **ISET** (Isolation by Size of Epithelial Tumor Cells) is another enrichment method that allows the collection of carcinoma cells by filtration, because of their larger size when compared to PB leukocytes. Further studies, focusing on additional cell lines and patients with a variety of carcinoma types, have to be performed to define the size threshold of ISET application. Apparently, the most valuable advantages of ISET is its ability to isolate epithelial cells without damaging cell morphology and its applicability in a wide range of solid tumors [329]. Clearly, however, this methodology should not be suitable for studies on small neuroendocrine tumor cells. Although the method has great potential, so far there are no data available which have evaluated the performance of filtration methods on a relevant number of clinical samples [330].

These above methods have been extensively used in the past for the recovery of rare cells from whole blood, as for example Cytomegalovirus (CMV) infected granulocytes, pluripotent hematopoietic progenitor cells, and fetal cells in maternal blood, etc. [326, 331-333]. Except for the DG separation method, which is enriching for MNCs, there is less experience on the effect of the above mentioned techniques for CTC recovery.

2.11.3.2 Immunological separation methods

To increase the sensitivity and specificity of CTC detection, several approaches of cell enrichment have been developed that are based on immunological characteristics of disseminated epithelial cells. These methods exploit the presence of epithelial specific

antigens (positive selection) or the absence of hematopoietic antigens (negative selection) on the surface of carcinoma cells. The most commonly used immunological separation method employed for CTC detection is IME. Two different approaches, the negative and positive IME, have been developed for CTC detection.

2.11.3.2.1 Negative IME

The basic principle of negative selection is the depletion of White Blood Cells (WBCs) with anti-leukocyte antibody coupled magnetic beads. The advantage is that cancer cell separation is not dependent on the expression of cancer or tissue specific antigens by the disseminated cancer cells. However, trapping of CTCs within the WBC fraction is often problematic [334]. In most studies negative IME tends to have lower yield than positive IME, and no more than a 3-4 fold enrichment of cancer cells is achievable [335, 336].

2.11.3.2.2 Positive IME

With positive concentration, antibodies reactive against epithelial cell surface antigens are conjugated on the surface of magnetic microbeads. CTCs attach to the beads and are separated from the cellular medium with magnetic fields. This is the most direct and specific way to isolate the target cells from a cell suspension and a number of companies are offering ready-to-use products for the separation of blood borne carcinoma cells. These immunomagnetic beads differ in their size, the amount of antibody coating and the prior preparation procedure necessary before IME. Although, some of these companies offer custom-coated magnetic beads, except Miltenyi Biotech's Carcinoma Cell Enrichment Kit™, in which the target antigens are intra-cellular CKs, the positive IME technology solely depends on the use of the cell-surface antigen, EpCAM. EpCAM is a homophilic cell-cell adhesion molecule, also called 17-1A antigen, which is encoded by the GA-733-2 gene [213]. The protein is found to be present in the vast majority of carcinomas, and has attracted attention as a tumor marker. A serious concern about the reliability of this method is that cancer cells have been observed to show considerable heterogeneity in the expression of cancer-specific, cell-surface antigens. *Braun et al.* found that CK+ cells in the BM of breast cancer patients co-express EpCAM, HER-2/neu (c-erb-B2), and MUC-1 in only 47%, 41%,

49%, respectively [241]. *Kasimir-Bauer et al.* reported even lower co-expression frequencies of EpCAM (15%) and HER-2/neu (c-erb-B2) (2.5%) on the surface of CK+ cells in the BM [337]. In terms of the tumor cells perspective down-regulation of EpCAM expression would permit these cells to be released from contact-mediated controls within the primary tumor, a key aspect of the metastatic process. The sensitivity of IME is strongly influenced by the level of expression of the enrichment target protein and thus the absence or down-regulation of these cell surface molecules can result in substantial reduction of assay sensitivity.

The method of IME produces an enriched sample of CTCs that can be subsequently used for DNA separation, mRNA purification, development of immunoassays, capture of biomolecules, and protein purification. It is compatible with downstream applications, such as flow cytometry, microscopic analysis, molecular biology experiments, cell culture and *in vivo* experiments. The overall sensitivity of the IME assay thus depends on the subsequent detection method. In cell line experiments, sensitivities between $1:10^6$ and $1:5x10^7$ have been reported for cancer cell detection by IME techniques and consecutive immunocytochemical staining [338, 339].

Most enrichment protocols use a combination of DG centrifugation and immunomagnetic separation or red blood cell lysis and filtration. The net yield of these approaches is low because of the relative low yield of the DG centrifugation step. The only advantage of this combination strategy is the analysis of a larger sample specimen in an acceptable time. Studies using IME with for detection of CTCs in the PB of breast cancer patients are summarized in ***Table 12***.

In summary, the methods applied to the separation of blood-borne cancer cells are time-consuming and rather expensive. Each method of concentrating has advantages and disadvantages and adds time and costs to the process of analyzing a fluid specimen. Currently available enrichment technologies require careful balancing between ‘minimal damage’ and ‘maximal concentration’ techniques.

2.12 Difficulties of CTC detection

Despite the clinical relevance and various methods of detection of solid tumor cells in hematological tissues, there not yet exists a consensus on the reliability of the methods that should be used for CTC detection. This is due to the fact that creating such a clinical test is extremely challenging.

The criteria, put together by the International Society of Cell Therapy Working Group for Standardization of Tumor Cell Detection, for a good tumor cell detection method are listed below [202]:

- High specificity and sensitivity
- Reproducibility
- Robustness
- Objective read out
- Potential for automated analysis
- Quantitation of tumor load
- Characterization of tumor load
- Proven clinical significance

It can be deduced from the previous chapters that these criteria are not yet fully met with any of the existing CTC detection methods. Therefore, it is not very surprising that results from different studies are highly discordant. This is well represented by the fact that just the mere detection rate of DTCs in the BM from non-metastatic breast cancer patients is in the range from 0 [340] to 100% [341]. But even if studies that examine a homogenous patient population who carry a high tumor cell burden are compared, results are highly discrepant and a significant proportion of patients are tested negative. ***Table 13*** contains relevant studies in metastatic breast cancer patients showing that irrespective of the enrichment technology used 35%-63% of patients remain to be tested negative.

The most probable reasons for the discrepant results are the substantial methodological variation between studies. Factors that are divergent in the above mentioned studies and could influence the sensitivity and specificity of a CTC assay are summarized below.

- Patient group
- Sample size
- Sampling method
- Sample handling
- Sample preparation procedure
- Method of detection
- Marker of detection
- Criteria/threshold for positivity

Besides there are intrinsic, confounding factors of a CTC assay that can influence the results regardless of the selected detection method. These common pitfalls of CTC detection are the following:

- The random disposition of tumor cells

Because tumor cells are not necessarily distributed uniformly in the BM a single sample or a small volume sample may not reflect the extent of tumor spread. It has been demonstrated that BM taken at several sites rather than only a single one improves sensitivity [251]. Studies have been performed where samples were taken at six or eight different sites [171, 342]. Likewise, the amount of tumor cells in the PB fluctuates. Serial blood samples may therefore give different results [343].

- Lack of quantification of tumor burden

Most studies on CTCs report whether a sample does contain tumor cells or not, without quantifying the tumor burden. Quantification of tumor burden could however have an important clinical value. As for example the actual amount of tumor cells detected in BM from patients with breast cancer, SCLC and prostate cancer seems to have a prognostic significance [172, 344, 345]. Furthermore, quantification of tumor cell burden during treatment might correlate with the efficacy of anti-cancer therapy and

thus effective therapy can be continued and ineffective therapy discontinued in a timely fashion [312, 346].

- Tumor-cell heterogeneity

The principle of CTC detection relies on the recognition of particular markers (e.g. antigens, gene transcripts, etc.) that distinguish tumor cells from surrounding cells, principally hematological cells. The ideal tumor-cell marker expressed by all tumor cells and not by other cells has not yet been found.

In summary, the clinical impact of CTC detection in solid epithelial cancer patients is still a matter of debate. Studies have yielded inconsistent results, most likely caused by the variety of methods applied to detect CTCs and the limited statistical power of single studies with small patient series. Because of the lack of consensus for a benchmark technology for tumor cell detection, the implementation into tumor classifications is pending, and awaits confirmatory studies (e.g. meta-analyses, more powerful validation studies) which are currently underway. Large multi-center trials based on a validated benchmark methodology, appropriately designed and carried-out by an international collaborative study group are needed to investigate the true clinical significance of CTCs.

Regarding methodological heterogeneity, a similar situation occurred in detection and evaluation of minimal residual disease in lymphoma, which, in an international effort, was successfully overcome some 10 years ago [347]. In patients with lymphoma standardized detection procedures now contribute to a refined staging system, resulting in individualized treatment options and an improved outcome for such patients [348]. This example clearly highlights the efforts that are now necessary in order to implement screening for CTCs into current risk classification systems and treatment protocols for patients with solid tumors.

3 Aims

3.1 General aims

A substantial body of literature exists evaluating the biological significance of CTCs from cancer patients. Many studies have concluded that the presence of CTCs provides important prognostic information predictive of disease-free and overall survival in various malignancies. By contrast, other reports have found no statistically significant relationship between CTCs and prognosis. This discrepancy is most likely because even in metastatic patients, the number of CTCs in BM or PB is extremely low. The frequency of CTCs in BM preparation from cancer patients has been estimated to be in the range of 10^{-5}–10^{-6} [262]. When present in PB, CTCs are generally found at a frequency of one cell per $1x10^{6–7}$ PB MNCs or in number 1-10 CTCs/ml [349]. While existing technology can identify these CTCs and correlate them with disease, no method has sufficient sensitivity to reliably measure a statistically significant number of cells, especially at early stages of the disease where treatment can be most effective. The issue of varying conclusions regarding the prognostic significance of CTCs may be attributed to the technical difficulties associated with the reliable detection of such rare events. In order to achieve accurate and reproducible results and determine the true clinical significance of CTCs the evident technical difficulties, however, have to be solved.

The main goal of our work was to develop and improve approaches for CTC detection in the PB of cancer patients and to use this technology in patient studies to investigate the prevalence of CTCs in cancer patients. The aims were to build methods that provide improved sensitivity and specificity of CTC detection while meets the requirements of a practical clinical CTC assay.

To overcome current technical pitfalls of CTC detection, novel methods and concepts of detection as well as thoroughly evaluated and standardized pre-analytical and analytical procedures were employed. Both cell-based and molecular methods of CTC detection were investigated for areas of improvement. In addition, steps of pre-analytical and analytical procedures were dissected in order to identify potential sources of inaccuracy and irreprocubility of CTC detection. The developed instrumentations and preparation

methods were used to investigate the prevalence of various rare cells, such as CTCs and CMV infected leukocytes. Furthermore, together with four different European laboratories a pilot study was conducted to assess the variation and inconsistencies of CTC detection and to formulate the basis of a standardized, quantitative CTC detection protocol.

The overall goal of the work described herein is the development and validation of a more sensitive CTC detection capability that is not subject to the same fundamental limitations as other CTCs assays. Achieving this goal would enable us to have an improved understanding of the biological relevance and diagnostic value of CTCs.

The subject of this thesis is based on the following published studies:

a) Study#1: Ladanyi A, Soong R, Tabiti K, Molnar B, Tulassay Z. Quantitative reverse transcription-PCR comparison of tumor cell enrichment methods. Clin Chem. 2001 Oct;47(10):1860-3.

b) Study#2: Soong R, Ladanyi A. Improved indicators for assessing the reliability of detection and quantification by kinetic PCR. Clin Chem. 2003 Jun;49(6 Pt 1):973-6.

c) Study#3: Vlems FA, Ladanyi A, Gertler R, Rosenberg R, Diepstra JH, Roder C, Nekarda H, Molnar B, Tulassay Z, van Muijen GN, Vogel I. Reliability of quantitative reverse-transcriptase-PCR-based detection of tumor cells in the blood between different laboratories using a standardised protocol. Eur J Cancer. 2003 Feb;39(3):388-96.

d) Study#4: Ladanyi A, Sher A, Kraeft SK, Herlitz A, Bergsrud D, Jafri N, Salgia R, Chen LB. Detection of occult tumor cells in the PB of Small Cell Lung Cancer Patients with the Rare Event Imaging System. American Society of Clinical Oncology meeting, 2003, Chicago, IL. Proceedings of ASCO 2003:22:A3404.

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e) Study#5: Ladanyi A, Sher AC, Herlitz A, Bergsrud DE, Kraeft SK, Kepros J, McDaid G, Ferguson D, Landry ML, Chen LB. Automated detection of immunofluorescently labeled CMV-infected cells in isolated peripheral blood leukocytes using decision tree analysis. Cytometry A. 2004 Apr;58(2):147-56.

3.2 Specific Aims

3.2.1 Specific aims of Study #1

1. To assess the effect of various sample preparation procedure on the real-time quantitative RT-PCR (qRT-PCR)-based detection of CTCs.
2. To assess the effect of various sample preparation procedure on the real-time qRT-PCR-based quantification of CTCs.

3.2.2 Specific aims of Study #2

1. Define the features and boundaries of conventional and real-time qRT-PCR detection.
2. Compare the capabilities of conventional PCR controls and Crossing Point (CP) values to identify false negativity.
3. To validate real-rime qPCR detection at its most sensitive level.
4. Investigate the value of various sample preparation to improve CK20 detection sensitivity, specificity.

3.2.3 Specific aims of Study #3

1. To test reproducibility of CTC detection between different laboratories using a standardized real-time qRT-PCR-based protocol.

2. To investigate the influence of sample processing, RNA isolation, cDNA synthesis and PCR amplification in different laboratories.
3. To compare qualitative and quantitative results of real-time qRT-PCR-based detection between laboratories.
4. To test the feasibility of standardization of pre-analytical and analytical methods across laboratories.

3.2.4 Specific aims of Study #4

1. To analyze PB samples from SCLC patients for the presence of CTCs with the Rare Event Imaging System (REIS).
2. To characterize CTCs with dual-marker immunoflourescence technique.
3. To correlate the number and qualities of CTCs with disease stage and clinical outcomes.

3.2.5 Specific aims of Study #5

1. To evaluate the feasibility of combining automated microscopy with classical image processing and machine learning to detect fluorescently labeled rare cells.
2. To attempt to improve the performance of rare event image cytometry through the incorporation of artificial intelligence methods for data classification.
3. To test the accuracy of this novel detection methodology on CMV-infected cells in isolated PB leukocytes.

4 Methods

Highly sensitive and accurate methods are a prerequisite for the effective detection of rare cells in the BM or in the PB. As described earlier (see *Chapter 2.11*) several CTC detection methods have been explored but each of the existing technologies has critical limitations for CTC detection and quantification. Prior to cell- or molecular -based methods to detect CTCs today's best practices apply some sort of cell-separation procedure to enrich for nucleated cells and/or CTCs.

4.1 Cell-separation methods

After obtaining or creating a suitable blood specimen with a small number of CTCs in it, the next challenge is concentrating the scant population of nucleated cells present in PB, which are dispersed in a large volume. Unlike highly cellular BM aspirations or other types of tissue biopsies, most of the volume of whole blood consists of anucleate red blood cells and plasma. To improve detection of CTCs in most studies some sort of tumor cell enrichment protocol is employed before analysis. These consist of an initial enrichment step of DG centrifugation, buffy coat preparation or other physical separation method of nucleated cell isolation (see *Chapter 2.11.3*). This initial step of enrichment may be followed by a specific isolation step using magnetic beads coupled to antibodies, filter techniques or multi-parametric flow cytometry.

In this thesis we analyzed PB samples, and therefore we focused on optimizing the sample concentration step for this particular type of specimen. We systematically evaluated several different methods, with the hypothesis that damage accrues to individual cells during each step of sample processing, such that each episode of centrifugation, washing, vortexing, exposure to various chemicals, permeabilization and fixation, is a possible episode of cell loss. In our studies four different enrichment procedures were used, two of which belong to the group of physical separation methods (DG separation and ammonium chloride-mediated RBC lysis) and two IME techniques that belong to the group of positive IME. The general principles, advantages and disadvantages of these procedures were summarized earlier in *Chapter 2.11.*. The

workflows of the physical separation methods examined in this study are summarized in *Figure 7* and *8*. The characteristics of the positive IME strategies applied in these studies are summarized in *Table 14*.

4.2 Molecular methods

4.2.1 Quantitative real-time RT-PCR

RT-PCR has been used to identify CTCs through their expression of epithelial or cancer-associated mRNA transcripts. Generally, it is considered to be more sensitive than antibody-based techniques, but has also been hampered by false positive results in samples from normal volunteers and from patients with other patho-physiological processes. These false positives stem from multiple sources, including issues with laboratory technique, primer selection, illegitimate expression of the target genes in normal cells, the presence of pseudogenes, or contamination (see *Chapter 2.11.2.2.1.2*).

To improve the reliability, especially the specificity of RT-PCR assays qRT-PCR may be used. In addition, qualitative marker information, qRT-PCR uses cut-off values of marker transcript numbers, above which transcripts can be considered as tumor cell derived. Moreover, when compared with 'conventional' RT-PCR, qRT-PCR relies not only on primers, but also on internal probes that specifically hybridize to the amplified sequences. In addition, due to the continuous measurement of the amplified signal, false-positive results, which could produce an abnormally shaped, non-linear amplification curve, could be easily identified and removed [350].

The principle of the real-time PCR technology is that during each PCR cycle the content of amplification products within a sample is photometrically determined in a special thermocycler. The number of PCR cycles required to obtain detectable amounts of PCR products is inversely correlated to the number of template molecules present within a sample before amplification. Thus, having standardized the assay conditions, the number of target transcripts within a sample can be quantified. The application of fluorescence techniques to the RT-PCR, together with suitable instrumentation capable of combining amplification, detection and quantification, has led to the development of

kinetic RT-PCR methodologies that are revolutionizing the possibilities for quantitating nucleic acids [351]. Conceptual simplicity, practical ease and the promise of high throughput have made the homogeneous real-time fluorescence detection assay the most widely used mRNA quantification method.

4.2.1.1 Hybridization probe format assay

This method uses two hybridization probes to maximize specificity [352]. One of the probes carries at its 3' end a fluorescein donor, which emits green fluorescent light when excited by the instrument's light source. Its emission spectrum overlaps the excitation spectrum of an acceptor fluorophore that is attached to the 5' end of the second probe. This probe must be blocked at its 3' end to prevent its extension during the annealing step. Excitation of the donor results in fluorescence resonance energy transfer to the acceptor and the emission of red fluorescent light. In solution, the two dyes are apart, and because the energy transfer depends on the spacing between the two dye molecules, only background fluorescence is emitted by the donor. After the denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement during the annealing step. This brings the two dyes in close proximity to one another and the fluorescein can transfer its energy at high efficiency. The intensity of the light of longer wavelength emitted by the second dye is measured, with increasing amounts of measured fluorescence proportional to the amount of DNA synthesized during the PCR reaction. A fluorescent signal is detected only as a result of two independent probes hybridizing to their correct target sequence. This increases specificity and generates additional flexibility for probe design. The only disadvantages of hybridization probe format assays are the strict optimization of probe design and the cost of the dye-based methods.

4.2.1.2 Crossing point

The concept of the CP is at the heart of accurate and reproducible quantification using fluorescence-based RT-PCR [353]. Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background [354]. This point is defined as the CP, and

will always occur during the exponential phase of amplification. Therefore, quantification is not affected by any reaction components becoming limited in the plateau phase, which results in a systematic bias against the more abundant templates and makes any quantification based on measurements of overall product yield intrinsically unreliable. Studies have demonstrated the CP is a more reproducible point in the PCR than the plateau phase measured in conventional PCR and it is also a reliable point for quantification with a log-linear inverse relationship existing between CP and sample concentration [355].

4.2.1.3 Calibrator normalized relative-quantification with efficiency correction

In many experimental studies with a quantitative approach an absolute value for the sample under investigation is not relevant. In such cases relative quantification that determines the changes in steady-state transcription of a gene is often adequate. However, errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples. This is especially relevant when the samples have been obtained from different individuals, and will result in the misinterpretation of the expression profiles of the target genes. The accepted method for minimizing these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalized [356]. The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. In addition, an endogenous control should also be expressed at roughly the same level as the RNA under study.

RT-PCR of gene expression is often performed using housekeeping genes as reference against which the expression level of a gene under investigation can be normalized. The housekeeping genes are a large group of genes that code for proteins whose activities are essential for the maintenance of cell function. Detection of housekeeping gene mRNA is routinely used to control several variables that may affect RT-PCR. These endogenous controls are present in each experimental sample and therefore serve as

perfect candidates for the normalization of the final result [295]. During the RT-PCR assay, the target CP is compared directly with the reference gene CP and is recorded as containing either more or less mRNA. Thus the target concentration is expressed relative to the concentration of a reference gene from the same sample material omitting the need for a standard with known concentrations and the result is expressed as a target/reference ratio [357]. This approach corrects the sample for differences in quality and quantity caused by:

- Variations in initial sample amount
- Variations in nucleic acid recovery
- Possible RNA degradation of sample material
- Differences in sample and/or nucleic acid quality
- Variations in sample loading/pipetting errors
- Variations in cDNA synthesis efficiency

In the relative quantification method the result is expressed as the target/reference ratio of each sample divided by the target/reference ratio of a so-called calibrator (***Figure 9***). The calibrator can be any nucleic acid, as long as its concentration and length of amplicon are known. Typically, the calibrator is a positive sample with a stable ratio of target to reference and is used to normalize all samples within one run, but in addition provides a constant calibration point between PCR runs [358]. In addition to the correction of sample quality differences, normalization to a calibrator corrects the results of the PCR for different detection sensitivity that might be caused by variable probe annealing efficiency and the template dependent fluorescence resonance energy transfer efficiency.

Almost all variables influencing the final result, like variations in sample amount or different hybridization probe annealing, are eliminated by the normalization to a reference and to a calibrator. Thus the accuracy of this method, based on calibrator normalized relative quantification remains only influenced by different PCR efficiencies of target and reference gene. According to this basic PCR equation, the generated copy number at a certain cycle is a function of the initial copy number, the PCR efficiency

and the cycle number (***Figure 10***). The final ratio resulting from the calibrator normalized relative quantification is only a function of PCR efficiency, and of the determined CPs.

However, PCR efficiency differences can be corrected to achieve exact results. The correction of PCR efficiency is needed as the amount of DNA only theoretically doubles with every PCR cycle. In reality, the efficiency is never equal to 100%, meaning that there will not be a perfect doubling of the amount of DNA at each cycle due to various factors, e.g. high GC-content. As for example at 90% of efficiency the amount of DNA will increase from 1 to 1.9 at each cycle, so the factor is 1.9 for each cycle. As per the equation of PCR a small difference in efficiency makes a lot of difference in the amount of final product. Furthermore, the changes in efficiency have a major effect on the CP value. The efficiency-corrected quantification is based on relative standard curves describing the PCR efficiencies of the target and the reference gene. These standard curves are determined once and can be used for each analysis run. The efficiency can be calculated as $10^{-1/slope}$, where the slope of the line is determined from the relationship between the PCR CP and the logarithm of concentration. With these determined efficiencies, the relative ratio needs to be adjusted. This procedure allows maximum reproducibility and controls for factors like:

- Primer annealing
- Efficiency of cDNA synthesis
- Amplicon sequence specificities, fragment length

4.2.1.4 LightCycler-CK20 Quantification Kit

CK20 is a member of the intermediate filament protein family involved in cell structure and differentiation (see *Chapter 2.11.1.2.2.1.)*. Early immunological and Northern blot studies found its expression is restricted to gastrointestinal tissue, bladder transitional and Merkel cell carcinoma [300]. Other adenocarcinomas, such as those from breast, endometrium, and lung, as well as squamous cell carcinomas were found not to express CK20. This restricted expression profile in gastrointestinal cancers, bladder transitional and Merkel cell carcinoma has formed the basis of its use to detect CTCs in PB, BM

and LNs of patients suffering from tumors and to use it as a surrogate marker [359, 360]. Furthermore, no processed CK20 pseudogenes have been found. For these reasons CK20 is one of the most widely used CTC markers in colorectal tumors. Even more, some studies have found associations between CK20 detection and stage, grade, and tumor recurrence [361-363]. In other studies CK20 was detected in PB, BM and urine from healthy donors or patients with nonmalignant disease [364-366]. This was later explained by the expression of CK20 in cells that were positively identified to belong to the granulocyte fraction of leucocytes, which appear to express the gene on a background level [367]. A further confounding factor has been the recent RT-PCR-based detection of CK20 in samples from breast, endometrial, lung and oral squamous cell carcinoma [368-372]. Due to technical variations between studies and a lack of quantitative assessment, the reasons for these discrepancies are poorly understood. These discrepancies have made it difficult to interpret the relevance of detecting CTCs by CK20 RT-PCR. As a result there is an important need to continue further studies on CK20 to involve standardized techniques with an ability to discriminate expression levels in a quantitative manner.

Although, the number of studies using qRT-PCR approach is increasing, few have examined the factors affecting the interpretation of results obtained by real-time PCR, and suitable approaches for quantification are yet to be established. To ensure correct interpretation, we used a used a newly developed LightCycler-CK20 Quantification assay (Roche Diagnostics, Basel, Switzerland) that is standardizable and applies a quantification concept with high reproducibility. The major feature of this hybridization probes format assay is the ability to monitor and quantify PCR amounts with using the LightCycler™ instrument. Furthermore, the reliable quantification of housekeeping gene expression allows an excellent quality control on a per-sample basis and relates marker concentration to sample quality [373]. This assay provides a more informative analysis and may help to unravel reasons behind discrepancies between studies on CTC detection.

4.3 Cell-based methods

So far, ICC is considered to be the gold-standard method for detection of rare CTC (see *Chapter 2.11.1.2.*). However without cell enrichment, an enormously large population of cells needs to be analyzed to identify a single prospective CTC. While the most promising enrichment method, IME (see *Chapter 2.11.3.2.*) has been used to demonstrate medical value, scrutinized analysis of the phenotypical heterogeneity of CTCs suggest that this approach has serious sensitivity issues for detecting all of the CTCs present in PB. An alternative approach is to scan un-enriched immunocytochemically labeled cells using optical scan technologies.

4.3.1 Automated Digital Microscopy

The major drawback of standard ICC is that searching for rarely occurring CTCs under the manual control of the microscope is extremely slow and, due to fatigue, prone to human error. To improve the throughput of the ICC assay and eliminate human error, we and others have developed ADM systems for CTC detection. The principle of this technology is that either an automated brightfield or fluorescent microscope is coupled to a sensitive Charged-Couple Device (CCD) camera and image analysis workstation [374-377]. Identification of CTCs is achieved through capturing and analyzing images of cells deposited on a substrate. Microscopy using automated image analysis for recognition of rare cells has been demonstrated to be the most accurate method currently available for rare cell detection [378]. By providing objective computer-based analysis that can reduce the subjectivity inherent in manual microscopic interpretation, along with images of cells that are classified, automated cellular imaging holds considerable potential for improving both the sensitivity and inter-laboratory consistency of the CTC assay.

Brightfield microscopy analyzes slides that are labeled with immunoenzymatic techniques. Such preparations are usually stable for long periods of time and an analysis can be performed on archived material. However, in contrast to immunofluorescent techniques, they can be influenced by endogenous enzyme activity that can cause high background and nonspecific staining. On the other hand, fluorescently labeled specimens are subject to photobleaching, caused by light illumination and/or oxidation

by room air and must therefore be especially protected and analyzed within a shorter period of times (usually within weeks). This main disadvantage of fluorescence microscopy is probably the reason why most studies of CTC detection use brightfield microscopy. Nevertheless, fluorescence-based analysis has several important advantages over brightfield techniques. First, it offers the potential of multimarker assessment of CTCs, while with the brightfield technique only a few markers can be used simultaneously. This capability is of particular importance if one considers the reported heterogeneity of CTCs (see *Chapter 2.9*). Second, fluorescent labeling provides a superior signal to noise ratio compared to brightfield methods. With properly optimized labeling steps very good contrast with minimal noise solely contributed by background auto-fluorescence can be achieved.

Despite the fact that ADMs are considered to be very accurate the analytical requirements for identifying and enumerating very rare CTCs is still very highly laborious. Firstly, because that even with the best ADM specificity reported, a pathologist would need to review and examine many hundreds of false positive events to identify a small number of true positive CTCs. Second, presently however, ICC with imaging analysis is constrained by the time required for image acquisition. Detecting a statistically valid number of CTCs is directly related to the number of cells analyzed. Since the highest reported acquisition rate is 860 cells/sec-1, the minimum time to examine a few million cells would take almost a day, which is prohibitively long for a clinical assay [379]. Therefore, improvements in ADM technology that facilitate the specific identification of a statistically relevant number of CTCs in a timely fashion are urgently needed.

4.3.1.1 The Rare Event Imaging System

We developed an automated microscopic system, the REIS, for the detection and analysis of cancer cells in PB or BM preparations. The REIS consists of an automated epifluorescence microscope (Nikon Eclipse 1000, Nikon Corp., Japan) with a computer-controlled stage (Ludl, Hawthorne, NY, USA), automated objective turret (contains dry objectives: 4x, 10x, 20x, 40x and 60x), automated filter cube changer with five positions, automated shutter, a high resolution black-and-white cooled CCD-camera

(SVGA Sensicam, Cooke Corp., Romulus, MI, USA) and a personal computer. For rare event detection, the REIS make use of proprietary software allowing for fast and highly sensitive fluorescent color detection, along with the capability for the analysis of a variety of morphometric features. The workflow of the REIS assisted analysis is shown in ***Figure 11***.

For the detection of fluorescing cells, the slide is first scanned at low magnification (4x) using one or more user defined filter set(s). The area to be scanned on the slide can be freely-defined by the user. CTCs are identified based on the parameters: light intensity, area, mean density and roundness. For every object of interest that falls within certain values for these parameters as specified by the user, the coordinates are saved for revisiting in a second scan.

Since the identification and quantification of CTCs are based on morphometric object analysis the availability of high-quality images are essential. Therefore a fast and reliable autofocus module was incorporated into the scanning software. The autofocusing module can compensate for the surface irregularity of the cell preparations by directing the Z-axis motor of the microscope. The focal plane is determined automatically by searching for the highest contrast image along the focusing curve by an autofocusing algorithm. This algorithm is able to focus even in extremely low contrast images with high background conditions and insensitive to optical artifacts and dust on the surface of glass slides. Even though, this was a good solution to acquire high-quality images the speed of automated focusing was of concern. To reduce the total scanning time and the time frame spent on autofocus operation a focal plane prediction feature was designed and implemented in the REIS system. This feature calculates the focal plane for the whole scan area based on three distinct focal points. This eliminates the need to perform autofocusing on each and every field of view as the Z stage is moved to the pre-calculated focal position automatically. Due to the depth of field of the scanning objective this feature can tolerate a small anomaly in precision.

CTCs in patient samples can be of variable size, shape and fluorescence intensity/density. Occasionally they can occur as clusters. Therefore the scanning

parameters of the REIS were set to cover a broad range in order to obtain maximal scanning sensitivity possibly at the expense of specificity. This leads to a considerable number of objects of interest (raw object count) that have to be stored and revisited in the second scan. To increase specificity of the automated scan and decrease the raw object count, we introduced a second imaging step using the DAPI channel in each image where positive events were identified. Once a target object is identified a nuclei image is automatically obtained and overlaid to the original image. Overlaying both images, it could be verified that the positive signal originated from a cell (which would contain a DAPI labeled nuclei) and not from debris or dirt particles. This so-called 'nuclear verification' feature was intended to decrease the raw object count.

Slides are automatically scanned at low magnification for the detection of tumor cells (positive events), which is based on CK labeling, and the total cell count, which is based on nuclear DAPI labeling. CK, a cytoskeletal component of epithelial and carcinoma-derived cells, is the most widely used and best characterized marker of cancer cell contamination (see *Chapter 2.11.1.2.2.1*). The first-round of identification of prospective CTCs is based on parameter criteria that were set-up for the CK label. As the total number of cells analyzed from each sample is one the most important factors influencing the probability of CTC detection, in parallel of the identification of prospective positive cells a total cell count was performed. The total cell count is necessary as the cell count prior to the assay can drastically deviate from this value. This is because technical factors during sample processing (dilution inaccuracies, loss of cells, cellular damage) can significantly influence the end differences in the total cell content per preparations [380]. The total cell count is performed on the 4x DAPI channel images using a dynamic, intensity-based histogram analysis thresholding method. When the total cell count feature is activated, DAPI channel images are automatically obtained at every field of view on the slide. Image analysis algorithms were designed to exclude dirt particles from the total cell count and to split clumps or touching cells into individual objects. The precision of the total cell count algorithm was validated in a separate study [376]. Once the scan is accomplished the scan results are expressed as the number of identified prospective positive cells/total number of cells analyzed. The pertinent information for each sample is entered via a client program to a

PostgreSQL database. This information is later associated with the scanning results obtained by the REIS. The scanning results include morphological measurements, total cell counts, and automatically obtained high-magnification images. Once all information is stored in the database, it can be reviewed from multiple client computers using a custom software application that provides the ability to search, sort, and filter data.

After the automated analysis is completed, the user can review all positive events on the computer screen and manually confirm them using higher magnification. During the review process, the user can accept images of true positive cells and delete images of debris or other false positives. In addition, cells can be viewed with different fluorescence filters for multiple-marker analysis: for increased detection specificity and phenotype characterization of residual tumor cells, we established a double-labeling protocol that combines the labeling of CK with that of surface antigens reported to be expressed in specific types of cancers, e.g., the EpCAM for breast, ovarian, colon, and lung carcinomas and the disialoganglioside GD2 (GD2) for SCLC, neuroblastoma, glioma, and melanoma [381]. Furthermore, a CD45 label was developed as a negative marker for CTCs.

The REIS was developed with the intention to combine the advantages of other methods capable to detect rare cells or cell components but without their inherited drawbacks.

- High-through put - Flow Cytometry
- Simultaneous constituent measurements - Laser Scanning Cytometry
- Single cell sensitivity - PCR
- Visualize the specimen conventionally and reanalyze the same cells - Microscopy

4.3.1.1.1 Decision Tree Algorithm

The identification of target cells in rare event cytometers is traditionally performed by applying a combination of preset ranges of discriminating image analysis parameters, or finding parameters, on digitally acquired microscope images. If the target cells have a

uniform staining pattern, cellular shape, and marker expression, this approach can reliably and efficiently distinguish between true-positive cells and artifacts. However, if the rare cells display a marked heterogeneity of these characteristics, the ability of this method to separate true-positive cells from false-positive detection is limited. Therefore, we tested a novel concept with combining ADM with classical image processing and machine learning to detect fluorescently labeled rare cells. Since the development of this approach require many hundreds of true-positive events instead of using CTCs we decided to evaluate the feasibility of this approach on CMV-infected leukocytes. This is because that CMV-infected cells are generally present in PB at several magnitudes higher frequency than CTCs [382, 383]. Nevertheless, the proof of principle presented herein could easily be converted to other rare cell applications.

The immunofluorescently stained slides were scanned by the REIS with the hardware and scanning software described previously [377]. The objects were segmented from their background by a dynamic thresholding method based on image intensity histogram analysis. Cytomorphometry measurements were performed on the segmented objects with the Image-Pro software (MediaCybernetics, Bethesda, MD, USA), which is using 49 different measurements to characterize the objects.

A two-step procedure to detect rare, fluorescently labeled CMV-infected cells among normal leukocytes was developed. The operation of the detection system is summarized in ***Figure 12***. The first step consisted of applying previously discovered finding parameters to acquired microscope images to differentiate cells and cell-like objects from obvious artifacts. The system then automatically collected a gallery of objects that met the finding criteria and performed an in-depth analysis of digitally measured cytomorphometric and densitometric features. This second step was achieved using the Decision Tree (DT) analysis, a supervised inductive learning algorithm developed by Ross Quinlan [384]. The objects detected by classical image processing were classified via DT analysis to true- or false-positive classes.

DT analysis enabled the construction of a classification tree from a training data set that predicted which category (true- or false -positive) an object that passed the finding

criteria fell into. The 49 ImagePro-Plus image analysis parameters measured on each object that passed the finding parameter criteria were recorded in text files. Post-scan, the text files were fed into the See5 software (RuleQuest Research Pty Ltd, St Ives, Australia) to identify patterns that delineated their categories via DT analysis. The classification rules consisted of a DT model of the ImagePro parameters and a breakpoint measurement that determined which branch of the tree to travel down when that parameter was above or below a given value. Each branch of a tree ultimately culminated in a leaf node that consisted of the final decision about that case, as well as an accuracy ratio stating how often the cases in that node were misclassified. Three properties of See5 - (a) boosting, (b) pruning, and (c) misclassification costs- were used to optimize the probability of correct classification.

(a) Boosting

Boosting refers to the ability of See5 to create multiple classifiers (called a boosting level) from one set of raw data [385]. We used a 10-trial boosting in this study, where ten separate DTs are combined to make predictions. Each of the 10 classifiers constructed sought to correct error made by the previous classifier. In making these corrections, new errors were produced that served as the basis for the following classifier. After the classifiers were constructed, they were used in combination to predict the outcome of a new case. Specifically, each of the 10 classifiers made a prediction on the new case along with an accuracy rate. Using a voting methodology, a final classification on the positive type (true- vs. false -positive) and a confidence measurement was returned.

(b) Pruning

DTs are usually created according to a two-step scheme (for details see ref. [386]). First, the tree is grown to construct the classification rules. To avoid over-fitting of the data, the DTs are simplified (pruned) using an error-based criterion with a binominal error approximation. The default global pruning option of the See5 program was enabled in all experiments of this study.

(c) Misclassification Costs

The concept of misclassification cost comes from the notion that some case misclassifications are more serious than others. By varying the costs assigned to false-negatives (Cfn) and false-positives (Cfp), the sensitivity and specificity of the assay can be adjusted to the required needs. The Cost Ratio (CR) that we used in this study was generated by expressing the ratio of Cfn/Cfp misclassification costs.

4.3.1.2 Spiked sample preparation with micromanipulation

In order to accurately detect and quantify CTCs, pre-analytic variables in the sample preparation procedure must be adequately addressed. For mimicking the *in vivo* situation of rare CTCs we spiked a variable number of cell culture-derived carcinoma cells into whole blood or MNC isolates from healthy donors. This method of modeling provides for a roughly known number of rare cells, which can be compared to the number of cells detected in order to get coarse statistics and comparisons about cell loss during the various stages of processing.

Before spiking cancer cell lines, the viability of cell cultures were assessed with trypan blue exclusion, and only if the viability of the culture was above 95%, it was used for spiking. Viable cultures were thoroughly washed, resuspended and, if necessary, trypsinized to prepare single cell suspension. For the preparation of high concentration samples (>10 rare cells/ml of whole blood) conventional dilution techniques were used. Micromanipulation was used to prepare low concentration samples (<10 rare cells/ml of whole blood). Samples for micromanipulation were prepared as follows; tumor cell lines were diluted to low concentrations in PBS (Phosphate-Buffered Saline) and observed with phase-contrast imaging under an inverted microscope (Nikon, TE2000, Nikon Corp, Japan). Glass capillaries were pulled with Narishige micropipettes puller (PC10, Narishige, East Meadow, NY, USA) and connected to a large-volume microinjector (IM-5B, Narishige) through teflon tubing. The syringe and micropipette will be filled with silicon oil (Sigma, St. Louis, MO, USA) and air bubbles were expelled by turning the micrometer syringe until oil drips out the front. The micropipette was then placed into a holder held by a three-axis joystick micromanipulator (MN-151, Narishige). Under the observance of a 60X objective

individual cells were counted as they are sucked up into the micropipette. Once the required numbers of cells were extracted, the micropipette was removed from the holder and placed above the opened blood container. To ensure that all cells were added to the blood samples the micrometer syringe was advanced until the oil-PBS border reached the tip of the micropipette.

5 Experimental Designs

5.1 Experimental design of Study #1

To assess the effect of sample preparation on the RT-PCR-based detection and quantification of disseminated tumor cells, we studied healthy donor blood samples with added colorectal cancer cells. We used a newly developed, quantitative RT-PCR assay for the putative gastrointestinal epithelia-specific marker, CK20 to evaluate the sensitivity, specificity, and quantitative PCR effects of DGE, two IME techniques, and a Non-Enriched (NE) preparation. For each series, 80 ml of PB were drawn from the ante-cubital vein of healthy volunteers with a Vacuette™ into heparinized tubes. To avoid epithelial cells from skin puncture, the first 5ml were always discarded. We added, per ml, 1,000, 100, 10, 1, and 0 HT29 colorectal carcinoma cells to triplicate 5-ml blood aliquots. The 1,000 and 100 cells/ml samples were prepared by dilution and the 10 and 1 cells/ml samples by micromanipulation. Each series was processed by either DGE of PB MNCs with the Ficoll Histopaque®-1077 (Sigma), IME with Epithelial Enrich® Ber-EP4-coated microsize beads (Dynal, Biotech, Oslo Norway), IME with a CellSearch Epithelial Cell Enrichment Kit® and anti-EpCAM-coated nanosize beads (Immunicon Corp, Huntingdon Valley, PA, USA), or without enrichment (NE series). The dilution series were repeated three times for each method with blood from different healthy donors. This gave 9 preparations (triplicates, 3 donors) of each of the 5-cell concentrations and 45 determinations for each of the 4-sample preparations, although 1 sample (0 cell/ml) of a DGE series was lost. The replicates served to compensate for the sample-to-sample variation of tumor cell loss during the procedure and also the general sampling problem arising from the problematic detection of low copy numbers.

At the end of each enrichment protocol, samples were resuspended in 300µl of PBS, and total RNA was isolated with the High Pure RNA Isolation Kit (Roche Diagnostics). Total RNA for the NE series was isolated directly after cell lysis in Stabilization Buffer for Blood/Bone Marrow (Roche Diagnostics). In all cases, the manufacturer's instructions were rigorously followed. We performed RT-PCR analysis of RNA samples with the commercially available CK20 Quantification Kit for the real-time

LightCycler PCR Instrument (Roche Diagnostics). From the input of a single RNA aliquot, this method provides a relative quantification of CK20 concentrations in a sample that is comparable between PCR runs. The quantities are expressed as the ratio of CK20 to the housekeeping gene Porphorynbilinogen Deaminase (PBGD) divided by the CK20-to-PBGD ratio in a calibrator sample. Measurement of PBGD is used as a control for RNA loading and integrity, whereas the calibrator provides an inter-run normalizer. RT-PCR analysis was performed according to the manufacturer's instructions with one exception: the usual reagent calibrator was substituted with 10ng/ml HT29 colorectal cell-line RNA, thereby setting the ratio of CK20 to PBGD in HT29 cells to 10^6. Therefore, samples with a relative ratio of 100 have a CK20-to-PBGD ratio 10^4-fold less than the calibrator. For each enriched sample, 10µL of RNA, equivalent to 500µL of whole blood, was analyzed. In the NE series, the 10µL of analyzed RNA corresponded to only 25µL of whole blood because of the reduced loading capacity of the procedure.

5.2 Experimental design of Study #2

In order to test our hypothesis that CP values might be more accurate indicators of the reliability of a PCR analysis than conventional indicators and to devise new models for use of CP values as indicators for assessing the reliability of analysis, we simulated the analysis of low concentrations of the putative colorectal micrometastasis marker CK20 and the reference genes PBGD and β2-microglobulin (β2M) and compared the capabilities of conventional indicators (total nucleic acid content and qualitative reference gene detection) and CP values to identify samples with unreliable analysis. We prepared, by serial dilution, four sample series comprising 10,000, 1,000, 100, 10, and 1 plasmid DNA copy for CK20, PBGD, and β2M and 20,000, 2,000, 200, 20, and 2 pg of SK29-Mel-1 (melanoma cell line) RNA. Five replicates of each concentration were analyzed for CK20, PBGD, and β2M by kinetic RT-PCR on the LightCycler instrument (Roche Diagnostics) according to the protocol in the LightCycler CK20 Quantification Kit (Roche Diagnostics). A reverse transcription reaction was performed for each SK29-Mel-1 RNA replicate, amounting to 25 reverse transcription reactions. The plasmid DNA and SK29-Mel-1 cDNA series for each gene were analyzed in

separate PCR runs, amounting to six individual runs of 25 samples each. Detection mixtures for CK20 and PBGD were obtained from the LightCycler CK20 Quantification Kit, and the detection mixture for β2M was obtained from the Light-Cycler β2M Housekeeping Gene Set. CP values were determined by the LightCycler Analysis Software (Roche Diagnostics, Ver. 3.5), using the data-driven second-derivative maximum function.

5.3 Experimental design of Study #3

In our pilot study, the LightCycler-CK20 Quantification Kit (Roche Diagnostics) kit was used to assess the inter-assay variation of RT-PCR-based detection of tumor cells in the blood between four different laboratories that all used the same standardized protocol. By this approach, the influence of pre-analytics like blood-sample processing, RNA isolation, and cDNA synthesis was evaluated. Blood samples of healthy donors spiked with various numbers of cultured colon tumor cells were examined. To study at which level the variation was introduced, three kinds of sample sets were generated in which (i) tumor cell RNA was spiked in RNA of MNCs, (ii) tumor cells were spiked in isolated MNCs, and (iii) tumor cells were spiked in whole blood. The experimental design is shown in ***Figure 13.***

Samples were prepared by two of the four laboratories. In the first laboratory, 10ml of blood was collected from a healthy volunteer and pretested for CK20 expression according to the protocol as described below. No CK20 expression was detected in this pretested sample, and 300ml blood was subsequently collected into a heparin-containing bag. This blood sample was divided into three aliquots of 100ml. The first aliquot of 100ml blood was used for the preparation of a set of samples in which RNA from the blood is spiked with RNA from cultured HT29 colon tumor cells. Therefore, MNC were isolated from 100 ml blood by DG centrifugation through Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). MNC were washed twice with PBS, snap frozen in liquid nitrogen, and stored at -80°C until use. For isolation of RNA from MNC and HT29 cells, the High Pure RNA isolation kit (Roche Diagnostics) was used according to the manufacturer's instruction. The quantity of RNA was measured by spectrophotometry at 260nm. Blood RNA was divided into 20 aliquots representing 5ml

blood each. HT29 RNA was added to 16 aliquots in quantities equivalent to 1, 5, 10 and 100 HT29 cell(s)/ml (four samples each). Four aliquots were not spiked with HT29 RNA. Thus, four identical series consisting of five samples each were generated. The second aliquot of 100ml blood was used for the preparation of a set of HT29-cell spiked MNC samples. Therefore, 100ml blood was divided into 20 aliquots of 5ml. MNC were isolated by DG centrifugation through Ficoll-Hypaque and washed twice with PBS. HT29 tumor cells were added to 16 MNC samples in quantities equivalent to 1, 5 and 10 cells/ml by micromanipulation and 100 cell(s)/ml by serial dilution resulting in four identical sets of spiked-MNC samples. Four MNC samples were not spiked with HT29 tumor cells. Cell pellets were snap frozen and stored at -80°C. The third aliquot of 100ml blood was used for the preparation of a set of HT29-cell spiked-blood samples (blood-A). Again, 100ml blood was divided in 20 aliquots of 5ml. HT29 cells were added to 16 aliquots of blood in quantities as described for the second series. Four blood samples were left unspiked. Thereafter, MNC were isolated and washed. The cell pellets were snapfrozen and stored at -80°C. Four identical sets consisting of five samples each were generated. A second laboratory collected 100ml heparin blood of another healthy volunteer who showed no CK20 expression in a previously pretested 10ml blood sample. This aliquot of 100 ml blood was used to prepare spiked-blood samples (blood-B) in the same manner as the third set of samples of the first laboratory (blood-A). Both laboratories used the same batch of HT29 cells to spike the blood samples. All samples were coded for blinded analysis and distributed on dry ice by courier to the other participating laboratories. Each laboratory individually isolated RNA from the distributed samples using the High Pure RNA isolation kit according to the manufacturer's instruction, except from the first series of samples distributed by laboratory 1 that already consisted of RNA samples. RNA quantity of individually isolated samples was measured by spectrophotometry at 260nm at the different laboratories separately. The LightCycler-CK20 Quantification Kit (Roche Diagnostics), the Light-Cycler Instrument (Roche Diagnostics), and the Relative Quantification Software 1.0 (Roche Diagnostics) were used for quantitative RT-PCR according to the manufacturer's instruction with minor modifications. In short, cDNA was synthesized from 1.5µg RNA isolated from the spiked-MNC and spiked-blood samples and from 20µl RNA from the spiked-RNA samples (representing 1.5µg RNA) in a total volume

of 40µl. Reverse transcription was performed in duplicate for the spiked-RNA samples and in single for all other samples. In each PCR, 5µl cDNA was analyzed. Each LightCycler run consisted of triplicate analysis of the 0, 1, 5, 10 cell(s)/ml samples and single analysis of the 100 cell/ml sample, the calibrator sample (supplied with the kit and consists of RNA from HT29 cells), the RT H_2O negative-control sample and the PCR H2O-negative control sample. Per sample both the target gene CK20 and the reference gene PBGD were analyzed in separate reactions. Per Light-Cycler run, one sample set was analyzed. A normalized ratio of the CK20 CP to the PBGD CP in a sample relative to the CK20:PBGD ratio in the calibrator sample was calculated automatically for each test sample. The calibrator ratio is always set to 1,000,000 according to the manufacturer's instructions. Data from these samples were then loaded into the Relative Quantification Software with the single loading scheme according to the manual of the kit, and the mean normalized ratio of a sample was calculated manually from the CK20-positive tests.

5.4 Experimental design of Study #4

All blood specimens were obtained with informed consent from healthy donors or cancer patients and were processed for microscopic analysis within 24 hours of collection. A very gentle single-tube sample preparation procedure was optimized for the recovery of CTCs from the PB. The first step of this procedure is an isotonic ammonium chloride based lysis of RBCs and platelets with isotonic ammonium chloride buffer [155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA (pH 7.4)] at room temperature for 5min. After centrifugation, the remaining cell pellet was washed, resuspended in PBS, and the total number of living cells was counted using trypan blue exclusion. Then the appropriate number of cells are loaded and attached to custom designed adhesive slides (Paul Marienfeld Co., Bad Mergentheim, Germany) at 37°C for 1hr, and the slides were then blocked with cell culture medium at 37°C for 20min. For the labeling of tumor cells we developed a dual marker indirect fluorescence procedure. For the double labeling of SCLC patients samples with CK and the cell surface antigen GD2, the cells were fixed in 1% paraformaldehyde in PBS (pH 7.4) at room temperature for 5min, washed in PBS, and blocked with 20% human AB-serum (Nabi Diagnostics, Boca Raton, FL, USA) in PBS at 37°C for 20min. Subsequently,

primary antibody directed against the surface antigen GD2 (14.18 mouse-MoAb, Lexigen Pharmaceuticals, Lexington, MA, USA) were applied at 37°C for 1hr. Cells were then washed, fixed in ice-cold methanol for 5 min., blocked again with 20% human AB-serum, and incubated with anti-CK antiserum (rabbit polyclonal, Biomedical Technologies, Stoughton, MA, USA) at 37°C for 1hr. Secondary antibodies (Alexa488-conjugated anti-mouse and rhodamine-conjugated anti-rabbit antibodies; Molecular Probes, Eugene, Oregon, USA and Jackson ImmunoResearch, West Grove, PA, USA) were mixed and applied at 37°C for 30min, followed by counterstaining of the nuclei with 0.5 µg/ml DAPI (Molecular Probes) in PBS. Doubly labeled cells were mounted in ProLong mounting medium (Molecular Probes). Slides were stored at 4°C and analyzed microscopically within a week. For labeling the breast carcinoma specimens the cells were fixed in 2% paraformaldehyde in PBS (pH 7.4) at room temperature for 20min., washed in PBS, and blocked with 20% human AB serum in PBS at 37°C for 20min. Subsequently, primary polyclonal rabbit antibodies directed against HER-2/neu were applied at 37°C for 1hr (DakoCorporation, Glostrup, Denmark), followed by incubation with Rhodamine-Red-X-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) at 37°C for 30min. Cells were then washed, fixed in ice-cold methanol for 5min, blocked with 20% human AB-serum, and incubated with the anti-pan-CK antibody (mixture of clones: C-11, PCK-26, CY-90, KS-1A3, and A53-B/A2; Sigma) at 37°C for 1hr. Secondary AlexaFluor488-conjugated antibody was applied at 37°C for 30min, followed by counterstaining of the nuclei with 0.5 µg/ml DAPI in PBS. Doubly labeled cells were mounted in ProLong mounting medium. Slides were stored at 4°C and analyzed microscopically within a week.

For finding parameter discovery and sensitivity, specificity, and reproducibility testing of the REIS, several sets of spiked samples were prepared from normal human blood and breast carcinoma cell line cells (BT-474, MDA-MB 435, SK-BR-3 and MCF-7; American Type Culture Collection, Rockville, MD, USA) and SCLCcell line cells (SW-2, HTB-119, and H82, American Type Culture Collection). Blood specimens were added to 0.1–10 carcinoma cells/10^6 PB MNCs. For the clinical validation of the REIS, slides from a large collection of blood samples from small cell lung and breast cancer patients were used. SCLC samples were drawn at diagnosis (before treatment) from

patients with LD or ED disease. Breast cancer samples were collected from patients with metastatic disease.

For the clinical testing our study population consisted of 55 histologically proven SCLC cancer patients, 35 of then were staged with ED, 20 with LD. In addition, blood samples were collected from 22 healthy volunteers (***Table 15***). At the time of diagnosis 5ml heparinized PB specimens were obtained from cancer patients prior to treatment and from healthy donors on institutionally approved protocol. The two markers we used in this study were CK and GD2. CKs are intermediate type filaments that are almost always abundantly present in the cytoplasm of SCLC cells. GD2 was identified as the predominant ganglioside expressed on the surface of SCLC cells [387]. This tumor associated antigen has also been used as a target of antibody therapy and immunolocalization [388]. The 14.18 MoAb reacts strongly with the cell surface of small cell carcinoma of the lungs and are uncreative with most normal tissues and other neoplasms with the exception of tumors derived from cells of neural crest origin [389].

5.5 Experimental design of Study #5

PB samples (n=81) were collected and were prepared according to the CMV Brite™ Turbo Kit (IQ Products, Groningen, The Netherlands) protocol. After erythrocyte lysis, 100µl of the recovered leukocyte suspension (~2 x 10^5 cells) was deposited on a microscope slide by cytocentrifugation. The cells were then fixed, permeabilized, and stored at -80°C until immunofluorescently stained and analyzed. In this study, 8 of 81 (9.87%) slides were deemed to be of insufficient quality for automated analysis because of the presence of large dirt particles that had detached from the filter card of the cytofunnel unit and interfered with autofocusing and the cell identification process. Among the remaining 73 slides analyzed, 10 slides were used for finding parameter discovery, 10 for validating the finding parameters, 32 for DT training and 21 for the external validation of the DT analysis. CMV-infected leukocytes were detected by using a cocktail of two mouse MoAbs (clones: C10/C11) targeting the CMV lower matrix phosphoprotein (pp65), an early antigen in virus replication that is abundantly present in most antigen positive cells [390, 391]. The secondary antibody of the kit was replaced with a goat anti-mouse Alexa 488 conjugated antibody (Molecular Probes) with which the slides were incubated in a humid chamber at 37°C for 20 min. For nuclei

counterstain, 0.5 μg/ml DAPI (Molecular Probes) was used. The slides were mounted in ProLong mounting media (Molecular Probes) and stored at 4°C until analysis (within 2 days). CMV-infected cells showed bright nuclear staining when examined under green fluorescence emission and retained their fluorescence signal for several days.

The stained slides were scanned by the REIS using a 4x magnification lens with a 0.2 numerical aperture (Nikon, Plan Apo). During the scan, a Fluorescein Isothiocyanate (FITC) filter cube (Chroma Technology, Brattleboro, VT, USA) with a 480nm bandpass excitation filter, a 505nm dichroic mirror, and a 510nm longpass emission filter was used for the acquisition of fluorescent images. The integration time was 2.1s for each microscope field at the camera gain setting of three. The scanning area covering the whole cytospin spot consisted of a mosaic of 30 (6x5) fields of view. After the slides were scanned, the system returned to the identified objects of interest and autofocused images were automatically taken at a higher magnification (10x, Nikon, S Fluor). The saved object coordinates allowed for relocation and further inspection of identified objects. All slides analyzed by the REIS were coded under blinded experimental conditions and analyzed under the manual control of the microscope. To ensure accurate enumeration of CMV-positive cells, a 20x (Nikon, S Fluor) objective lens was used for manual scanning. Manually detected cells were also inspected at the nuclei channel (DAPI), and only overlapping FITC and DAPI channel signals were considered as true-positive cells.

As described (see *Chapter 4.3.1.1.1)* a two-step procedure was used to detect rare, fluorescently labeled CMV-infected cells among normal leukocytes. The first step consisted of applying previously discovered finding parameters to acquired microscope images to differentiate cells and cell-like objects from obvious artifacts. The system then automatically collected a gallery of objects that met the finding criteria and performed an in-depth analysis of digitally measured cytomorphometric and densitometric features using DT analysis. The objects in the image gallery were then classified according to the readout (true- or false -positive) of the DT analysis. To discover an appropriate set finding of parameters a novel thresholding method of acquired microscope images was developed for the purposes of this study. Based on

intensity histogram analysis, the thresholding algorithm identified the peak of background intensity and determined the appropriate cell segmentation threshold level relative to that position. The algorithm was verified to segment the boundaries of the labeled cells reliably from their background. A conjunctively linked set ('ANDed') of image analysis parameters (finding parameters) was applied on the segmented images in order to filter out objects that did not meet the finding criteria. The data set used to discover the appropriate finding parameters and their ranges consisted of 10 slides from 10 different CMV+ patients. CMV+ cells were manually located on the slides and the area of the slide where the positive cells were sited was digitally photographed by the REIS. After performing the segmentation step, data using 49 ImagePro measurements were gathered of 3,288 objects (135 true- and 3,153 false-positive detections) and compiled into one set of raw data that contained the proper manual classification (true- or false-positive) of each object. A custom-built Visual Basic software application was developed to determine the combination of finding parameters and ranges that were most efficient in discriminating CMV-infected cells (true positives) from non-specifically fluorescing artifacts. The prerequisite of the desired parameter combination was to keep 100% of true-positive cells while removing as many false-positive objects as possible. Distinguishing parameters were identified by comparing the ranges per parameter of true-positive and false-positive subclasses of the raw data set. The application generated different distinguishing parameter combinations and 'virtual scanning' of the raw data set was performed. Because of the computing power needed to test these permutations, we limited the tests to parameter combinations that varied in length from 1 to 7 parameter(s). A parameter combination size of five, close the plateau of the maximal removal rate (78.4%) attainable by conjunctively linked finding parameters, was chosen for use in further experiments. The multiple five parameter sets discovered were ranked according to their false-positive removal efficiency and the one that removed the most false-positive objects (76.82%) was selected for further use. To validate the parameter set's retention of true-positive cells, an additional set of 10 CMV+ slides, containing 196 antigen-positive cells, was tested (results not shown). The final parameter set used in this study was successful in removing 76.66% of false-positive detections.

6 Results

6.1 Results of Study#1

To examine the impact of sample preparation on CK20 results, we assessed their effects on CK20 sensitivity, specificity, and PCR quantification. The results of this study are summarized in ***Figure 14***. All methods detected 1,000 and 100 cells/ml in 100% of cases, but the detection of 10 and 1 cell/ml samples was inconsistent for all preparation methods. Furthermore, for both IME techniques, this inconsistency also extended to PBGD detection in the samples with low cell numbers. Overall, sample preparation appeared to have little effect on the lower limit of detection with all preparations allowing sensitive detection of 1 cell/ml. Although the detection frequencies of 1-10 cells/ml were greater in enriched than non-enriched series, the inconsistent detection at these low levels and the inequality of loading volumes make it difficult to make direct comparisons. Nevertheless, from the results of this study, it appears for the detection of low cell numbers, analysis of triplicate preparations are required to approach a 100% probability of detection. In samples without added tumor cells, the CK20 signal was seen with the Immunicon IME (1/6, 17%) and NE (2/9, 22%) series, whereas it was undetectable from DGE (0/8, 0%) and Dynal IME (0/4, 0%) preparations. To assess the effects of sample preparation on PCR quantification, we restricted our analysis to the 1,000 and 100 cells/ml samples to avoid complications arising from poor quantitative reproducibility at low copy numbers [392]. Analysis of these samples demonstrated two distinct quantitative results (***Figure 15***). For DGE and NE samples, the relative CK20 ratio decreased with decreasing cell number. In contrast, the relative CK20 ratio for the IME preparations remained constant at around 10^6 for the same series. Closer inspection of the individual CK20 and PBGD quantities helped to provide an explanation for these two quantitative patterns. Whilst CK20 quantities decreased in proportion with cell number for all preparations, PBGD quantities remained constant for DGE and NE and decreased in proportion with CK20 and cell number for the IME preparations.

6.2 Results of Study#2

The summary of results of Study#2 is provided in ***Figure 16***. Detection frequencies, mean CP values, for each gene in each sample were derived from 5 replicates of each concentration for each gene. In accordance with previous findings, in samples with high concentrations, gene detection was consistent, with high CP reproducibility and linearity (R^2 >0.999) between concentrations and CP values, whereas in those with low concentrations, genes were detected inconsistently or not detected, CP reproducibility was decreased, and linearity was lost. When we assessed analysis reliability, using conventional indicators, there was little correlation between the occurrence of an unreliable analysis and the sample nucleic acid content or among the three genes. Detection was inconsistent or absent in samples containing 200, 20, or 2pg of CK20 RNA and samples containing 2pg of PBGD RNA, whereas β2M was detected consistently in all samples (***Figure 17***). With increasing dilution, CP reproducibility markedly decreased for CK20, PBGD, and β2M in samples containing 2,000, 20, and 2pg of RNA, respectively. Linearity was lost (R^2<0.999) in the same samples in which RNA was inconsistently detected, i.e., samples containing 200 and 2pg for CK20 and PBGD, respectively (***Figure 16*** and ***Figure 18***). Hence, for this sample series, nucleic acid content or qualitative reference gene detection provided a poor indication of the reliability of detection and quantification. The correlation between the occurrence of unreliable analysis and copy numbers among the genes was better. Inconsistent detection and loss of linearity occurred in samples with single copies of CK20 and PBGD. Reduced CP reproducibility occurred in samples with 10 copies for all three genes, highlighting that the inadequacy of conventional indicators was likely attributable to their lack of direct correlation with specific gene quantities. Assessing CP values as potential indicators showed that inconsistent detection (***Figure 19***, Panel A), poor CP reproducibility (***Figure 19***, Panel B), and loss of linearity between CP values and concentration (***Figure 19***, Panel C) occurred within specific ranges of CP values. Importantly, these correlations were independent of the gene or the nucleic acid type being analyzed, suggesting that CP values could be useful indicators for reliability of analysis of other genes and samples of unknown concentration. To define models for the use of CP values as indicators, we considered that four zones of PCR reliability indicating consistent detection with high CP reproducibility (white zone), poor CP

reproducibility (gray1), inconsistent detection and loss of linearity (gray2), and unlikely detection (black) could be mapped from the coincidence of the different types of PCR reliability with CP values (***Figure 21***). Using the lowest mean CP value for poor CP reproducibility, we defined the white/gray1 boundary at CP 33.06. We chose the CP value of 37.01, corresponding to the first observation of inconsistent detection, as the gray1/gray2 boundary and the theoretical CP value at which CK20 in 20pg of RNA should have been detected to indicate the gray2/black boundary. Extrapolating the CP difference of 3.73 between the mean CP values for 20,000 and 2,000pg of CK20 RNA, we determined that detection of CK20 in 20 pg of RNA should have occurred around CP 42.80 [31.61+(3.73x3)]. Thus, using this model, we could determine the reliability of detection or quantification of a test sample by its CP value and its corresponding zone. Apart from defining the reliability of a result, indicators also identify the adequacy of samples to provide a reliable result, particularly when detection is likely to be inconsistent. To define a model for this, we observed that the difference in CP values between two genes (ΔCP) remained relatively constant, independent of sample concentration (***Figure 19***, Panel C and ***Figure 21***), although this was valid only for reliably analyzed samples. We surmised that addition of a reference gene CP to an expected ΔCP(target-reference) would enable the CP of the target gene, and hence its reliability of analysis from its detection zone, to be estimated. At a ΔCP(CK20- β2M) of 11.88, based on the analysis of 20,000pg of RNA, this model accurately predicted the reliability of CK20 detection for each respective β2M CP and sample concentration (***Table 16***). Using this model, we could thus determine the likely adequacy of a sample to provide reliable analysis for a given target gene from the CP for a reference gene and knowledge of an expected ΔCP(target-reference).

6.3 Results of Study#3

Analysis of identical RNA samples resulted in small differences between laboratories regarding the median CPs (***Table 17***). Each laboratory detected PBGD expression within a range of one cycle except for laboratory 2 that showed a broader range of 1.97–3.38 cycles. PBGD CPs were comparable in the first and second RT reactions of the RNA samples. PBGD RT-PCR of RNA isolated at the different laboratories for the 10

MNC samples (five samples of the MNC and five samples of the blood-A experiment) distributed by laboratory 1 resulted in quite comparable median crossing points (26.62–27.94 cycles) for laboratories 1, 3 and 4. These CPs were only slightly increased compared with the median PBGD CPs of the distributed RNA samples (24.91–26.75). However, laboratory 2 showed a relatively high median PBGD crossing point of 29.06 cycles in the blood-A experiment. Median PBGD crossing points in blood-B samples (28.84–32.45) are somewhat increased compared with PBGD crossing points in the blood-A samples (27.04–29.06). All laboratories detected CK20 expression in all HT29-RNA spiked-RNA samples without failure (***Figure 22***, Panel A). Normalized ratios were comparable between the different laboratories and all laboratories showed a clear positive correlation between amounts of spiked HT29 RNA and the normalized ratio (***Figure 23***, Graph A). In the HT29-cell spiked MNC samples, again all laboratories detected CK20 expression in all spiked samples without failure (***Figure 22***, Panel B). Variation in normalized ratios between laboratories was somewhat increased compared with the results of the spiked-RNA samples (***Figure 23***, Graph B). Two of three laboratories reported a positive correlation between the number of spiked tumor cells and the normalized ratio. In the two sets of HT29-cell spiked-blood samples generated by laboratory 1 (blood-A) and 2 (blood-B), only the 100 HT29 cells/ml blood samples were reported CK20-positive without failure by all laboratories (***Figure 22***, Panel C and D). In total, CK20 expression was detected in 16 out of 20 samples spiked with 1–10 HT29 cells/ml blood. In 10 out of these 16 positive samples, CK20 expression was detected inconsistently. In four HT29-cell spiked-blood samples, no CK20 expression was detected, and the sensitivity of the experiment was limited to 5 cells/ml for laboratory 2 in the blood-A samples and to 5 and 10 cells/ml for laboratories 1 and 4 in the spiked-blood-B samples, respectively. The detection frequency in the blood-A samples compared with the blood-B samples was 69% (24/35) compared with 40% (12/30), respectively. The variation in normalized ratios between the laboratories was considerable, and in the blood-A samples, only laboratories 1 and 2 showed increasing normalized ratios in samples with increasing numbers of HT29-cells spiked. However, the linearity between the number of spiked tumor cells and normalized ratio was not evident (***Figure 23,*** Graph C). Of all un-spiked samples tested, CK 20 expression was detected three times in the MNC sample analyzed by laboratory 1 and once in the

blood-A sample analyzed by laboratory 4. Both of these samples were generated by laboratory 1.

6.4 Results of Study#4

The REIS has been extensively evaluated on spiked samples in comparison to manual microscopy and these results have been presented elsewhere [377]. In summary, the results showed that REIS-assisted analysis provides excellent sensitivity and reproducibility for the detection of rare cancer cell-line cells relative to manual microscopy. The performance of REIS was also evaluated on 40 slides of patients with SCLC and 40 slides of patients with breast cancer. Again, all specimens were blindly evaluated manually and using REIS-assisted analysis. The user-interface of the REIS software and the results of these experiments are shown in ***Figure 24*** and ***Table 18***, respectively. In 14 of 37 slides containing cancer cells (37.8%), the reviewer detected one or more tumor cell(s) using REIS-assisted analyses that were not detected by manual microscopy. In two slides, manual microscopy detected the presence of tumor cells, whereas REIS concluded the absence of positive cells. At a later date, the specimens were manually reanalyzed, blinded to the original classification. Careful manual screening at higher magnification (using the 20x lens) revealed that there were no tumor cells in either specimen, findings consistent with the REIS result but contrasting with the original manual microscopic analysis. It can be speculated that in these two cases, the originally identified cells exhibited a very weak fluorescence signal that further weakened during storage. We concluded that despite successful efforts to reduce photobleaching by embedding cells in ProLong mounting medium (Molecular Probes) and storing slides at 4°C, cells occasionally lose their fluorescence during prolonged storage.

In samples from SCLC patients, interestingly almost equal proportions were tested positive for CTCs in the ED and in the LD group (13/35, 37% versus 7/20, 35%, respectively) with using CK and GD2 as markers (***Table 19*** and ***Figure 25***). In contrast in the healthy control group only one patient sample was tested positive because of the presence of CK-positive cells (1/22, 4.5%). Except for one case in each disease group all patients who were tested positive for CTCs have had keratin-positive cells in the

circulation (1/13, 7.7% in the ED and 1/7, 14.3% in the LD group). In addition to the keratin positive cells, both markers were detected in almost half of the positive patients in the ED group (6/13, 46.1%) and only in a single case in the LD group (1/7, 14.3%). GD2 positive cells alone were detected infrequently, 1/13 (7.7%) and 1/7 (14.3%) in the ED and in the LD group, respectively. In the control group GD2 positive cells were completely absent (0/22). A gallery of images of CTCs recovered form these patients demonstrating CK+ (***Figure 26***, A-C) and GD2+ (***Figure 26***, D-E) single cells and cell clusters as well as a CK/GD2 double-positive cell (***Figure 26***, F) is shown. Quantitative results from this study are presented in ***Figure 27***, with the numbers of CTCs/million WBCs are displayed in each patient group. Using The Mann-Whitney U test there was no statistically significant difference between the ED and LD group, but there was a trend showing more CK+ cells from the advanced stage disease patients (ED group). The numbers of GD2 positive cells were almost completely overlapping in the two stage groups. The correlation with clinical outcome could not be determined yet, as the follow-up of these patients is still ongoing. The most current follow-up data is summarized in ***Table 20***.

6.5 Results of Study#5

To prevent the largest class from being favored in the development of the classification rules, we obtained a balanced distribution of rare (true-positive cells) and common events (false-positive objects) in the training data set. This balanced distribution was created from 13 highly CMV+ slides containing a range of 1-530 CMV-infected cells per slide (median: 27) and 19 CMV-negative slides for our training data set. The slides were scanned by the REIS using the adjusted five parameter set and a high magnification image gallery of the 4,075 objects that passed the system's finding criteria was collected. By manually reviewing the image-gallery, 2,047 objects were classified as true-positive cells (specific nuclear labeling) and 2,028 as false-positive objects (auto-fluorescing particles or cell debris). The ImagePro measurements of these 4,075 instances and their appropriate designations (true- versus false- positives) were entered into See5 for DT analysis. This data set contributed to the development of the classification rules (learning phase) that were used to determine the class of a given new case (classifying phase). The DT's performance was tested first on the training data set.

From the confusion matrix (***Table 21***), we obtained 89.1% sensitivity, 91.8% specificity and 9.5% misclassification rate (% incorrectly classified/total number of objects analyzed). The performance of DT analysis on the training cases affirmed the system's capacity to classify new cases. To assess the "real world" classification accuracy, two different methods, both relying on the fact that the validating sets are separated from the training set, were used in this study:

(a) 10-fold stratified cross-validation and
(b) Independent external data-set validation

(a) As expected, the 10-fold cross validation performance of the system was lower than it was on the training data set. The classification accuracy was 82.4%, the misclassification rate was 18% and the sensitivity and specificity were 82.51% and 82.4%, respectively. The mean cross validation matrix is shown in ***Table 21***. By using asymmetrical misclassification costs, we extended the 10-fold cross validation analysis, generating classification trees optimized for a range of cost ratios (***Figure 28***) We were especially interested in examining sensitivity and specificity values when false-negative misclassifications were designated more costly than false-positives (right from the vertical uniform cost line on ***Figure 28***). To better study sensitivity and specificity relations, a Receiver Operating-like Curve (ROC) was created from these results (***Figure 29***). In contrast to the ROC curves regularly used to determine optimal cutoff value for a diagnostic test, this curve contains the information of sensitivity-specificity combinations at varying-cost ratios. This ROC-like curve can be used in ways similar to regular ROC curves for selecting a suitable misclassification cost. Results from the 10-fold cross validation process indicated that a CR of 3 (false negative misclassifications were three times more costly as false-positive misclassifications) provided a good balance point between high sensitivity (94.3%) and an acceptable level of specificity (56.2%), and was therefore selected for use in further experiments. By assigning a CR of 3, the misclassification error of the DT increased to 25%.

(b) Because our data set was limited in size and the 10-fold-cross validation utility estimate is based on a smaller training data set than the full data set that was available

for DT induction, a second validation step was conducted on an external, completely new data set. This independent data set consisted of 21 additional patient samples that were scanned first by manual microcopy. By manual analysis, 12 slides were found to contain one or more CMV-infected cells and 9 slides tested negative. The slides were then scanned in the REIS and the collected objects were classified using a DT created from the complete training set (***Table 21***). REIS-assisted scanning with DT analysis correctly identified 12 of 12 positive samples with a 91.64% mean sensitivity (range: 66-100%) of true-positive cells. In two of the 12 cases, the automated approach detected more cells than manual screening, while manual microscopy detected a higher number of CMV-infected cells in 6 of 12 cases. Concordant results were obtained in four cases. There was no statistically significant difference ($p=0.065$) between the two methods with the Wilcoxon signed-rank test. Interestingly, manual counts exceeded automated counts in preparations with higher numbers of positive cells. We observed that the major error source of losing cells in these highly positive samples was the misclassification of unusually shaped cells not presented previously to the DT. A less frequent error was due to the incomplete segmentation of dim cells' boundaries and therefore their loss at the first step of the analysis process. In our observation, preparations with higher CMV-infected cell counts tended to contain more irregularly shaped and dimly stained cells. On the negative slides, an average of 23.89 objects (range: 15-35) were collected into the image gallery by the automated system. With the current mode of operation the average false alarm rate (i.e. artifacts counted as cells; (collected objects-manual counts)/total number of cells analyzed) of the system was 0.015%. DT analysis correctly classified 64.11% (range: 50-71.43%) of these detections as false-positive objects.

7 Discussion

In this thesis we addressed the methodological problems associated with CTC detection from different perspectives. Our goal was to design studies that were strongly built upon each other and increase our knowledge and understanding on CTCs in a step-by-step fashion. Beyond the mere technical problems associated with the reliable detection of rare cells we were investigating clinical samples from various sources to improve our learning on the biology of cancerous and infectious diseases.

In our first study (Study#1) we investigated the value of various sample preparation procedures to improve the detection sensitivity and specificity of a recently developed quantitative real-time RT-PCR assay (LightCycler-CK20 Quantification assay, Roche Diagnostics) and assessed the effect of sample preparation on the RT-PCR based quantification. Results from this study suggested that the detection frequencies of 1–10 cell(s)/ml were greater in the enriched than NE series; however, the inconsistent detection at these low concentrations and the inequality of loading volumes made it difficult to make direct comparisons (***Figure 14***). Nevertheless, from the results of this study, it appears for the detection of low cell numbers, analysis of triplicate preparations are required to approach a 100% probability of detection. The improved CK20 specificity of DGE over NE complies with the finding of *Jung et al.*, who found that DGE was essential for the removal of the CK20-expressing granulocyte fraction in un-enriched blood [291]. The result of specific CK20 detection after DGE agrees with many CK20 studies [280, 393, 394], although this has not been a unanimous finding [364]. The ability of IME to improve CK20 specificity has not been reported previously. These results suggest that Dynal IME but not Immunicon IME provides improved specificity, although caution should be observed when making this conclusion because of the single-case nature of the disparity and the inconsistency of detection at this low concentration. Nevertheless, differences in procedure, bead size, and the epitopes targeted between the two methods could potentially influence the number and types of cells entrapped by the two methods, thus influencing specificity (***Table 14***). Further studies performing these IME procedures on healthy-donor blood samples to

characterize CK20-expressing cells will help to clarify this disparity. Analysis of these samples demonstrated two distinct quantitative results. For DGE and NE samples, the relative CK20 ratio decreased with decreasing cell number, suggesting that results from these preparations may be useful for indicating tumor cell load (***Figure 15,*** Panel A). In contrast, the relative CK20 ratio for the IME preparations remained constant at ~10^6 for the same series (***Figure 15,*** Panel A). The relative ratio of 10^6 in the analyzed IME preparations suggests that the RNA contained in the samples had the same CK20 to PBGD ratio as the calibrator. Because the added cells targeted by IME were HT29 cells and the calibrator in this study was HT29 cell RNA, these results suggest that relative quantification of IME preparations may be useful for providing an indication of tumor cell identity. Closer inspection of the individual CK20 and PBGD quantities helped to provide an explanation for these two quantitative quantitative patterns (***Figure 15,*** Panel B and C). Whereas CK20 quantities decreased in proportion to cell number for all preparations, PBGD quantities remained constant for DGE and NE and decreased in proportion with CK20 and cell number for the IME preparations. This is consistent with detection of PBGD from background non-epithelial cells in DGE and NE preparations and only the isolated epithelial cells in IME preparations.

An important finding from Study#1 is that, rather than sample preparation, the statistical chance of capturing low cell and/or copy numbers may be a more significant determinant of assay sensitivity. Whether these problems are causes of cell loss during preparation or inconsistent target-copy capture were examined in our subsequent study (Study#2), using replicates of various amounts of DNA and RNA samples.

In Study#2, we prepared sample series to simulate the analysis of gene levels at the lower limits of assay sensitivity. In agreement with previous findings, our results showed inconsistent detection and reduced CP reproducibility for the detection of samples at low concentrations (***Figure 16***). Moreover, our results also demonstrated a loss of linearity between CP values and concentration at low levels for which, to our knowledge, experimental evidence has not been provided for previously (***Figure 19***). The detection and quantification of low levels presents a number of problems to the reliability of analysis. Mathematical models have shown that, at the limits of assay

sensitivity, stochastic sampling effects become a large determinant of the outcome of analysis and that multiple sampling at this level results in outcomes that follow a Poisson distribution [395-397]. In support of this, numerous studies have characterized inconsistent detection and poor CP reproducibility for the analysis of low levels ([343, 398-401]. In this study, we hypothesized that CP values may be more accurate controls of analysis reliability than conventional controls due to their quantitative nature and direct relationship to specific gene levels. In support of our hypothesis, the onset of poor CP reproducibility, loss of linearity between CP values and concentration and inconsistent detection each coincided within specific ranges of CP values (***Figure 16*** and ***Figure 19***). Importantly, these correlations were independent of the gene or the nucleic acid type been analyzed, suggesting CP values could be useful controls for indicating the reliability of analysis of other genes and samples of unknown concentration. In contrast, our results showed little correlation between the features of low-level analysis and conventional PCR controls. The nucleic acid content of samples had little correlation with occurrence of unreliable analysis between genes. The qualitative detection of reference genes (PBGD, β2M) provided a poor indication of the reliability of analysis of our target gene (CK20). Indeed, the use of conventional controls led to frequent false CK20 negative interpretation in the lower concentrated samples in our sample series (***Figure 17***). Hence, these results suggested CP values may be more accurate indicators for assessing analysis reliability than conventional controls. Two important roles for controls are in defining the reliability of a result as well as the adequacy of a sample to provide a result. Conventionally, qualitative reference gene detection and measurement of the total nucleic acid content have performed these roles. To present ways in which CP values could be used as controls, we devised two models. In the first model, we showed the different features of unreliability could be mapped into ‘zones’ of PCR reliability with CP values as boundaries for each zone (***Figure 20***). By matching a sample's CP value with its zone, the likely reliability of its analysis could be determined. Hence, from our results, this model would imply CP values before 33.06 would define samples consistently detectable with high CP reproducibility, after 33.06 quantitatively unreliable, after 37.01 inconsistently detectable and after 42.80 unlikely to be detected at all. We devised the second model for using CP values to assess the adequacy of a sample to provide a reliable analysis of a specific target gene, particularly

when the target gene was likely to be inconsistently or not detected. To develop this model, we demonstrated that, in samples reliably analyzed, the ΔCP(target-reference) of a sample remains relatively constant independently of sample concentration (***Figure 21***), hence showing it could be used as a constant to represent the relationship between reference and target gene for a given sample of unknown concentration. Indeed, the constancy of the ΔCP(target-reference) has been shown previously and is an element integral to quantification concepts such as the ΔΔCP method [402]. From this relationship, we surmised that a target gene CP (and through the first model, its likely analysis reliability) could be predicted by determining a reference gene CP for a sample and adding it to an expected ΔCP(target-reference). Testing of this model in our sample series demonstrated its validity (***Table 16***). Hence, these results demonstrated a model whereby reference gene CP values could be used as quantitative controls for determining the adequacy of a sample for analysis.

In addition to conventional controls, other systems for ensuring sample and result reliability have been described. Recommendations from a round-table debate involving 26 investigators included the evaluation of ribosomal RNA to define RNA integrity and the use of multiple reference genes to provide a broader assessment of template quality [403]. *Degan et al.* demonstrated improved quantification could be obtained by adjusting sample loading to specific ranges of β2M level [404]. Other studies have used internal controls, ensuring the reliability of analysis by the addition and detection of specific levels of mimic copies within samples [354, 405]. In principle, systems using standard curves to measure copy levels could also provide a reliable assessment of detection reliability, given the inclusion of sufficiently low standard copy. Nevertheless, the direct relationship of CP values to copy levels gives using CP values as controls a number of potential advantages. It circumvents the need for the additional analytical steps, sample preparation and reactions required by ribosomal RNA, internal control and standard curve systems. It eliminates the reliance on spectrophotometry for the preparation of these standards or the adjustment of sample loading. Moreover, using CP values potentially allows for a larger dynamic range of analysis than those often provided by standard curves as well as validation over a larger spectrum of concentrations than internal controls and multiple reference gene systems.

The use of kinetic PCR combined with our sample series has provided a number of insights into the nature of low-level analyses. With the quantitative capabilities of kinetic PCR, our results have shown the inadequacy of conventional controls may derive from their failure to indicate the gene-specific copy levels. Our results showed that, rather than with the total nucleic acid content, inconsistent detection coincided with detection of individual copy levels of the three genes. We determined the reference genes, PBGD and β2M had higher copy levels than CK20 in our RNA samples, providing a scenario of inconsistent CK20 detection in the presence of consistent reference gene detection at sufficiently low sample concentrations. Together, these results have provided experimental evidence of the potential limitation of conventional controls to provide an accurate assessment of result and sample reliability and caution against the assumption that these controls validate the reliability of analysis. A second insight concerns the use of replicates. Replicate analysis has commonly been considered as a potential solution to overcoming problems associated with low-level analysis. However, while using replicates may allow reliable qualitative detection of samples likely to be inconsistently detected, our results suggest, this may not be useful for other unreliability features. Qualitatively, even with quintuplicate analysis, 2pg RNA samples would have been considered falsely CK20 negative using conventional controls and β2M as a reference gene in our sample series. Quantitatively, even with quintuplicate analysis, linearity between mean CP values and sample concentration was reduced for samples with low levels, suggesting replicate analysis would be unable to restore quantitative accuracy at these levels. This residual inaccuracy of replicate analysis highlights the importance of identifying such samples with adequate controls rather than applying a protocol of replicate analysis. This would allow for a more informed assessment of the reliability of the analysis results, a potentially more accurate exclusion of these samples from analysis or re-analysis at higher concentrations. The final insight comes from our use of two reference genes. Our results suggest the choice of reference genes would rely on the type of system used for analysis. For qualitative detection using conventional controls, the reduced false-negative interpretation of 2pg samples obtained using PBGD suggests using reference genes with low expression such as PBGD may be preferable. However, for using reference genes as quantitative

controls as described in our second model or for quantification, it may be more desirable to use highly expressing reference genes such as β2M. This would ensure that the reference gene used to estimate lower target gene CP values itself is not affected by the imprecision of low-level quantification.

Based on the insights that we gained from Study#1 and Study#2 we designed and performed an inter-laboratory study (Study#3) to assess the influence of pre-analytics on the variation of RT-PCR-based detection of CTCs in the PB between four different European laboratories all using the same standardized protocol. Until now, comparative studies between laboratories were scarce and the large heterogeneity in the methods used for CTC detection makes different studies difficult, if not impossible to compare [290, 293, 294, 406]. There is therefore an urgent need for standardization to determine the true sensitivity, specificity and clinical relevance of RT-PCR-based detection of CTCs in blood of cancer patients.

We have used a commercially available kit for RNA isolation and a commercially available kit for quantitative RT-PCR on the LightCycler Instrument designed to detect CTCs and DTCs in PB and BM samples by measuring CK20 expression. Conflicting results regarding the sensitivity, specificity and clinical relevance of RT-PCR-based detection of CK20, which was the marker of interest in our study, was published in the literature [362, 407]. However, this is not only the problem with CK20, but also with the other frequently used RT-PCR markers of colorectal cancer cell dissemination, like CEA [313, 408] and guanylyl cyclase C [409, 410].

Our results indicate that when a standardized protocol for RNA isolation and RT-PCR is used, reasonable good results in samples analyzed by different laboratories can be obtained with respect to two points. First, in the two sets of samples in which tumor cell RNA was spiked in RNA of blood and in which tumor cells were spiked in MNC, valid data were obtained by three laboratories for both sets, and in all these runs, CK20 expression was detected at concentrations as low as one tumor cell per ml of blood. Second, in these runs, increasing concentrations of spiked tumor cells generally resulted in higher CK20 expression levels as detected by the different laboratories (***Figure 22***).

RNA isolation introduced limited extra variation in CK20 expression levels of comparable samples analyzed by different laboratories. However, this variation may also be partly due to variations in tumor cell spiking and in MNC harvest. Despite the uniformity of all spiked-RNA samples distributed, PBGD expression was not detected in all samples. Further analysis of the calibrator samples' fluorescence curves, which show the real-time formation of PCR product, suggests that this is most probably due to a sub-optimal run of the PBGD PCR. This stresses the importance of real-time fluorescence-curve analysis additional to the assessment of the final quantitative results.

Due to CK20 expression in granulocytes, these cells need to be depleted from blood samples when CK20 RT-PCR is used to study CTCs in PB samples [291]. To evaluate the effect of DGE on results between laboratories, two laboratories generated one set of samples each in which tumor cells were spiked directly into blood after collection and before sample processing. Analysis of these samples gave rise to larger variations between laboratories and a reduced sensitivity of the analysis. The lowest concentration of one tumor cell per ml blood could still be detected in four out of seven runs. However, the low tumor cell concentrations were inconsistently CK20+. This suggests that target transcripts are present in very low concentrations and amplification is a matter of chance resulting in stochastic effects. This phenomenon has been observed in our previous studies (Study#1 and Study#2) and has been described by others [343, 411, 412].

In the results of the spiked-blood samples, there was no clear relationship between the number of spiked tumor cells and the normalized CK20 ratio. However, in three of seven runs, the detected CK20 expression levels in the samples with 100 tumor cells per ml were elevated compared with the samples with 10 tumor cells per ml, and the expression levels in the samples with 10 tumor cells per ml were elevated compared with the samples with one and five tumor cells per ml (***Figure 23***). The detection frequency in the spiked-blood samples generated by laboratory 1 (blood-A) compared with the spiked-blood samples generated by laboratory 2 (blood- B) was 69% (24/35) and 40% (12/30), respectively. This suggests that sample preparation at the two laboratories may have affected the assay sensitivity. However, the compared number of

samples is too limited to evaluate the suggested small difference between the laboratories accurately.

More obvious is the difference in RNA quality between the two sample sets as determined by PBGD expression levels. In the blood-B sample set, three out of four laboratories report higher median crossing points for PBGD with a broader range compared with the blood-A sample set. As we have determined in Study#2 a low copy number house-keeping gene, PBGD is a good indicator for RNA quality, and low expression of PBGD as detected by high CPs may give rise to false-negative results [411]. Therefore, reduced RNA quality, which was confirmed by agarose gel electrophoresis of a random set of samples (data not shown), might have brought about the lower detection frequency in spiked samples generated by laboratory 2 compared with laboratory 1. As per our previously shown model of CP reliability zones (Study#2), further research is needed to define a maximum CP for PBGD that ensures minimal risk of false-negative results due to inferior RNA quality.

The results on background expression of CK20 in MNC isolated from blood samples of healthy donors and non-cancer patients are conflicting. Studies showing both an absence [363, 413] and presence [317, 364] have been published. Background expression of CK20 in MNC may be due to illegitimate expression [279], presence of a residual limited number of granulocytes [414], presence of non-malignant epithelial cells [415], and expression in CD34+ blood cells [410]. The discrepancies between studies may be explained by differences in the sensitivity of the method as a reduced sensitivity results in an improved specificity [416]. Despite the negative CK20 results in the pretested blood samples of healthy donors in this study, CK20 expression was detected in two out of 14 un-spiked samples. These inconsistent positive results in un-spiked healthy donor blood samples that had been shown to be CK20-negative in previous testing might be caused by very low concentrations of CK20 transcripts resulting in stochastic amplification. This is supported by the low levels of CK20 expression detected in these un-spiked samples, which were lower compared with the CK20 expression levels detected in the samples spiked with five tumor cells per ml.

The idea that the more sample handlings are performed, the more tumor cells are lost seems evident. This thought is nicely illustrated by our results showing gradually reduced CK20 expression levels when tumor cells were spiked to blood samples at an earlier stage in the sample processing (***Figure 23***). This stresses the necessity for standardized pre-analytical and analytical methods across laboratories during the whole course from blood sample collection to the final PCR result. Our results emphasize that even if standardized protocols are used for all of the steps, the total variation in results increases with the number of pre-analytical steps.

An alternative way of CTC detection is relying on the identification of whole cells rather than its molecular composition. As we have discussed earlier (see *Chapter 2.12*) cell-based detection is constrained by the absence of a means of detection that is both efficient and robust. Identifying and enumerating very rare tumor cells by microscopy is highly laborious, and the accuracy and sensitivity of the analysis is potentially impacted by the fatigue of the reviewer. In our subsequent studies (Study#4 and #5) we have focused our efforts on the development and improvement of a fluorescence-based rare event detection system, the Rare Event Imaging System (REIS).

Results from Study#4 show that REIS-assisted microscopy is a sensitive and highly reproducible method for the detection, enumeration, and characterization of CTCs in PB samples. Comparative cancer cell detection in 40 slides from breast cancer patients and 40 slides from lung cancer patients revealed that automated screening provides superior sensitivity over manual analysis. Specifically, 14 of 37 cancer containing slides (37.8%) were incorrectly classified as negative in the manual scan but classified positive by REIS-assisted analysis (***Table 18***). When the REIS system was tested in the clinical setting on PB samples from SCLC patients CTCs labeled with CK and GD2 were successfully recovered (***Figure 26***). In addition, not only single cells but clusters of CK+ and/or GD2+ cells were detected. Although, clumping of cells could simply be an artifact of the sample preparation process the observation of cell clusters in the PB is in line with our previous study where a different enrichment method was used on samples from colorectal cancer patients [417]. In animal studies the presence of circulating tumor cell clusters and tumor-lymphocyte mixed clumps was found to be a more

important prognostic factor in the metastatic process as compared with single circulating cells [87, 418]. However, in humans additional studies are required to determine precisely which tumor cell characteristics confer survival advantage and metastatic potential within the circulation.

Somewhat surprisingly we found that almost equal proportions of LD and ED SCLC patients (35 and 37%, respectively) were tested positive for CTCs in the PB. It is hard to put this finding in context with other studies, as in contrast to NSCLC, there have been only a few investigations that examined the presence of CTCs in the PB of SCLC patients. Methodological differences between these and our studies further complicate this issue. Nevertheless, our CTC positivity rate is within the range of other studies in both disease stages (35% for LD in our study versus 0-38% in other studies, 37% for ED in our study versus 20-58% in other studies, ***Table 23***). It is also noteworthy that even with carefully selected markers specificity problems, especially in the context of normal bloods, were encountered in all of these studies including ours. While, a single CK+ cell was detected in one of our healthy donors PB sample, GD2 was totally absent in healthy controls and proved to be a 100% specific marker. However, when the marker distribution is examined we found that CK is a more frequently present marker in the PB of SCLC patients (***Table 19*** and ***Figure 25***). Interestingly, with advanced stage of the disease GD2 positive cells were more frequently recovered from the PB of SCLC patients (10% in LD and 20% in ED patients, ***Figure 25***). At this point, the reason for the differential expression of markers at different stages of diseases is not known, but one might speculate that the appearance of GD2+ cells in the circulation could be connected to the metastasis and invasiveness promoting role of biologically active sphingolipids [419]. We were not able to show a statistically significant difference in the frequency of CTCs between the ED and the LD group but this could be due to the limited sample-size of our study. However, in comparison to another larger study that analyzed 7.5ml of PB sample from metastatic breast cancer patients with the IME technique the percentage of patients with ≥5 CTCs was 49% versus 14.3% in the more advanced ED group in our study [205]. Whether this is because of the limited sensitivity of our assay system or the different biology of these tumor types needs to be

investigated further. The analysis of the follow-up data from our study is still ongoing and correlation with clinical outcome will be presented later.

A major disadvantage of the REIS-assisted and other automated microscopic methods is that the number of objects identified in each slide (raw object count) can vary between tens and hundreds of objects. We observed that the raw object count depends on the prepared biological material (e.g., the kind of material analyzed, such as blood from healthy donors versus blood from patients before, during, and after treatment or the type of antibody used for labeling, such as monoclonal versus polyclonal) and on the computer's finding parameters. For the computer's finding parameters, the REIS was set to obtain a high level of sensitivity to detect all prospective tumor cells, including heterogeneously and/or weakly stained individual tumor cells and cell clusters. This high level of sensitivity leads to the concomitant pick up of fluorescent signals from debris, not originating from truly positive cells. To reduce the number of raw objects that have to be reviewed by the laboratory professional, we implemented a "nuclear verification feature" into the automated screening algorithm. This feature excludes fluorescing dirt and cell debris that do not contain a cell nucleus (no DAPI signal) and reduces the number of raw objects that have to be reviewed by >50%. Sometimes, even careful morphological analysis cannot support or exclude the true nature of a positive cell. One way to address this is to use multiple markers in the same specimen. Multiple marker analysis can be easily achieved in a fluorescence-based system, such as the REIS. We developed double-labeling protocols for patient specimens and implemented an automated multicount-scanning algorithm into the REIS-assisted analysis to detect cells labeled with more than one marker. Although the number of available antibodies for the characterization of tumor cells is limited at this time, the multicount feature will become more valuable as new markers are identified through high-throughput genetic analyses. Another option for the additional characterization of CTCs is the relocation feature of the REIS. This allows for the additional analysis of positive cells on the slide by FISH, in situ-PCR or other *in situ* methods. [420]. The advantage is that only the slides with a positive cell need to be prepared, saving expensive reagents and labor time. Furthermore, one could microdissect cells of interest by laser capture techniques and then subject them to molecular methods that have been designed for the analysis of

single cells or small cell populations [421]. This additional information will increase the specificity of tumor cell detection and give further insight into the biology of rare cancer cell dissemination.

Two commercial systems based on brightfield microscopy and single marker detection, the ACIS by ChromaVision Medical Systems, Inc. (San Juan Capistrano, CA; [374] and the MDS by Applied Imaging Corp. (Santa Clara, CA; [375] were recently introduced for the detection of CTCs in BM and PB. Both systems were reported to provide excellent sensitivity and reproducibility for disseminated tumor cell detection. Two automated microscope systems based on fluorescence imaging, the Laser Scanning Cytometer (Compucyte Corp., Cambridge, MA) and the Metafer 3.0 automatic image analysis system (MetaSystems GmbH, Altlussheim, Germany), have been demonstrated to detect small numbers of tumor cells in spiked blood samples [422] and in the BM of neuroblastoma patients [420]. Both systems showed a high level of sensitivity, although data on the reproducibility or day-to-day performance of the CTC detection assays were not presented in those studies. There is only one other system like ours, originally developed by the Immunicon Corp.® (Huntingdon Valley, PA, USA) that provides a blood-diagnosis protocol for CTC detection. This approach of CTC detection has recently demonstrated medical value with approval from the U.S. Food and Drug Administration for management of metastatic breast cancer patients. Based on their studies the presence of CTCs in the PB, as detected by the CellSearch™ Circulating Tumor Cell Kit, is associated with decreased progression free survival and decreased overall survival in patients treated for metastatic breast cancer. More recently, with using the same technology the company demonstrated that the evaluation of CTCs in the PB of measurable metastatic breast cancer patients is an accurate measure of treatment efficacy [346]. In fact, they demonstrated that the assessment of CTCs is an earlier, more reproducible indication of disease status than current imaging methods. These results suggest that CTCs may be a superior surrogate end point, as they are highly reproducible and correlate better with overall survival than do changes determined by traditional radiology. The importance of this is that more accurate determination of treatment effectiveness early in the course of therapy might spare patient toxicity from futile therapy and allow treatment to be changed to a more

effective regimen. Whether such an early assessment of response results in an improved overall outcome or quality of life will need to be prospectively assessed in clinical trials designed to investigate this question.

However, there is still a large room for improvement in CTC detection technologies. In two recent published reports of metastatic breast cancer studies, the Immunicon test was uninformative in a large percentage of patients (39% [205] to 63% [423]). For lung cancer, the number of metastatic cancer patients with a negative Immunicon test result was 80% [423]. The reported results for detecting CTCs in earlier stage cancer patients is even more inconsistent and no benefit in earlier stages of malignancy has been shown with this technology.

To improve the detection of CTCs and the performance of our REIS system in our last study (Study#5) we tested a novel approach for the detection of immunofluorescently labeled CMV-infected cells that combines classical image processing with the cost sensitive DT analysis of digitally measured cellular features. The data from this study showed that DT analysis can substantially reduce the number of false-positive detections (64.11%) that were otherwise indistinguishable by the classical image processing methods that was for example employed in our previous study (Study#4). This novel method of rare cell identification decreases false-positive detections without considerably diminishing the sensitivity of the assay (94.3% with 10-fold cross-validation, 91.64% with external validation). It should also be noted that in the external validation set, all 12 positive slides were correctly classified as positive, reflecting a diagnostic sensitivity of 100%.

In recent years, intelligent diagnostic systems using machine-learning algorithms have been applied to various medical problems involving the analysis of microscope image data [424-426]. The learning algorithms implemented in these systems can be categorized into three main groups: statistical methods (e.g., discriminant analysis, Bayesian classifiers, and k-nearest neighbors), neural networks and DT analysis. Comparative studies of machine learning methods conclude that in most applications these algorithms perform similarly in terms of classification accuracy [427]. However, it

has also been reported that there are learning problems where certain algorithms perform significantly better than others [428]. For the purpose of identifying rarely occurring CMV-infected cells, we used DT induction for the following reasons. First, the relatively faster running time for DT induction allows a large number of candidate objects to be evaluated by the algorithm. Second, unlike neural networks, DT analyses can perform accurately without the benefit of an extensive training data set. Third, the transparency of diagnostic knowledge obtained by DT analysis makes the results readily interpretable and the classification fully explainable [429]. Lastly, with modification of the misclassification costs, the sensitivity and specificity of DT analysis can easily be tuned to the required levels. Although our study concerns a relatively small data set for DT induction (n=32), the results show that DT classification of cells by digital cell image analysis is a feasible approach that can be applied for rare event cytometry. The feasibility of this approach is confirmed when classification accuracy from our study (18–25% misclassification rate) is compared to the results of others using DT analysis in non-rare event image cytometry applications. *Yeaton et al.* used 40 cytometric variables on brush cytology specimens to assist in the distinction between chronic pancreatitis and pancreatic adenocarcinoma [430]. These investigators reported of a 14.7% misclassification rate when using the leave-one-out validation process. *Decaestecker et al.* used 31 cytometry-generated variables to distinguish benign leiomyoma from malignant leiomyosarcoma and obtained a 13% misclassification rate [428]. In another study, the same authors extracted 9 DNA ploidy-related parameters of Feulgen-stained nuclei of superficial bladder cancer specimens in order to distinguish between grade I–III tumors and obtained a 42.6% misclassification rate of unseen cases [431]. *Perner et al.* measured 132 parameters on imunofluorescently labeled HEp-2 cells with the intention to recognize different antinuclear autoantibody staining patterns via DT analysis [432]. In this study the authors showed that their classifier operated with a 25% error rate.

8 Conclusions

In our investigations we intended to improve novel molecular- and cell -based methods of CTC detection as well as to increase our knowledge on CTCs. Our first study (Study#1) suggested that sample preparation may play a role in determining assay specificity and relative PCR quantification concentrations. Assay sensitivity at low cell numbers, however, appeared to be primarily determined by the probability of tumor cell and/or target gene capture. We speculate that the inconsistency of detection at these cell numbers and lack of replicates used by most studies may have made a significant contribution to the controversial results published to date. The lack of reproducibility and adequate guidelines for the standardization of novel molecular CTC assays continues to be a major obstacle to their clinical application.

Another problem has been that, with the increased sensitivity and dynamic range of molecular assays, there has been relatively little revision of the controls used with them [433]. In our second study (Study#2) we have used a selected sample series to demonstrate the concept that CP values may be more accurate controls of analysis reliability than conventional controls. In exploring the wider application of our models, we have observed similar trends with other genes and sample sources using the same assay system (results not shown). However, analyses using other assays and detection formats suggest these systems differ in assay sensitivity and hence the positioning of the zonal CP boundaries. Nevertheless, the concept of different zones of analysis reliability existing and the capabilities of CP values to indicate these zones has remained valid throughout these tests. These results have suggested a wider application for the models in our study; however indicate that with different assay systems a preliminary determination of the boundaries is required. This would require only an analysis of a replicated sample series serially diluted to the limits of assay sensitivity similar to that of this study. Another important element of our models is the derivation of an expected ΔCP(target-reference). Our experience shows this value can be determined by using cell lines or pilot samples representative of the samples to be analyzed. Alternatively, the

ΔCP(target-reference) can also be defined mathematically to designate a minimal threshold for reliable analysis of a desired target to reference level difference.

The observations of this study form the basis for four recommendations to improve the reliability of low-level PCR detection and quantitation (***Figure 20***). The first would strongly advocate the use of replicates for samples expecting detection in the gray zone. The 40% detection of single copy plasmid DNA samples for CK20 and PBGD (***Figure 16***) suggests at least triplicates should be analyzed. The second recommendation would be to consider quantities derived from CPs in the gray zone with caution. As CP values are used exponentially in most quantification concepts, poor CP reproducibility can significantly skew the quantities calculated and thereby the interpretation of many results. We observed mean CPs from samples in the gray zone maintained linearity with log concentrations, suggesting the analysis of replicates may be an approach for improving quantitative reliability from such cases. Thirdly, in the event of detection being expected in the black zone, exclusion of the sample or a re-analysis with an increase in its concentration would be appropriate measures. Considering the potential inadequacy of conventional quality controls, a fourth recommendation would be to use CP values as indicators of PCR reliability. Indeed, CP values may be more relevant indicators as they provide more quantitative precision and derive directly from the PCR. In this study, we have demonstrated CP values can provide an improved assessment of the reliability of samples and results to conventional controls. Through the application of the procedures and models described in this study, a more intuitive selection of samples and their results and potentially more accurate assessment of biological processes may be obtained. The potential value of these models was further validated in our third inter-laboratory study (Study#3).

In Study#3 we aimed to assess the variation of RT-PCR-based detection of tumor cells in blood between four different European laboratories using a commercially available kit with a standardized protocol of sample preparation and analysis. By this approach, the influence of pre-analytics like blood-sample processing, RNA-isolation, and cDNA synthesis was evaluated on different sets of samples prepared by central laboratories. In conclusion, quantitative PCR has been shown to be a powerful tool to evaluate both

qualitative and quantitative differences in the detection of CTCs between laboratories. Use of standardized methods for RNA isolation and quantitative RT-PCR as performed in this study resulted in fairly comparable data between different laboratories showing CK20 detection without failure in spiked samples even at low frequencies of tumor cells and increasing CK20 quantitative signals with increasing numbers of spiked tumor cells. More variation was introduced by the blood sample preparation method. The present study, which was the first concerted initiative to standardize RT-PCR methods for tumor cell detection across laboratories, indicated that comparable results between laboratories may be possible, but a high level of standardization was required, especially in the field of pre-analytics. The results of this pilot study stress the need for further comparative collaborative investigations of the pre-analytical procedures currently used. After pre-analytical procedures are further optimized in a standardized manner, the results from this pilot study can be used to define criteria for an acceptable level of inconsistency between laboratories, and consequently these criteria can be used to compare results between different laboratories in a more extended study.

In conclusions of our studies addressing molecular methods of CTC detection (Study#1-3) we believe that careful selection of sample preparation methods providing improved specificity, rigorous replicate and quantitative analyses and high level of standardization of pre-analytical and analytical protocols in future studies may provide a better understanding of the significance of RT-PCR-based CTC detection.

Our next set of studies (Study#4 and #5) was describing the development, testing and improvement of a fluorescence-based automated microscope system (the REIS) that offers a sensitive and reliable assessment of rare fluorescently labeled cells. The REIS approach presents a superior alternative to manual microscopic detection and provides an opportunity of high-content cellular analysis of rare cells. We demonstrated the methodological power of the new automated rare event analyzer using model systems, and first results analyzing samples of patients with SCLC. Analysis of SCLC patients' samples revealed an interesting but not yet clinically correlated pattern of distribution of CTC markers. We found that single cells as well as clusters of CTCs labeled with CK and/or GD2 were recovered from the PB of SCLC patients and almost equal proportions

of LD and ED SCLC patients (35 and 37%, respectively) were tested positive for CTCs in the PB (***Table 19*** and ***Figure 25*** and ***26***). We also found GD2+ cells were more frequently recovered from the PB of advanced stage SCLC patients (10% in LD and 20% in ED patients, respectively). Although, the follow-up of these patients is still ongoing it now appears feasible to use the REIS to conduct larger prospective clinical trials to explore the significance of the occurrence of rare cancer cells in cancer patient specimens. This approach may enable an improved method for screening of PB samples and for obtaining novel information about disease staging and about risk evaluation in cancer patients.

However, based on the findings of Study#4 we also realized the shortcomings of this generation of REIS system, the major one being the tedious manual review of the often enormously large image gallery set of collected objects of prospective positive cells. This was because the system's finding parameters were set to a high sensitivity to detect all possible tumor cells, including cell clumps and weakly or heterogeneously stained cells.

As the technology described herein is readily applicable for other types of malignancies or diseases where the enumeration of rare cells is of interest we designed our next study (Study#5) to another rare-cell problem, the detection of CMV-infected cells. To use CMV-infection as our model appeared to be a good choice for three main reasons. First, the development of more sophisticated image analysis algorithms to better distinguish between true positive and false positive hits require many hundreds of true-positive events. Since CMV-infected leukocytes are present in PB at several magnitudes higher frequency than CTCs they offer a good alternative to test the feasibility of our approach. Second, just like CTCs, CMV-infected cells consist of a largely heterogeneous cell population both from the morphological and immunochemical perspective. Last but not least, CMV infection poses serious risks to immunocompromised patients, such as solid-organ or BM allograft recipients, Human Immunodeficiency Virus (HIV)-infected individuals, and cancer patients. The preferred method of managing patients at high risk for CMV disease is the prompt initiation of pre-emptive antiviral therapy, based on the detection of the CMV lower matrix phosphoprotein (pp65), an early antigen in virus

replication that is abundantly present in most antigen positive cells (see references at *Ladanyi et al.* Cytometry A. 2004 Apr;58(2):147-56.) The pp65 antigen positive cells can be distinguished from the background normal hematopoietic cells similar ways as CTCs can be discriminated from normal blood cells. Thus for our purposes CMV-infected cells could very well be used as a model of disseminated rare cells.

We adapted the REIS to detect CMV-infected cells and devised a rapid and reliable computer-assisted method for their quantitative detection. We demonstrated that DT analysis of digitally measured cytomorphometry and densitometry features can significantly improve the recognition of rare events. We believe that the proposed methodology has the potential to reduce the technical errors common to the manual enumeration of rare cells. Furthermore, the possibility for screening more cells with an automated system may lead to earlier diagnosis and more accurate therapy monitoring of CMV infection. We are in the process of adopting this methodology for the enumeration of CTCs and test whether the earlier and more accurate determination of residual disease can potentially improve prognostication and through the modification of therapy survival of the disease.

In summary, the lack of a standardized method to detect CTCs is a clear barrier to the clinical implementation of CTC detection. As a result, there is an important need to continue further studies that involve standardized techniques. We hope that our studies provided additional insights into the molecular- and cell -based methods of CTC detection and this additional knowledge will be translated to useful clinical tests in the near future.

9 Summary

A considerable body of evidence indicates that tumor cells are shed from a primary tumor mass at the earliest stages of malignant progression. Some of these cells will travel via the peripheral blood to sites anatomically distant from the primary tumor and form metastases. As individual disseminated tumor cells present in low numbers, they can be occult to standard methods of investigation. However, these circulating tumor cells (CTCs) are understood to be a source of eventual lethal metastases, the major cause of treatment failure in cancer patients.

Many studies have concluded that the presence of CTCs provides important prognostic information predictive of disease-free and overall survival in various malignancies. By contrast, other reports have found no statistically significant relationship between CTC detection and prognosis. This discrepancy is most likely because even in metastatic patients, the frequency of CTCs is extremely low (estimated to be in the range of 1-10 CTCs/ml of PB). We hypothesize that the issue of varying conclusions regarding the prognostic significance of CTCs may be attributed to the technical difficulties associated with the reliable detection of such rare events.

The main goal of our work was to develop and improve approaches for CTC detection in the PB of cancer patients and to use this technology in patient studies to investigate the prevalence and characteristics of CTCs. The aims were to build methods that provide improved sensitivity and specificity of CTC detection while meeting the requirements of a practical clinical CTC assay. Both cell-based and molecular methods of CTC detection were investigated for areas of improvement. In addition, steps of pre-analytical and analytical procedures were dissected in order to identify potential sources of inaccuracy and irreprocubility. The developed instrumentations and preparation methods were used to investigate the prevalence of various rare cells, such as CTCs and Cytomegalovirus (CMV)-infected leukocytes. Furthermore, together with four different European laboratories a pilot study was conducted to assess the variation and

inconsistencies of CTC detection and to formulate the basis of a standardized, quantitative CTC detection protocol.

Results from our investigations provided novel insights into the molecular and cell - based detection and quantitation of rare cells. Through the application of the procedures and models described in this thesis a potentially more accurate assessment of level signals may be obtained. This would facilitate the earlier diagnosis and more accurate therapy monitoring of infectious and cancerous diseases.

10 Acknowledgements

Primarily, I would like to express my gratitude to my mentors, Dr. Zsolt Tulassay and Dr. Bela Molnar, for their continuous help and support in the conduction of this work and in the preparation of this thesis. I would also like to thank the doctors and researchers of the 2nd Department of Medicine (Semmelweis University, Budapest) for their valuable help with collection and preparation of clinical samples. I am particularly grateful for Dr. Agnes Ruzsovics, Dr. Ferenc Sipos and Karola Albert (2nd Department of Medicine, Semmelweis University, Budapest), members of the Cell Analytical Laboratory (Semmelweis University, Budapest). Dr. Richie Soong (Roche Diagnostics, Penzberg, Germany), Dr. Stine-Kathrein Kraeft (Dana-Farber Cancer Institute, Boston, MA, USA) and Dr. Lan Bo Chen (Dana-Farber Cancer Institute, Boston, MA, USA) co-authors of many of these studies, are acknowledged for their help and tremendous contribution. I also thank the Department of Dermatology (Semmelweis University, Budapest) for the use of the LightCycler Instrument and the 1st Department of Pathology (Semmelweis University, Budapest) for their generous gift of various cell lines. Last but not least, I dedicate this thesis to my family; without their support and encouragement, this work may not have been completed.

11 Tables and Figures

Table 1: *5-year relative survival rates by stage at diagnosis, 1995-2000. in the US.*
Rates are adjusted to normal life expectancy and are based on cases diagnosed 1995-2000, followed through 2001.

Site	All Stages	Local	Regional	Distant
Lung& bronchus	15.2	49.4	16.1	2.1
Colon& Rectum	63.4	89.9	67.3	9.6
Prostate	99.3	100.0	-	33.5
Breast	87.7	97.5	80.4	25.5

Local: An invasive malignant cancer confined entirely to the organ of origin.
Regional: A malignant cancer that 1) has extended beyond the limits of the organ of origin directly into surrounding organs or tissues; 2) involves regional lymph nodes by way of lymphatic system; 3) has both regional extension and involvement of regional lymph nodes.
Distant: A malignant cancer that has spread to parts of the body remote from the primary tumor either by direct extension or by discontinuous metastasis to distant organs, tissues, or via the lymphatic system to distant lymph nodes.

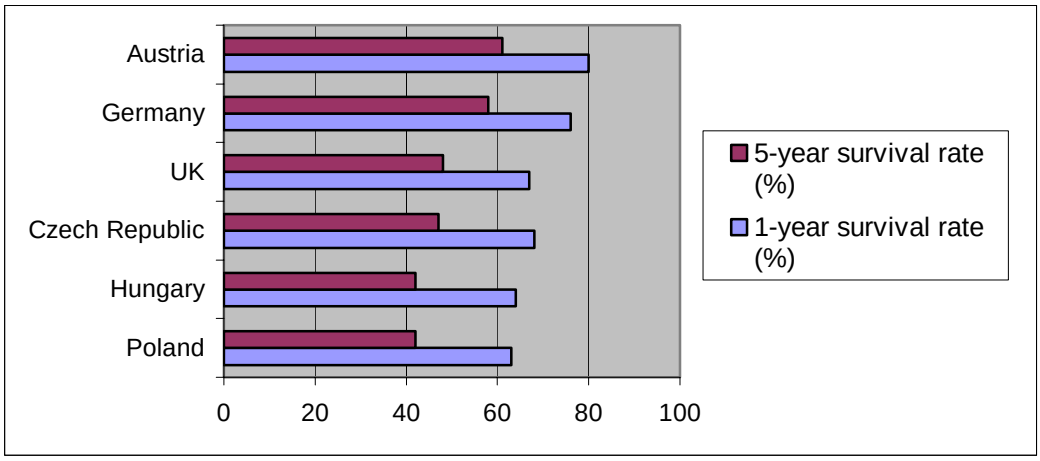
Source: Surveillance, Epidemiology, and End Results Program, 1975-2001, Division of Cancer Control and Population Sciences, National Cancer Institute, Bethesda, MD 2004.

Table 2: *5-year relative survival rates by the EUROCARE-3 study*

Site	All Stages, Mean	All stages, Range
Lung& bronchus	11	6-16
Colon& Rectum	50	28-57
Prostate	67	42-95
Breast	77	60-86

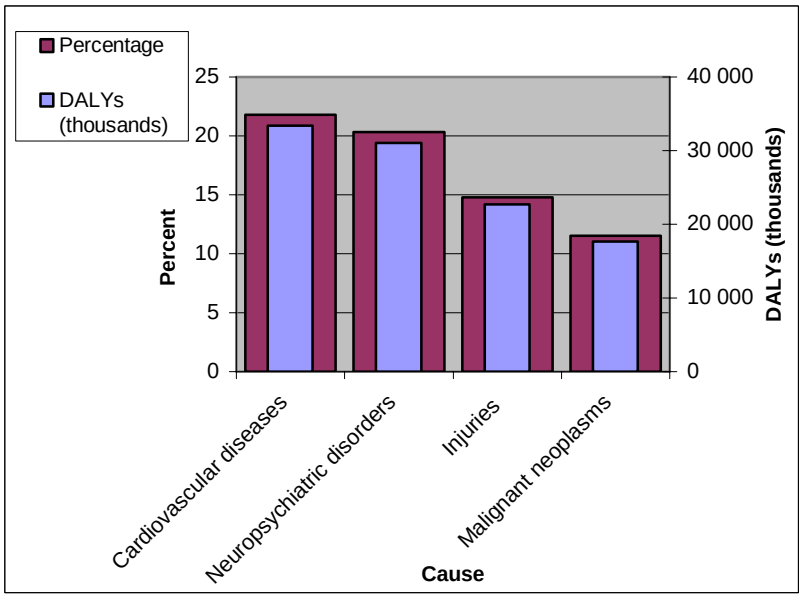
Source: Coleman MP, Gatta G, Verdecchia A, Esteve J, Sant M, Storm H, Allemani C, Ciccolallo L, Santaquilani M, Berrino F; EUROCARE Working Group. EUROCARE-3 summary: cancer survival in Europe at the end of the 20th century. Ann Oncol. 2003;14 Suppl 5:v128-49.

Figure 1: *1-year and 5-year survival rates for all tumor types combined (except skin) for selected European countries.*



Source: Karolinska Institute: Not All Cancer Patients are Treated Equally, a pan-European comparison regarding patient access to cancer drugs. Oct 2005 http://www.prnewswire.co.uk/cgi/news/release?id=1554890

Figure 2: *The top four causes of burden of disease in the WHO European Region in DALYs, estimates for 2000.*



Source: 1. The European Health Report, 2002. WHO Regional Publications, European Series, No. 97. http://www.euro.who.int/document/e76907.pdf, 2. The world health report 2001. Mental health: new understanding, new hope. Geneva, World Health Organization, 2001.

Table 3: *Deaths and DALYs attributable to the 10 leading causes in Hungary.*

	Causes	Total DALYs	% of Total
All causes		1 778 886	100.0
1.	Ischemic heart disease	186 226	10.5
2.	Cerebrovascular disease	121 473	6.8
3.	Unipolar depressive disorders	104 867	5.9
4.	Cirrhosis of the liver	93 358	5.2
5.	Alcohol-use disorders	82 576	4.6
6.	Trachea, bronchus and lung cancer	76 036	4.3
7.	Hearing loss, adult onset	54 648	3.1
8.	Chronic obstructive pulmonary disease	48 778	2.7
9.	Self-inflicted injuries	48 137	2.7
10.	Osteoarthritis	46 469	2.6

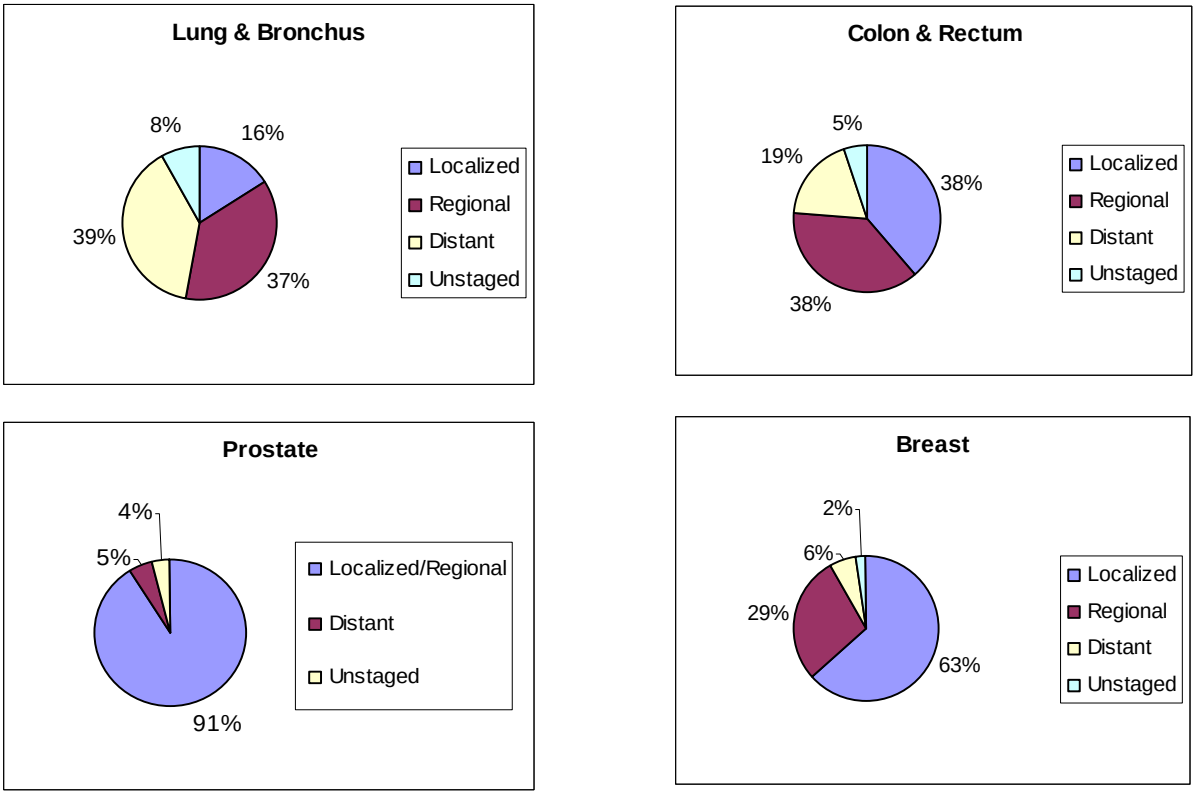
Source: The European health report 2005: public health action for healthier children and populations. WHO Regional Publications, http://www.euro.who.int/document/e87399.pdf
DALYs= Disability-Adjusted Life Years'

Table 4: *Direct cost for cancer in selected European countries in 2002/2003. Total in million Euro, per capita Euro and share of total healthcare expenditures.*

	Direct costs for cancer (€ million)	Direct costs for cancer per capita (€)	Cancer costs as % of total healthcare costs	Total healthcare expenditures (€ million)	Population (2003)
Poland	1,300	34	6,5	20,000	38,195,000
Hungary	566	56	6.5	8,700	10,124,000
Czech Republic	663	65	6.5	10,200	10,202,000
UK	10,823	182	10.6	102,100	59,544,000
Germany	12,100	154	5.4	224,000	82,502,000
Austria	923	114	6.5	14,200	8,067,000

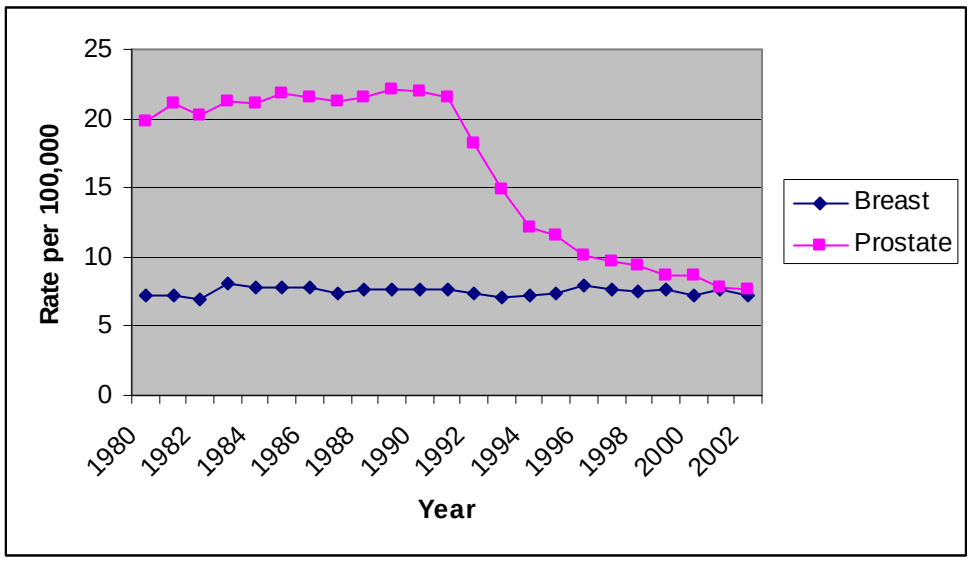
Source: 1. Karolinska Institute: Not All Cancer Patients are Treated Equally, Oct 2005, (ref. 31) http://www.prnewswire.co.uk/cgi/news/release?id=1554890
2. Organization for Economic Co-operation and Development. OECD Health Data 2005: Statistics and indicators for 30 countries. 2005. http://www.oecd.org

Figure 3: *Stage distribution of common cancers at diagnosis in the US.*



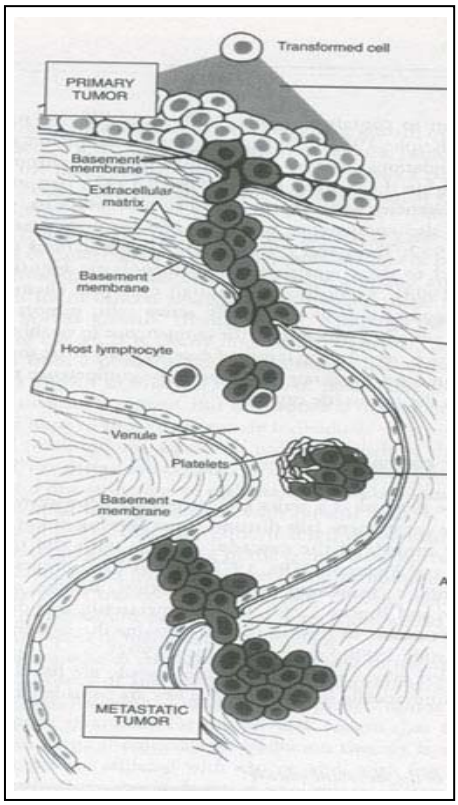
Source: 1. Surveillance, Epidemiology, and End Results Program, 1975-2001, Division of Cancer Control and Population Sciences, National Cancer Institute, Bethesda, MD 2004. Rates are from the SEER 9 areas (San Francisco, Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, Atlanta). Rates are based on follow-up of patients into 2002. 2. Seiffert J, editor. SEER Program Comparative Staging Guide for Cancer, Version 1.1. Bethesda, MD: National Cancer Institute, 1993. NIH Publication No. 93–3640.

Figure 4: *Rates of new cases of distant-stage prostate cancer disease in the US: 1980-2002.*



Source: SEER Program, National Cancer Institute. Incidence data are from the SEER 9 areas (http://seer.cancer.gov/registries/terms.html). Analysis uses the 2000 Standard population (Census P25-1130) as defined by NCI. http://seer.cancer.gov/stdpopulations/)

Figure 5: *Schematic diagram of the metastatic cascade.*



Source: McKinnell, R.G. The biological basis of cancer. Cambridge University Press, 1998.

Table 5: *Isolated tumor cells versus micrometastasis.*

	Isolated (disseminated or circulating) tumor cells	**Micrometastasis**
Size	Single tumor cells or small clusters	≤0.2cm in greatest dimension
Contact with vessel or lymph sinus wall	No	Yes
Extravasation	No	Yes
Extravascular stromal reaction	No	Usually yes
Extravascular tumor cell proliferation	No	Yes

Source: Hermanek P, Hutter RV, Sobin LH, Wittekind C. International Union Against Cancer. Classification of isolated tumor cells and micrometastasis. Cancer. 1999 Dec 15;86(12):2668-73.

Table 6: *Clinical significance of DTCs in the BM of breast cancer patients.*

Reference	No. of cases	Methods (markers)	Positive rate (Stage)	Clinical relevance
Cote et al., [172]	49	ICC (CK, mucin)	37% (Stage I-II)	Earlier and higher recurrence rate
Harbec et al., [173]	100	ICC (CK, mucin)	38% (Stage I-IV)	Independent prognosticator for DFS and OS
Diel et al., [171]*	727	ICC (mucin)	43% (Stage I-III)	Independent prognosticator for DFS and OS
Landys et al., [434]	128	ICC (CK)	19% (Stage I-II)	Independent prognosticator for DFS and OS
Mansi et al., [169]	350	ICC (mucin)	25% (N/A)	Associated with a shorter DFS and OS but not an independent prognosticator
Slade et al., [435]	23	RT-PC (CK-19)	61% (Stage I-III)	No prognostic value
Ikeda et al., [436]	117	RT-PCR (CK-19)	34% (Stage I-III)	Independent prognosticator of relapse
Braun et al., [164]*	552	ICC (CK)	36% (Stage I-III)	Independent prognosticator of DDFS and BCSS
Gebauer et al., [165]	393	ICC (CK, Ber-EP4)	42.4% (Stage I-III)	Independent prognosticator of DDFS and BCSS
Gerber et al., [170]	484	ICC (CK)	37.2% (Stage I-III)	Independent prognosticator of DDFS and OS
Jung et al., [437]	59	RT-PCR (CK-19)	49.1% (DCIS, Stage I- III)	Independent prognosticator of recurrence
Wiedswang et al., [176]	817	ICC (CK)	13.2% (Stage I-III)	Independent prognosticator of DDFS and OS
Pierga et al., [92]	114	ICC (CK)	59% (Stage I-IV)	No prognostic value
Ismail et al., [438]	50	RT-PCR (CK-19)	74% (Stage I-III)	No prognostic value
Schindlbeck et al., [439]	265	ICC (CK)	25.7% (N/A)	Independent prognosticator of OS
Braun et al., [168]*	4703	ICC (CK, mucin, Ber-EP4, TAG-12)	30.2% (Stage I-III)	Independent prognosticator of BCSS and OS

ICC= Immunocytochemistry, CK= Cytokeratin, DFS= Disease-Free Survival, DDFS= Distant Disease-Free Survival, OS= Overall Survival, DCIS= Ductal Carcinoma In Situ, RT-PCR= Reverse-Transcription-PCR, BCSS= Breast Cancer-Specific Survival, TAG-12= Tumor-Associated Glycoprotein-12.

* Prognostic value supported by multivariate analysis.

Table 7: *Clinical significance of DTCs in the BM of NSCLC and SCLC patients.*

Reference	No. of cases	Methods (markers)	Positive rate (Stage)	Clinical relevance
NSCLC				
Cote et al., [440]	43	ICC (CK)	40%, (Stage I-III)	Earlier and higher recurrence rate
Pantel et al., [441]	139	ICC (CK)	59.7%, (Stage I-III)	Independent poor prognosticator for N0 patients
Ohgami et al., [442]	39	ICC (CK)	39%, (Stage I-III)	Earlier recurrence
Passlick et al., [443]	139	ICC (CK)	59.7%, (Stage I-III)	Independent prognosticator for OS
Hsu et al., [444]	96	ICC (CK, Ber-EP4)	22%, (Stage I-IV)	No prognostic value
Mattioli et al., [445]	18	ICC (CK)	70%, (Stage I-III)	No survival data
Poncelet et al., [446]	99	ICC (CK)	22%, (Stage I-IV)	No prognostic value
Sugio et al., [447]	58	ICC (CK)	47% (N/A)	Independent prognosticator for OS
Yasamuto et al., [448]	315	ICC (CK)	31.9%, (Stage I-IIIA)	Related to a poor prognosis only for patients with stages II to IIIA
Hsu et al., [449]	212	ICC (CK, Ber-EP4)	34,4% (Stage I-IV)	No prognostic value
SCLC				
Zych et al., [450]	146	ICC (N/A)	19.2%, 28/146 (Stage LD, ED)	Independent prognosticator of time to progression and OS
Pasini et al., [451]	84	ICC (NCAM)	39%, 33/84 (N/A)	Related to shorter OS
Pasini et al., [344]	60	ICC (MLuC1)	38%, 23/60 (N/A)	Related to poor outcome
Pelosi et al., [452]	30	ICC (NCAM)	63%, 19/30 (Stage LD, ED)	Independent prognosticator of unfavorable prognosis

NSCLC= Non-Small Cell Lung Cancer, ICC= Immunocytochemistry, CK= Cytokeratin, OS= Overall Survival, SCLC= Small-Cell Lung Cancer, NCAM= Neural Cell Adhesion Molecule, LD= Limited stage Disease, ED= Extensive stage Disease.

Table 8: *Clinical significance of DTCs in the BM of prostate cancer patients.*

Reference	No. of cases	Methods (markers)	Positive rate (Stage)	Clinical relevance
Wood et al., [453]	86	RT-PCR (PSA)	45.3%, (Stage I-II)	Independent prognosticator of DFS
Weckermann et al., [345]	154	ICC (CK)	25.3%, (Stage I-II)	No prognostic value
Kollermann et al., [454]	45	ICC (CK)	37.7% (Stage I-II)	No prognostic value
Weckermann et al., [455]	82	ICC (CK)	N/A (Stage I-III)	Independent prognosticator of biochemical progression
Bianco et al., [456]	58	RT-PCR (PSA) and ICC (CK, MIB-1)	36%, (Stage I-II)	Independent prognosticator of DFS*
Lilleby et al., [457]	66	ICC (CK)	21%, (Stage I-III)	No prognostic value
Mitsiades et al., [458]	111	RT-PCR (PSA, PMSA)	26%, (Stage I-II)	Independent prognosticator of DFS

RT-PCR= Reverse-Transcription-PCR, PSA= Prostate-Specific Antigen, CK= Cytokeratin, DFS= Disease-Free Survival, PMSA= Prostate-Membrane Specific Antigen.

* Only the presence of proliferating cells (both RT-PCR and ICC positive) were prognostic for DFS.

Table 9: *Clinical Significance of DTCs in the BM of colorectal cancer patients.*

Reference	No. of cases	Methods (markers)	Positive rate (Stage)	Clinical relevance
Schlimok et al., [459]	156	ICC (CK18)	27%, (N/A)	Associated with higher relapse rate
Lindemann et al., [460]	88	ICC (CK18)	32%, (N/A)	Independent prognosticator of DFS and tumor relapse
Leinung et al., [461]	145	ICC (CK)	24%, (N/A)	Independent prognosticator of OS, DFS and tumor relapse
Vlems et al., [91]	32	RT-PCR (CK20)	25%, (Stage IV)	No prognostic value
Koch et al., [462]	25	RT-PCR (CK20)	16%, (Stage IV)	Independent prognosticator of tumor relapse
Schoppmeyer et al., [100]	47	ICC (CK)	32%, (Stage IV)	No prognostic value
Koch et al., [94]	71	RT-PCR (CK-20)	28%, (Stage II)	No prognostic value

ICC= Immunocytochemistry, CK= Cytokeratin, DFS = Disease-Free Survival, RT-PCR= Reverse-Transcription PCR, OS = Overall Survival

Table 10*: Probability (in %) of false-negative detection because of sample size at different CTCs prevalence where a positive detection is a sample containing ≥1 CTC(s).*

CTC prevalence	Sample size			
	10^6	10^7	$5x10^7$	10^8
10^{-6}	36.78	0.005	~0	~0
10^{-7}	90.484	36.788	0.7	0.005
$2x10^{-8}$	>99	82	36.788	13

Table 11: *Rationale for CTC markers with molecular detection.*

Rationale	Mechanism	Molecular Targets
Genetic alteration	Chromosomal translocation	T11:22, t14:18
	Point mutation	p53, ER, ras
	Deletion	BRCA1, BRCA2, DCC
	Alternative splicing	CD44, MUC-1
	Amplification	HER-2/neu (c-erb-B2), EGFR, N-myc, ras
	Altered ratios	Gelatinase A/TIMP2
Tissue specific		PSA, PSMA, muc-18, keratin, thyroglobulin, NPGP 9.5, PTH, tyrosinase, p97, MAGE-3, AFP, tyrosine hydroxylase, surfactant
Cancer specific		CEA, b-HCG, CK19, K-ras, telomerase, microsatellite, endothelin receptor

Source: Modified from Raj GV, Moreno JG, Gomella LG. Utilization of polymerase chain reaction technology in the detection of solid tumors. Cancer. 1998 Apr 15;82(8):1419-42.

ER= Estrogen Receptor, BRCA= Breast Cancer Susceptibility Gene, DCC= Deleted-in-Colorectal-Cancer gene, MUC-1= Mucine-1 gene, EGFR= Epidermal Growth Factor Receptor; TIMP2= Tissue Inhibitor of Metalloproteinase 2, PSA= Prostate Specific Antigen, PSMA=Prostate Specific Membrane Antigen, NPGP= Non-Penetrating Glycoprotein, PTH= Parathyroid Hormone, MAGE= Melanoma Antigen Gene, AFP= A-Fetoprotein; CEA= Carcinoembryonic Antigen, HCG= Human Chorionic Gonadotropin.

Table 12: *Comparison of results using different methods of IME for detection of CTCs in the PB of breast cancer patients*

Author (ref)	Number / Stage of Patients	Enrich-ment method	Sample size	Marker of Detection	Detection Method	% Positive
Bauernhofer et al., [463]	25/ Stage IV	IME	N/A	CK + EpCAM	ADM	25
Taubert et al., [464]	125/ Stage I-IV	DG + IME	16ml	CK	Manual Microscopy	26
Gaforio et al., [465]	92/ Stage I-IV	DG + IME	10ml	CK	Manual Microscopy	62
Witzig et al., [466]	84/ Stage I-IV	IME	20ml	CK	ADM	28
Cremoux et al., [467]	94/ Stage I-IV	IME	10ml	MUC-1	RT-PCR	37
Beitsch et al., [468]	34/ Stage I-IV	IME	10-20ml	CK + CD45	FACS	97
Kruger et al., [469]	25/ Stage I-IV	DG + IME	N/A	CK	Manual Microscopy	20
Engell et al., [193]	48/ Stage I-IV	DG + IME	N/A	CK + CD45	FACS	73
Racilla et al., [234]	30/ Stage I-IV	IME	10-20ml	CK + CD45	FACS	93

IME= Immunomagnetic Enrichment, CK= Cytokeratin, EpCAM=Epithelial Cell Adhesion Molecule, ADM= Automated Digital Microscopy, DG= Density Gradient enrichment

Table 13: *CTC detection in the PB of metastatic (Stage IV) breast cancer patients*

Author, (ref)	Number of Patients	Sample size	Enrich-ment method	Marker of detection	Detection method	% Positive
Cristofanilli et al., [205]	177	7.5ml	IME	CK+ CD45	CellSpotter Analyzer™	61
Allard et al., [423]	422	7.5ml	IME	CK + CD45	CellSpotter Analyzer™	37
Pierga et al., [92]	39	7-14ml	DG	CK	ADM	41
Baker et al., [470]	20	20ml	DG	CK19, MUC-1	RT-PCR	65
Stathopoulou et al., [471]	47	N/A	DG	CK19	RT-PCR	41
Slade et al., [435]	37	10ml	DG	CK19	RT-PCR	54
Zach et al., [472]	43	10ml	RBC lysis	Mamma-globin	RT-PCR	49
Soria et al., [473]	25	10ml	DG + IME	Telo-merase	PCR-ELISA	41

IME= Immunomagnetic Enrichment, CK= Cytokeratin, DG= Density Gradient enrichment, ADM= Automated Digital Microscopy, RBC= Red Blood Cell, ELISA= Enzyme-Linked Immunosorbent Assay

Figure 7: *Workflow of ammonium chloride-mediated erythrocyte lysis with the experimental parameters.*

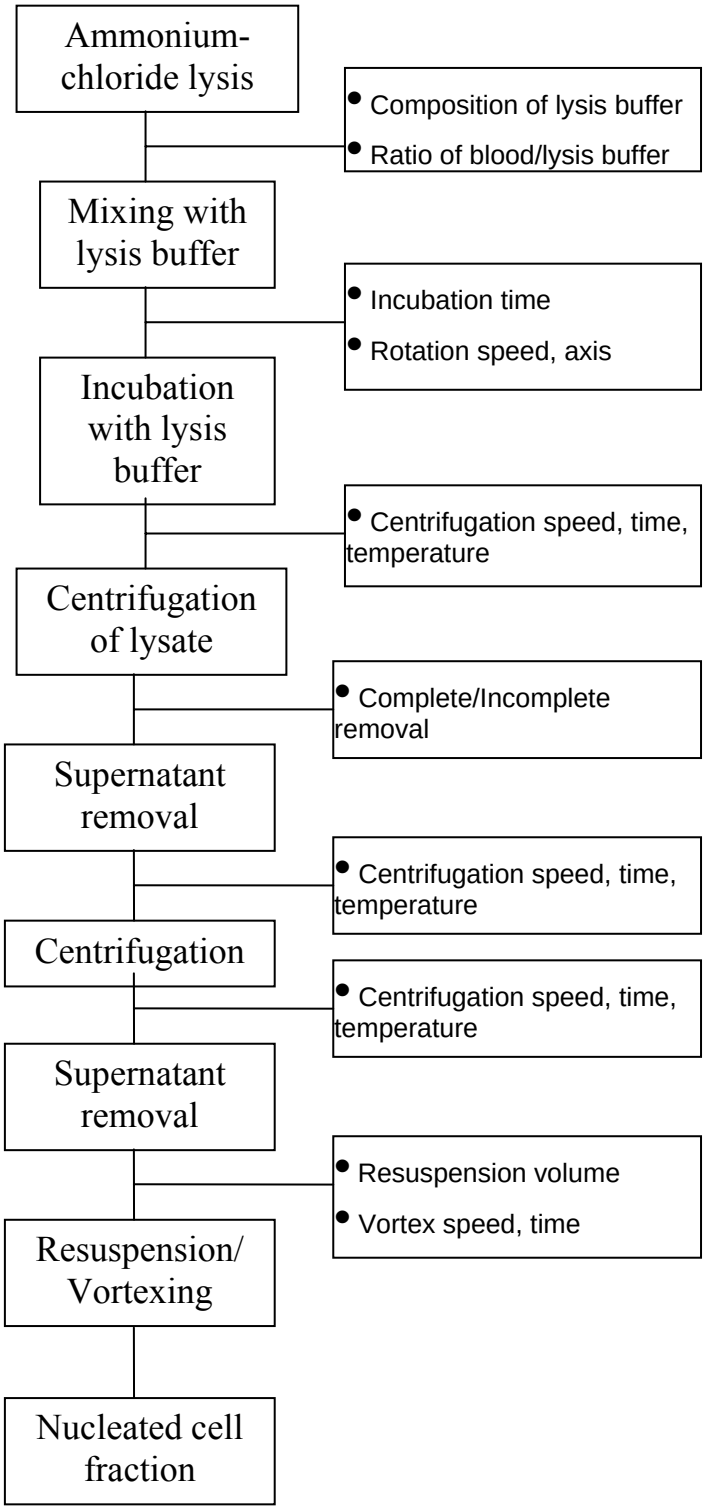


Figure 8: *Workflow of DG enrichment with the experimental parameters.*

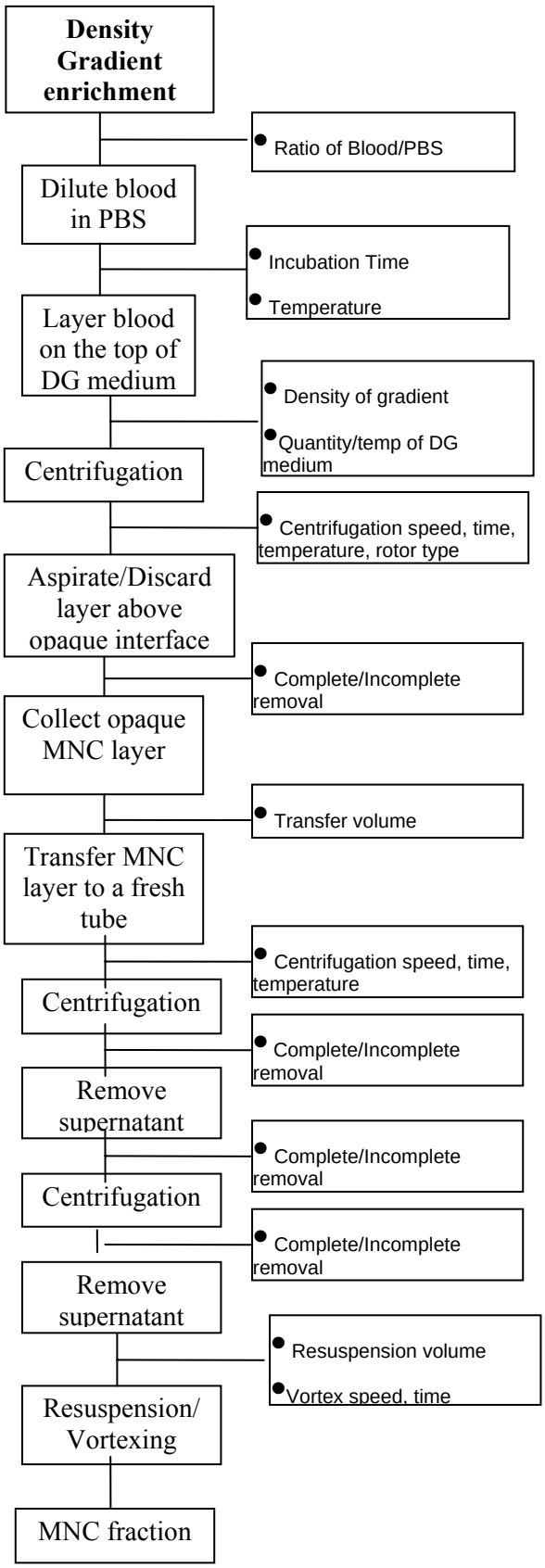


Table 14: *Positive IME strategies used in these studies.*

Company	Product name	Bead size	Starting Material	Target Antigen
Dynal Biotech	Ber-EP4-coated Dynabeads™	4.5 µm	Whole Blood/MNC	EpCAM
Immunicon	CellSearch Anti-EpCAM nanobeads™	150 nm	Buffy coat	EpCAM

Figure 9: *Normalization to a calibrator in qRT-PCR*

$$\text{Normalized Ratio} = \frac{\text{conc. target (sample)}}{\text{conc. reference (sample)}} : \frac{\text{conc. target (calibrator)}}{\text{conc. reference (calibrator)}}$$

Figure 10: *The basic equation of PCR amplification.*

$N = N_0 \times E^{CP}$

N: number of molecules at a certain cycle
N_0: initial number of molecules
E: amplification efficiency
CP: Crossing Point, cycle number at detection threshold

Figure 11: *Workflow of REIS analysis.*

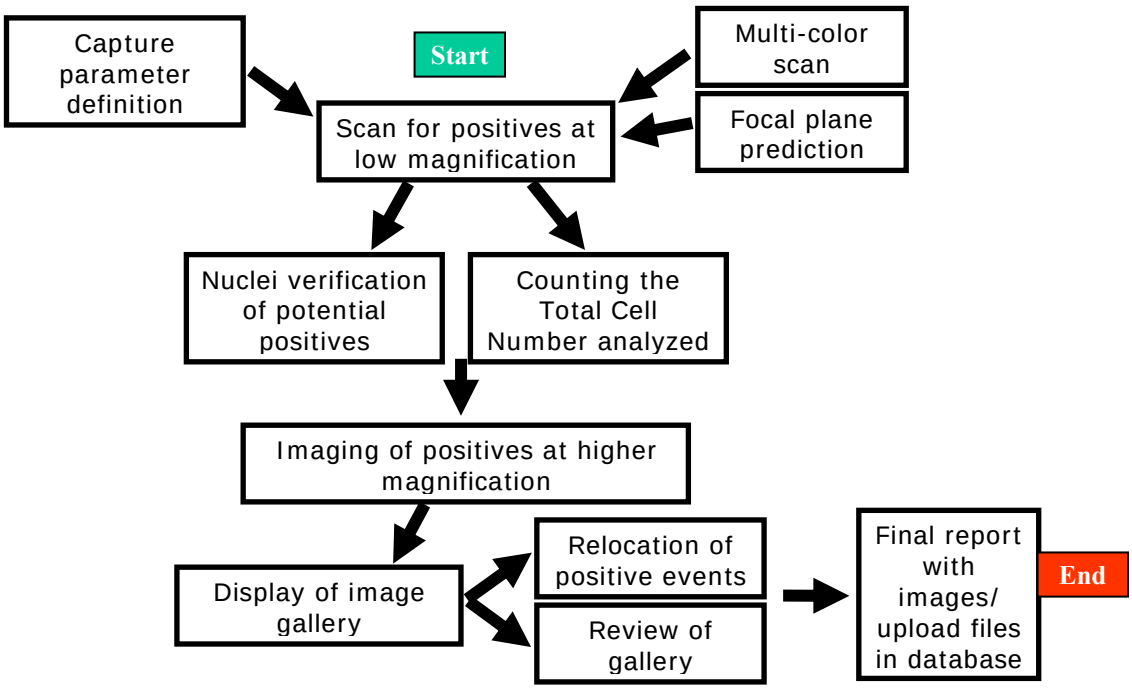


Figure 12: *Flow chart showing major steps for detecting CMV-infected cells.*

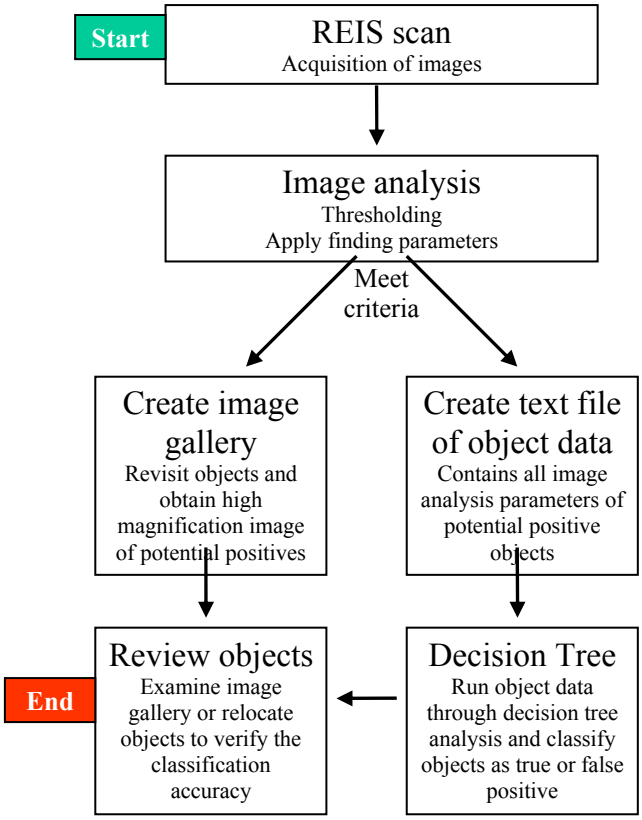


Figure 13: *Experimental design of Study#3.*

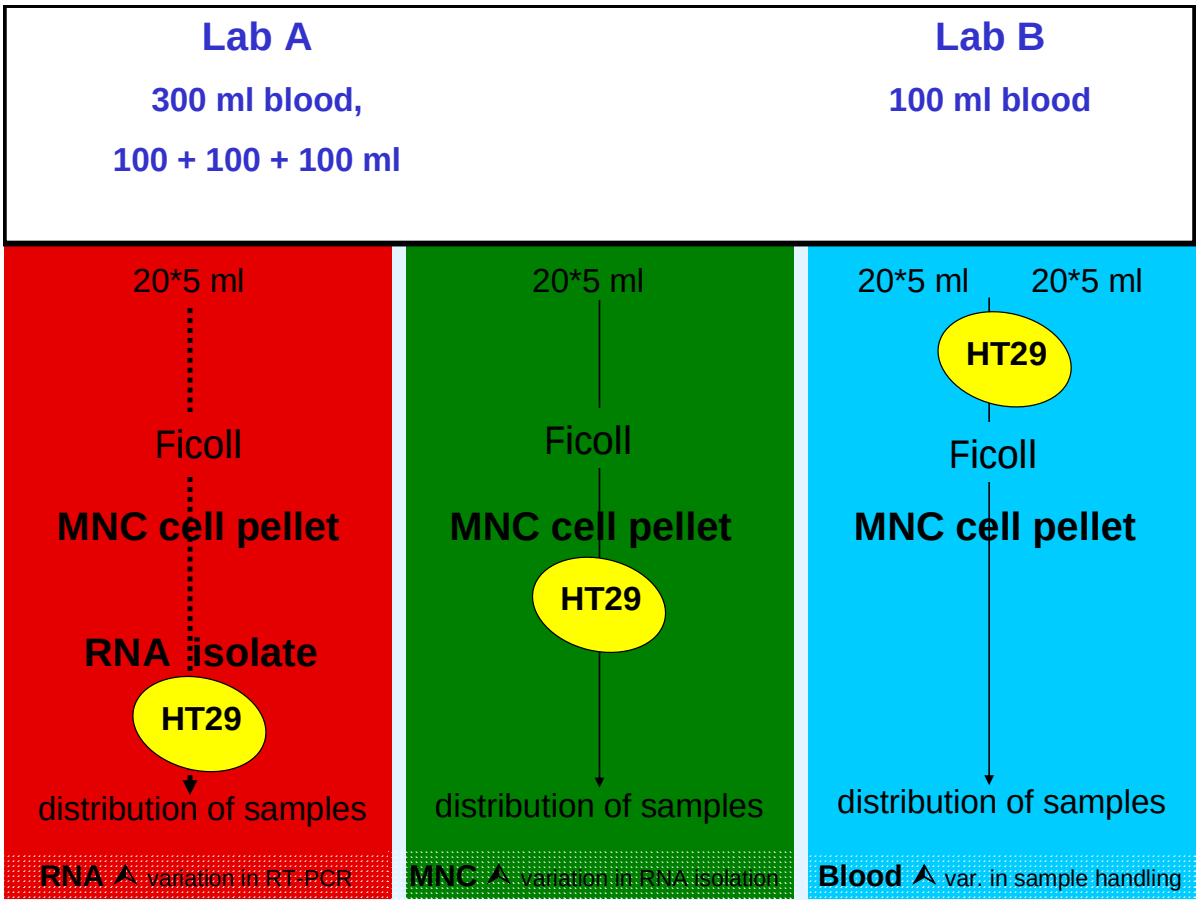
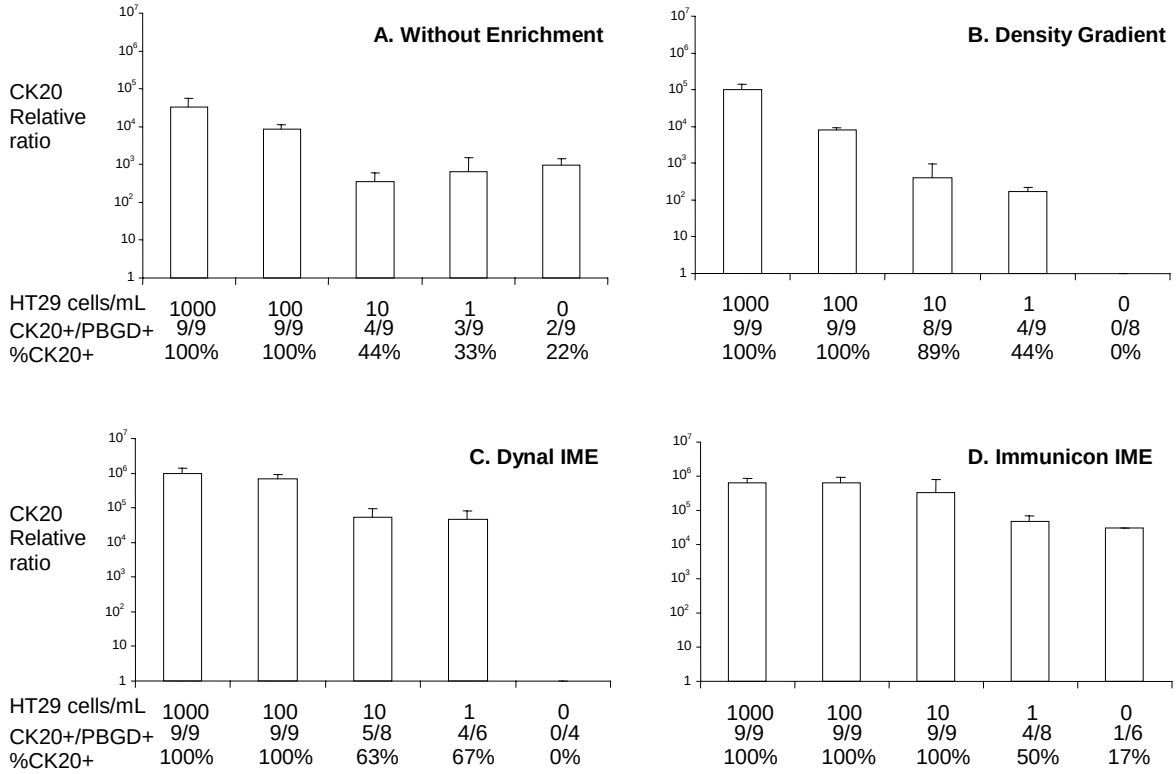


Table 15*: Patient population (n=55) of Study#4.*

Group	Number of patients	Age (median, range) yrs	Gender, M/F
ED-SCLC	35	59 (38-78)	14/21
LD-SCLC	20	59 (37-76)	6/14
Healthy Controls	22	36 (24-59)	5/17

ED-SCLC= Extensive stage Disease Small Cell Lung Cancer
LD= Limited stage Disease Small Cell Lung Cancer
M/F=Male/Female

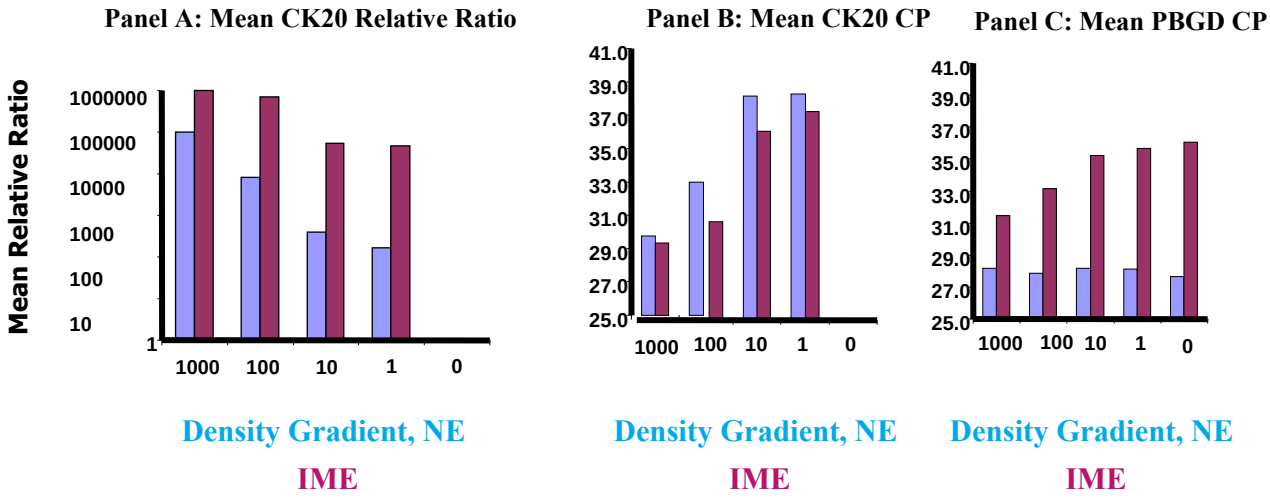
Figure 14: *CK20 quantification and detection frequency in Study#1.*[*]



[*] The CK20 relative ratio mean and SD of each supplemented cell concentration for each procedure are displayed. The quantities from only the CK20-positive samples were measured. The frequencies of detection from each sample preparation are indicated below each graph.

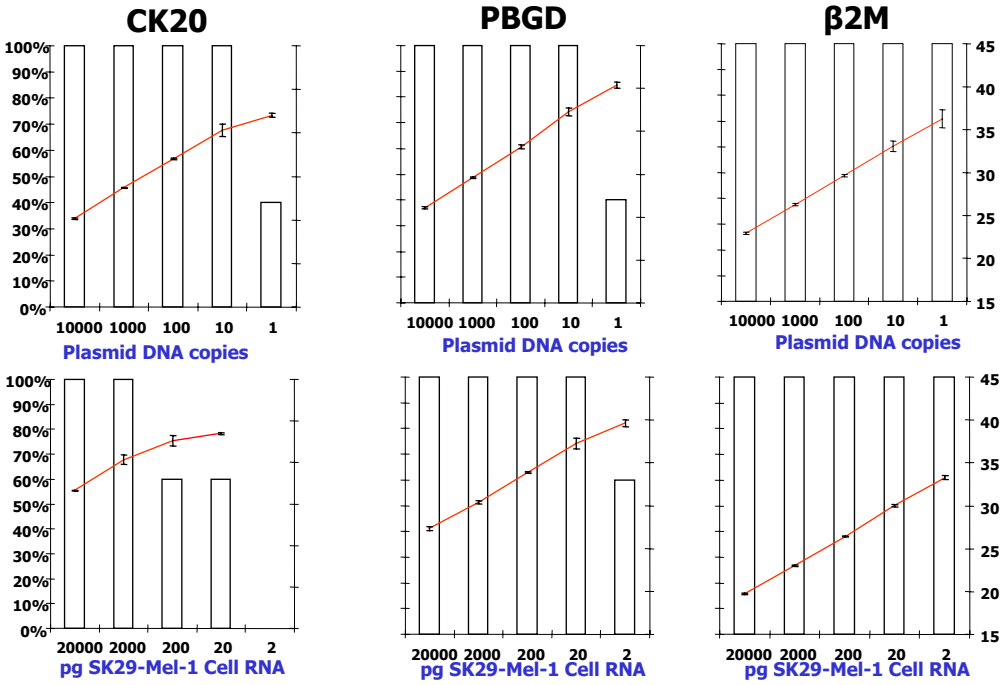
CK= Cytokeratin, IME= Immunomagnetic Enrichment, PBGD= Porphorynbilinogen Deaminase

Figure 15: *Quantitative results from Study#1. Panel A: Mean CK20 relative ratio, Panel B: Mean CK20 CP, Panel C: Mean PBGD CP; Blue: Density Gradient and NE preparations, Red: IME preparations*



CK= Cytokeratin, CP= Crossing Point, IME= Immunomagnetic Separation, NE= Non-Enriched, PBGD= Porphorynbilinogen Deaminase

Figure 16: *Summary of conventional PCR and kinetic PCR results from Study#2. X-axis: Number of plasmid DNA copies (upper row); Pg of SK29 Mel-1 cell RNA (lower row, Y-axis (left): Detection frequency with conventional PCR (bar graphs), Y-axis (right): Mean CP value and SD of kinetic PCR (line graphs).*



CK= Cytokeratin, CP= Crossing Point, IME= Immunomagnetic Separation, NE= Non-Enriched, PBGD= Porphorynbilinogen Daminase

Figure 17: *Conventional PCR results from Study#2.*

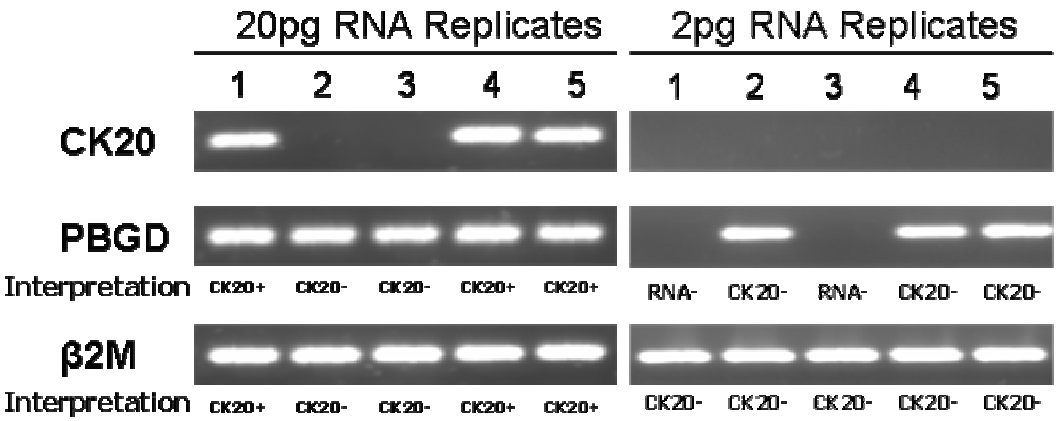


Figure 18: *Kinetic-PCR results from Study#2.*

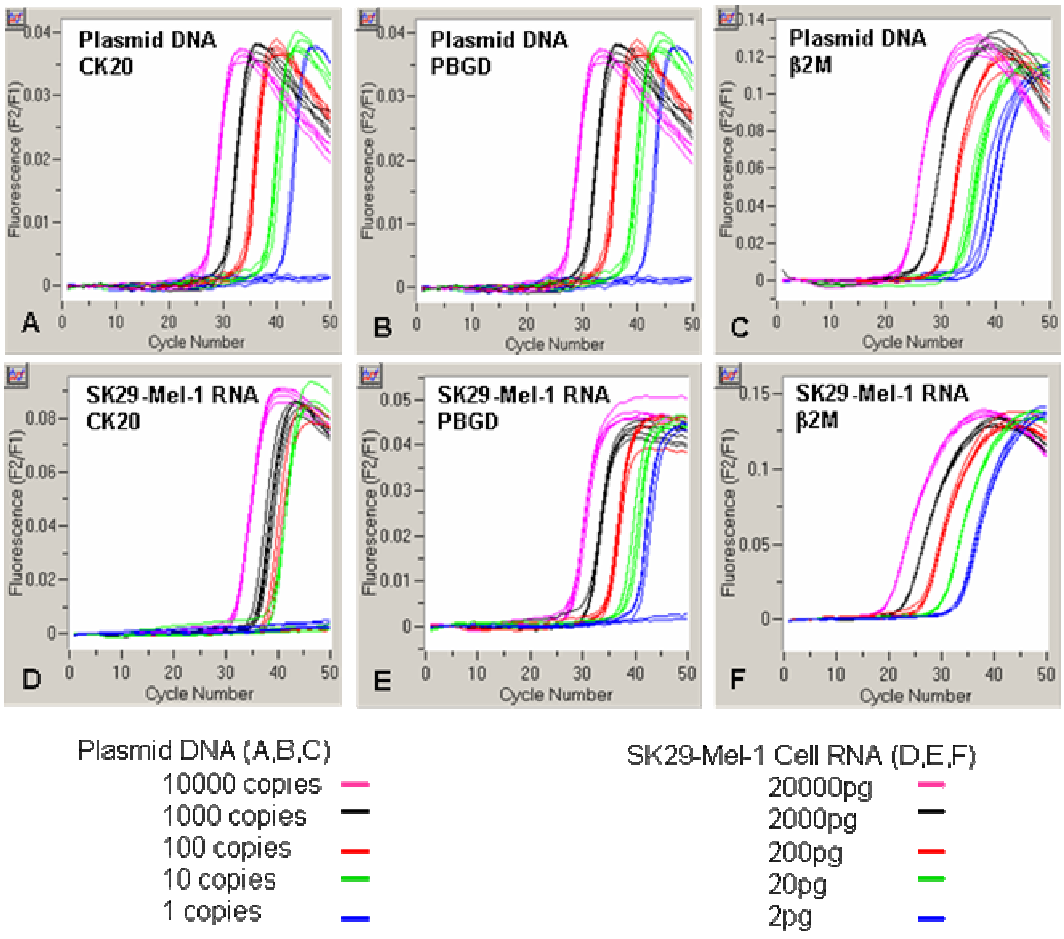


Figure 19: *Correlation between mean CP values and detection frequency (Panel A), SD of the CP (Panel B), and sample concentration (Panel C) for the detection of CK20 from Study#2.*

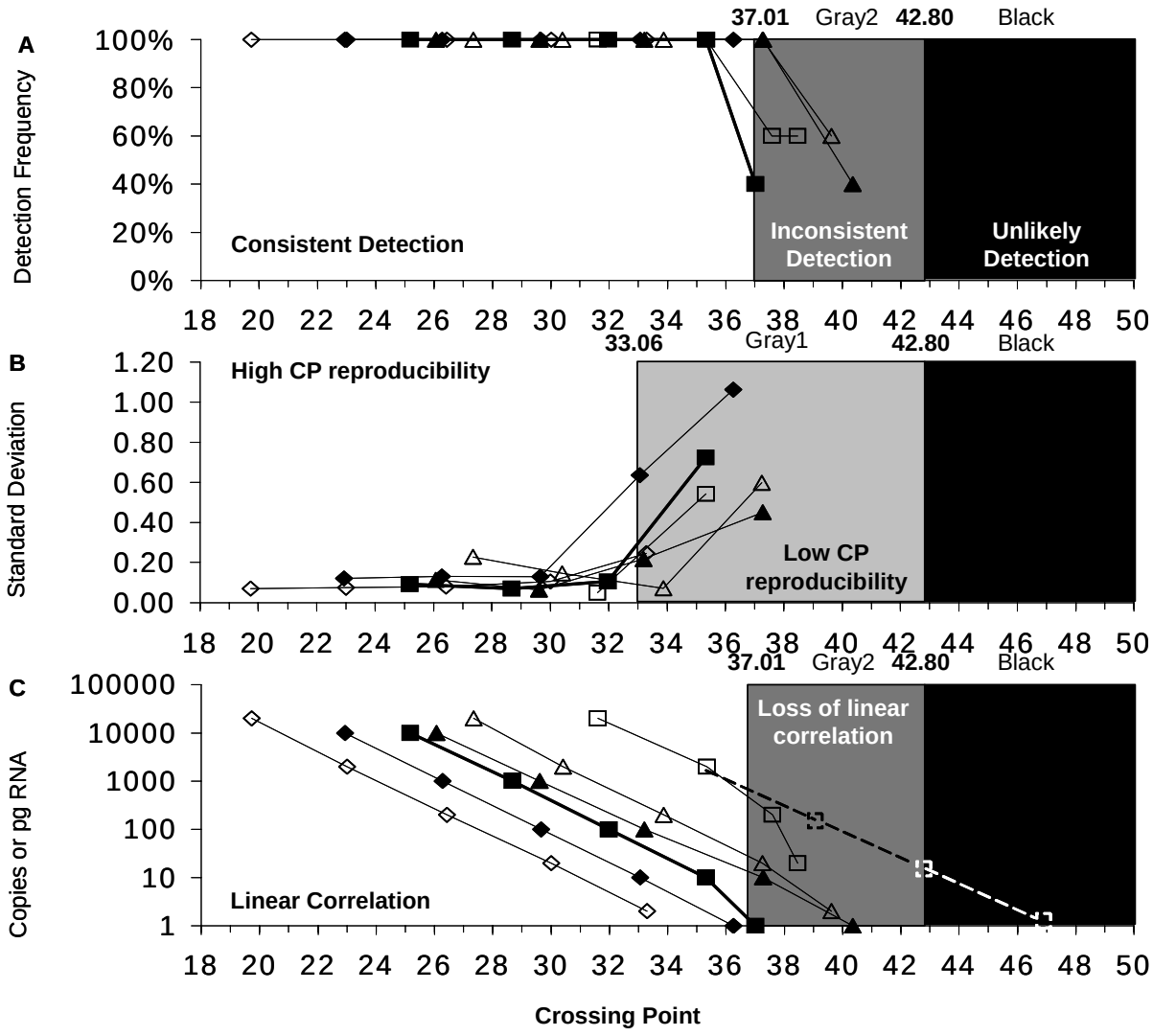


Figure 20: *Zones of PCR reliability and summary of conclusions from Study#2*

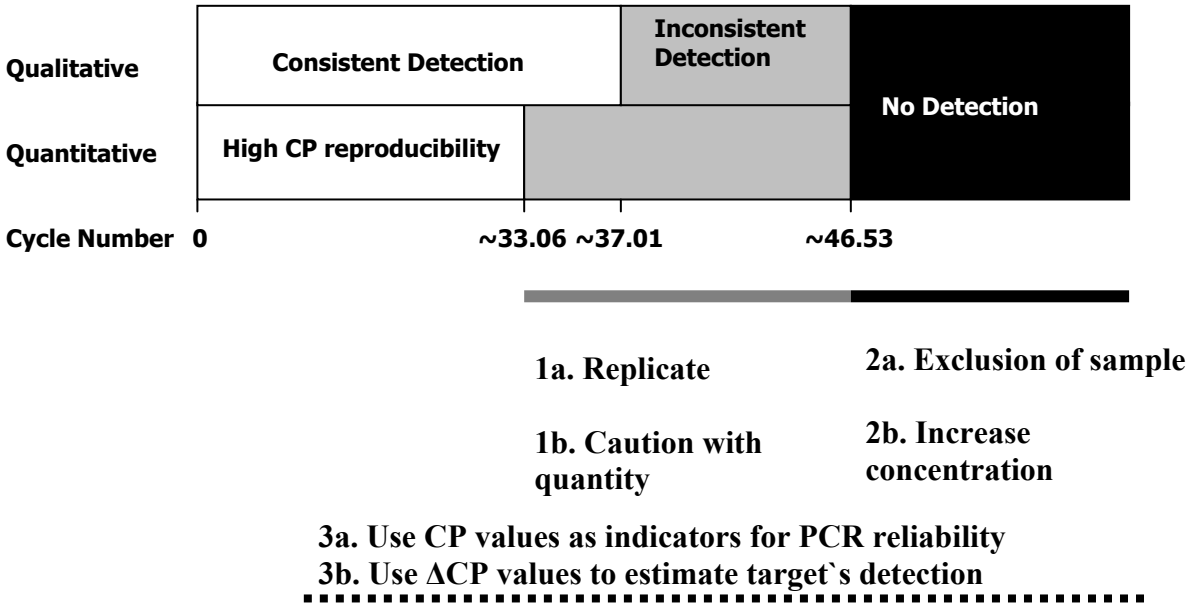


Figure 21: *Differences in crossing points (ΔCP) between PBGD and β2M (P-β), CK20 and PBGD (C-P) and CK20 and β2M (C-β) for the detection of CK20 (squares), PBGD (triangles) and β2M (diamonds) at different concentrations of SK29-Mel RNA from Study#2. The dashed line connects points from the three genes determined from the same sample concentration.*

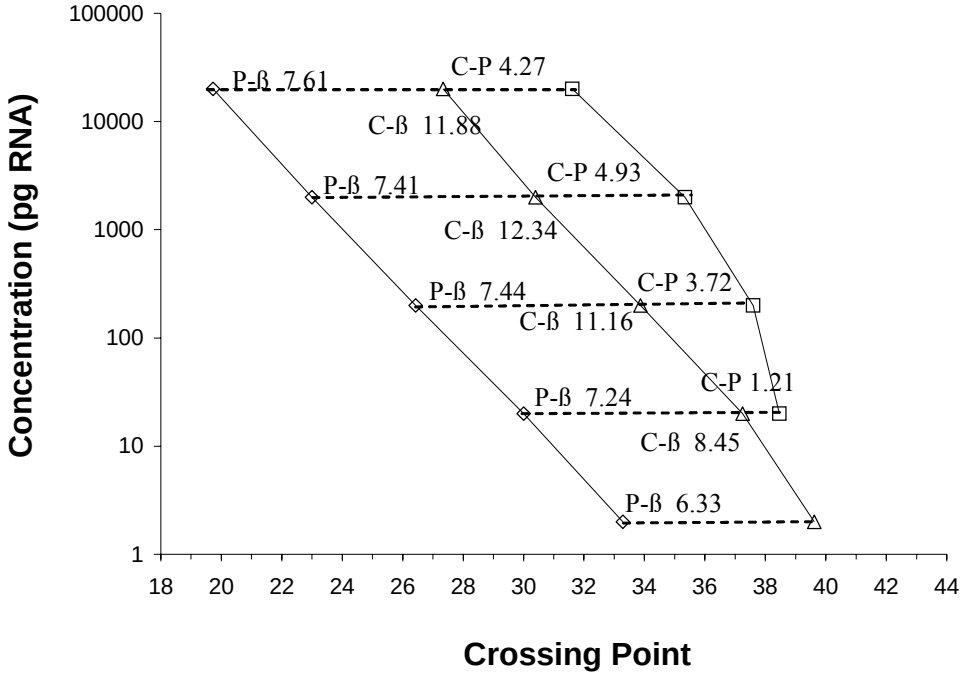


Table 16: *Estimation of CK20 CP and its reliability of detection based on β2M CP and ΔCP(CK20-β2M).*

β2M CP (sample)	**+ ΔCP (CK20-β2M)**	**Estimated CK20 CP*** **(expected feature)**	**Observed mean CK20 CP (observed feature)**
19.73 (20000pg)	+ 11.88	31.61 (consistent CP/detection)	31.61 (consistent CP/detection)
23.00 (2000pg)	+ 11.88	34.88 (poor CP reproducibility)	35.34 (poor CP reproducibility)
26.43 (200pg)	+ 11.88	38.31 (inconsistent detection)	37.59 (inconsistent detection)
30.01 (20pg)	+ 11.88	41.89 (inconsistent detection)	38.46 (inconsistent detection)

Estimated CK20 CP = β2M CP + 11.88 [ΔCP(CK20-β2M) for 20000pg SK29-Mel-1]. The expected feature was determined from PCR reliability zones.

Table 17: *PBGD crossing points specified by laboratory and experiment in Study#3.*

	Lab 1		**Lab 2**		**Lab 3**		**Lab 4**	
	Median PBGD CP	Range	Median PBGD CP	Range	Median PBGD CP	Range	Median PBGD CP	Range
RNA 1st RT	26.75	26.31–26.89	27.41 *	26.06–28.03	26.23	25.64–26.63	24.91	24.61–25.54
RNA 2nd RT	**		28.12 ***	26.43–29.81	26.20	25.69–26.51	25.29	25.05–25.52
MNC	27.58	27.06–27.89	27.43	25.93–31.80	26.62	26.20–27.12	27.29	26.06-29.08
Blood-A****	27.94	27.72–28.43	29.06	28.32–31.57	27.04*	26.21–27.92	27.47	26.53–28.00
Blood-B*****	32.45	31.68–38.12	29.17	28.75–30.16	28.84	27.57–31.87	31.71	29.79–35.20

* Only 4/5 samples were PBGD-positive
** Quantitative data lost
*** Only 2/5 samples were PBGD-positive
**** Blood samples generated by Laboratory 1
***** Blood samples generated by Laboratory 2

***Figure 22**: Summary of qualitative detection of CK20 in Study#3*
Axis-X: Number of HT29 cells/ml
Axis-Y: Number of laboratories with positive CK20 results

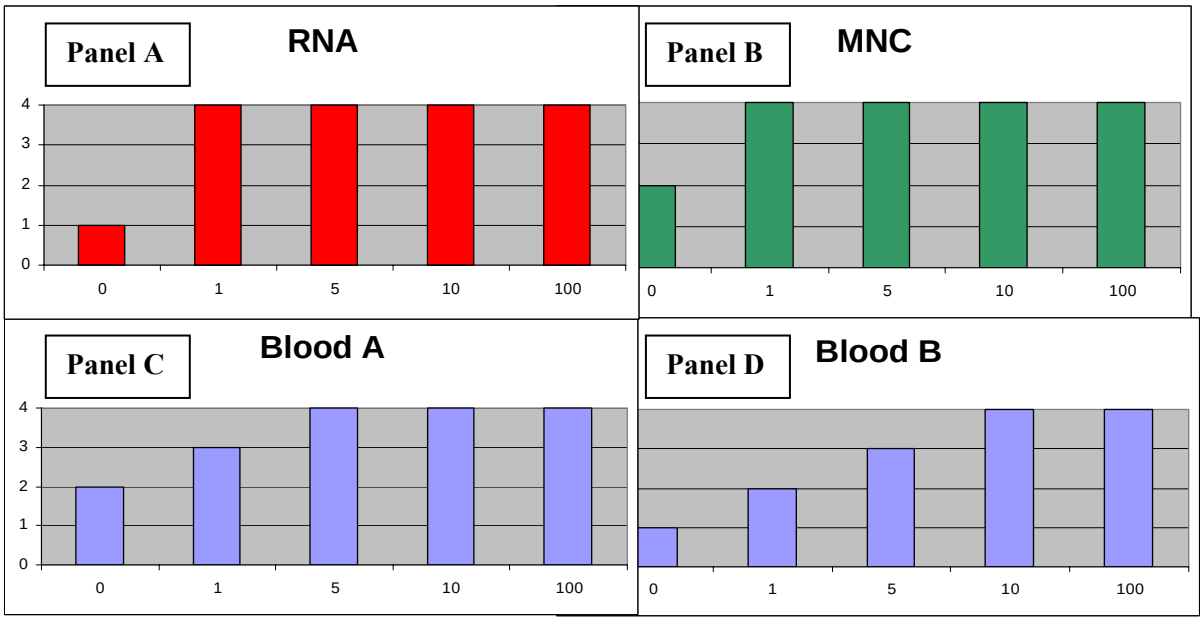


***Figure 23:** Quantification of CK20 expression in three sets of blood samples spiked with tumor cells at three different points of sample processing. The results in the samples produced by laboratory 1 are shown. Axis-X: Number of spiked HT29 cells/ml, Graph A-RNA samples, Graph B-MNC samples, Graph C-Blood samples; Axis-Y: CK20 relative ratio.*

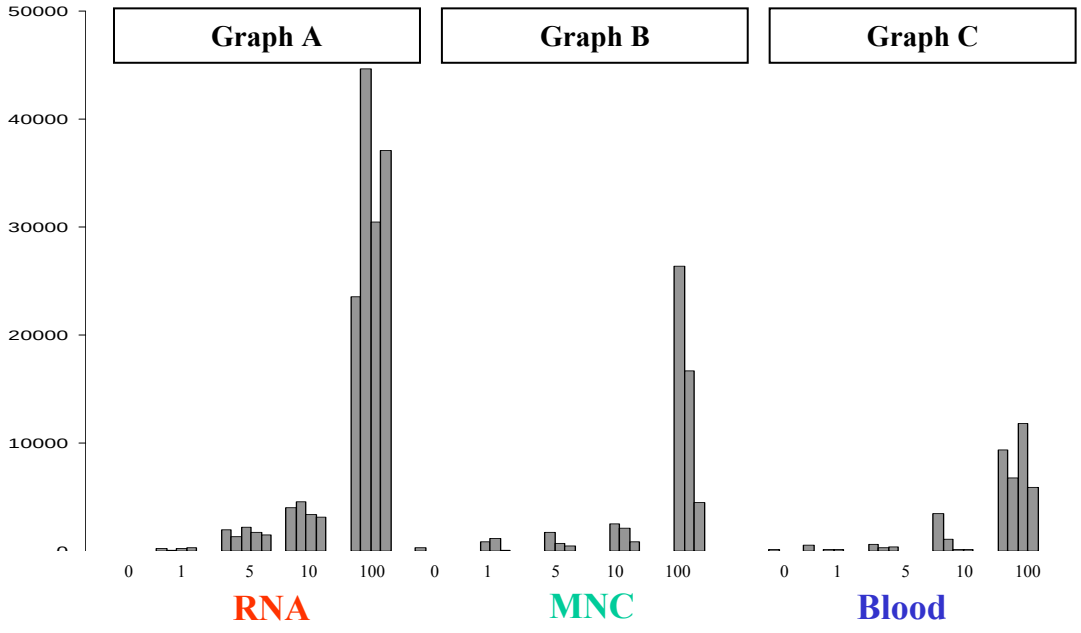


Figure 24: *The user-interface of the REIS software.*

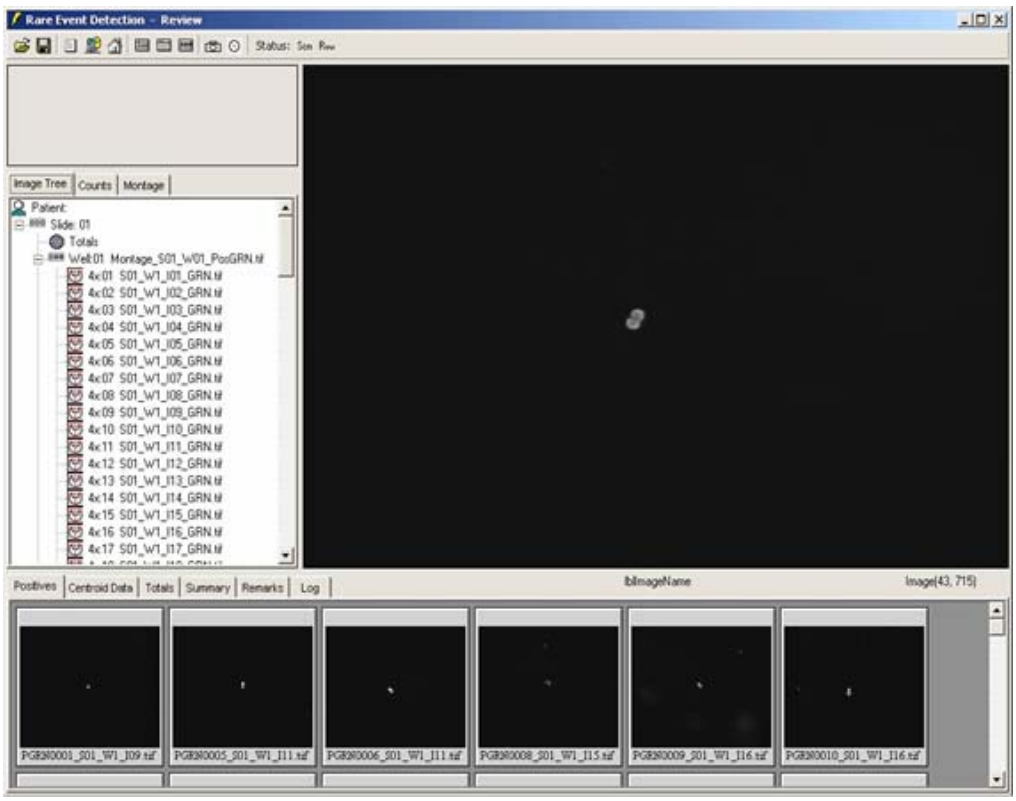


Table 18: *Validation of the REIS in comparison to manual microscopy (n=80). The performance of REIS was evaluated on 40 slides of patients with lung cancer (Table A) and 40 slides of patients with breast cancer (Table B).*

A	Manual microscopy		
REIS-assisted analysis	Cancer cell positive slides	Cancer cell negative slides	Total
Cancer cell positive slides	8	10	18
Cancer cell negative slides	1	21	22
Total	9	31	40

B	Manual microscopy		
REIS-assisted analysis	Cancer cell positive slides	Cancer cell negative slides	Total
Cancer cell positive slides	13	4	17
Cancer cell negative slides	1	22	23
Total	14	26	40

Table 19: *Summary of results from Study#4.*

No. of Patients	ED-SCLC	%	LD-SCLC	%	Control	%
	35		20		22	
Total +	13	37,1	7	35	1	4,5
CK +	12	34,3	6	30	1	4,5
GD2 +	7	20	2	10	0	0
CK/GD2 +	6	17,1	1	5	0	0

ED= Extensive stage Disease, SCLC= Small Cell Lung Cancer, LD= Limited stage Disease, CK= Cytokeratin, GD2= Disialoganglioside 2.

Figure 25: *CTC marker distribution in ED (Panel A) and LD (Panel B) SCLC patients' samples. Pie charts: Blue = % of CTC negative samples, Black=% of CTC positive samples. Pie of pie charts: Red =% of only CK-positive samples, Green= % of only GD-2-positive samples, Yellow = % of both CK- and GD2-positive samples.*

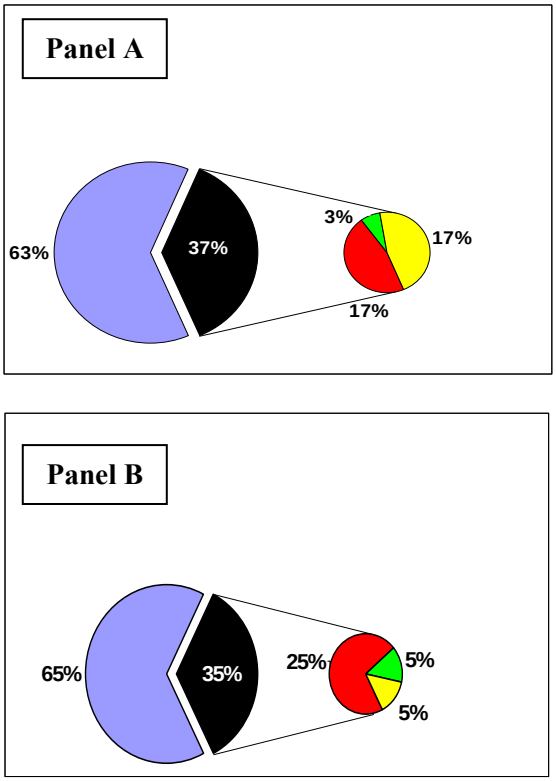


Figure 26: *Example of single cells and clusters of cells that had been recovered from the PB of SCLC patients. The specimen is labeled with mouse monoclonal anti-GD2 antibodies and anti-mouse AlexaFluor488 (green), and rabbit polyclonal anti-CK antibodies and rhodamine-conjugated anti-rabbit antibodies (red). Slides were counterstained with DAPI (blue).*

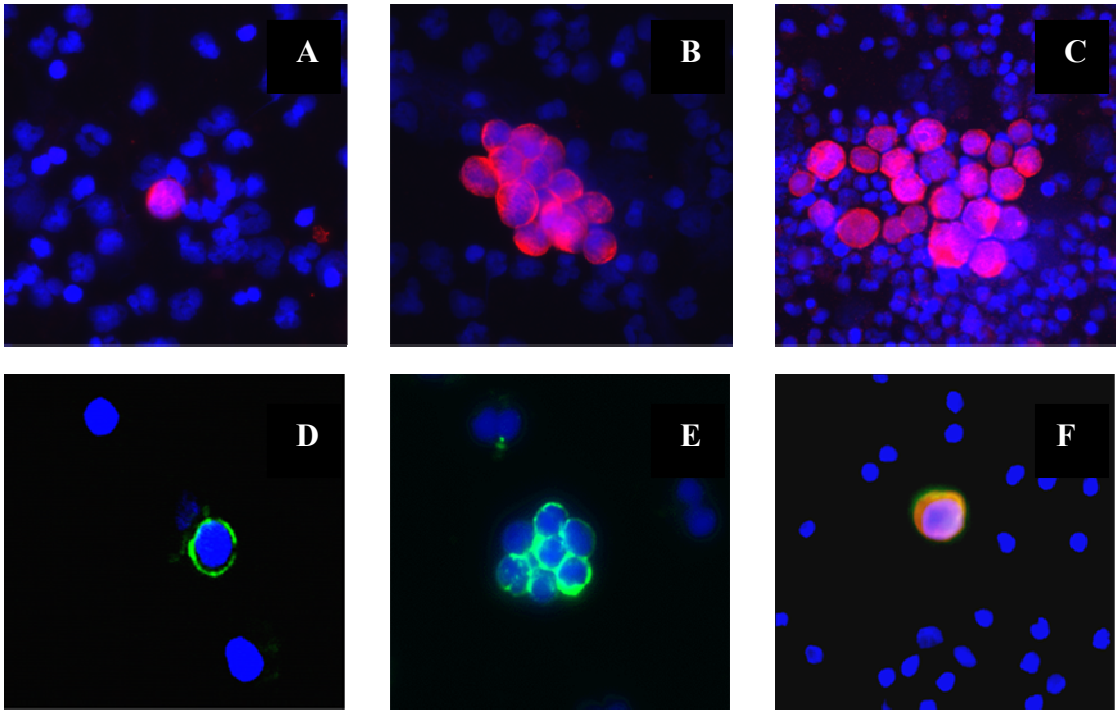


Figure 27*: The number of CTCs/10^6 WBCs in each disease group is displayed from Study#4.*

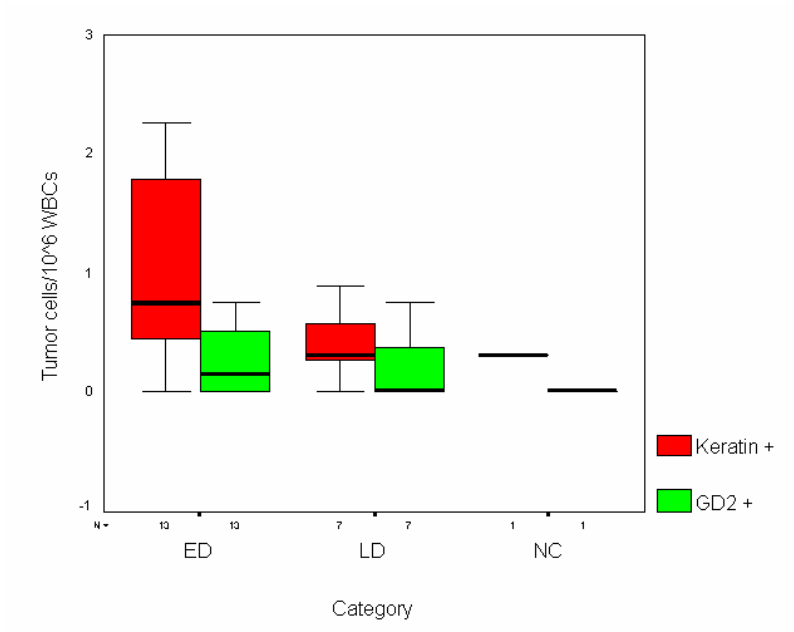


Table 20: *Follow-up data of SCLC patients (n=55) from Study#4.*

	ED-SCLC	LD-SCLC
Death	20	4
Alive	15	16
Mean Survival (Months)	12,6	16,7
Median Follow-up time and range (Months)	15.3 (2.3-25)	14.5 (3.6-26.6)

Table 21: *Performance of decision tree analysis on the training data set and with 10-fold-cross validation (n=32, total number of objects=4075) from Study#5.*

	Training data		10 fold-cross validation	
	Actual +	**Actual -**	**Actual +**	**Actual -**
Predicted +	1824	167	1688	357
Predicted -	223	1861	359	1671

Figure 28: *Sensitivity and specificity combinations obtained by varying cost ratios from Study#5. Left from the uniform cost line is in favor of specificity (Cfn/Cfp<1) and right is in favor of sensitivity (Cfn/Cfp>1).*

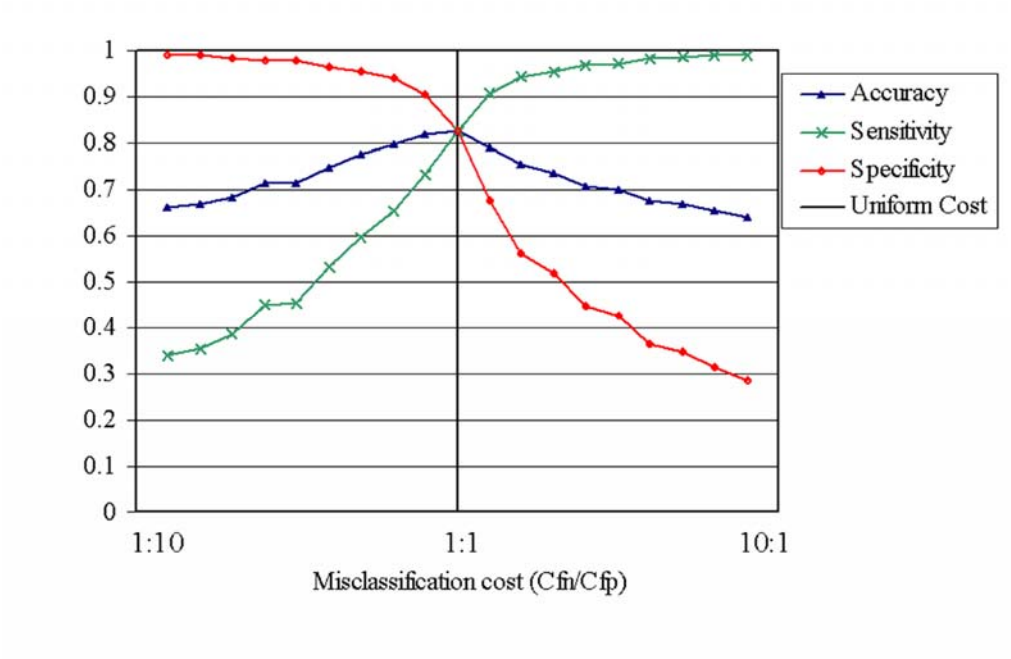


Figure 29: *Graph of a receiver operating-like curve (ROC) from Study#5.*

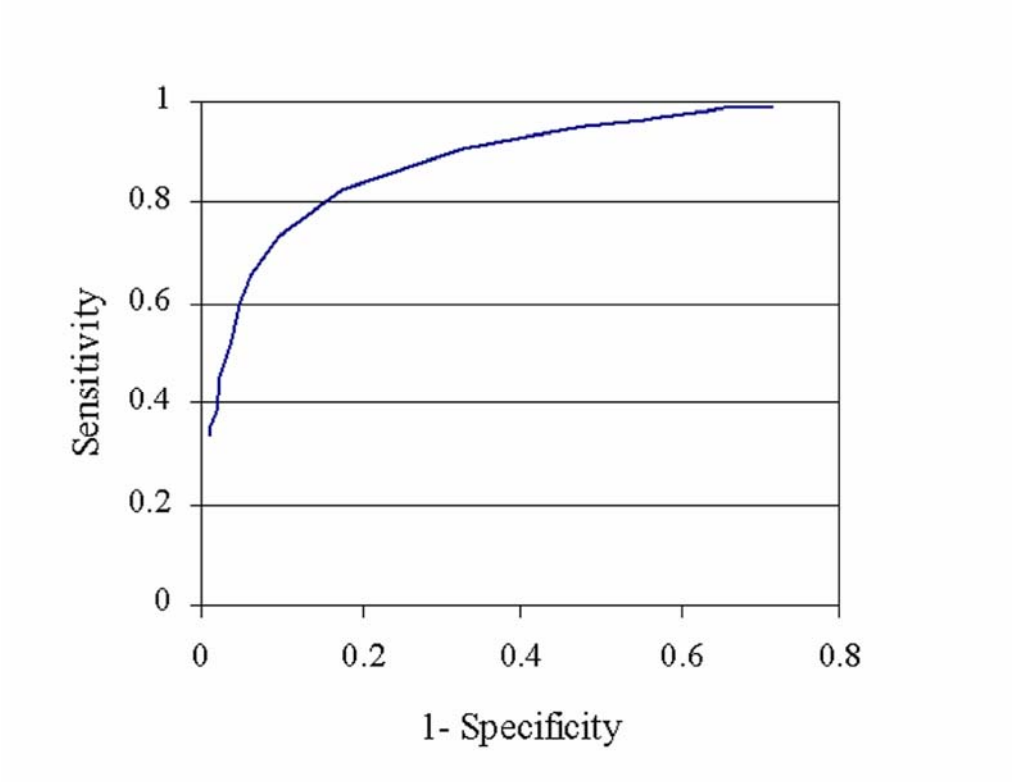


Table 22: *Manual versus REIS-assisted detection in the external data set (n=21) from Study#5. Slides 1–12 were positive for CMV-infected cells; slides 13–21 were negative.*

Slide Number	Slide ID	Manual Scan Positive Count*	Collected objects**	Decision Tree Positive Count***	% Sensitivity****
1	3908	1	23	1	100.00
2	2123	1	59	2	100.00
3	4215	1	46	2	100.00
4	2652	2	61	2	100.00
5	3018	4	36	3	75.00
6	2935	6	28	4	66.66
7	595	6	34	6	100.00
8	3875	6	29	6	100.00
9	3419	8	41	7	87.50
10	1862	59	105	52	88.13
11	962	118	175	113	95.76
12	3019	135	138	117	86.66
Mean		**28.92**	**64.58**	**26.25**	**91.64**

Slide Number	Slide ID	Manual Scan Positive Count	Collected objects	Decision Tree Positive Count	% Removal Rate *****
13	1808	0	15	5	66.67
14	1979	0	26	11	57.69
15	1982	0	33	10	69.70
16	1987	0	12	6	50.00
17	1994	0	22	8	63.64
18	2053	0	21	8	61.90
19	2222	0	20	7	65.00
20	2225	0	31	9	70.97
21	2233	0	35	10	71.43
Mean		**0.00**	**23.89**	**8.22**	**64.11**

* Number of true-positive cells that were detected by manual microscopy.
** Number of objects that met the finding parameters and therefore collected by the REIS in the image gallery as potential positives.
*** Number of cells that were classified as true positive by decision tree analysis. Of note, that on the positive slides (slide 1–12) only those cells that were verified to be true positive after manual review of the image gallery were sent to decision tree analyses.
**** Percentage of automated-counted cells to manual counted cells.
***** Percentage of reduction of the size of the image gallery that was achieved by decision tree analysis.

Table 23: *Studies analyzing PB samples from SCLC patients.*

Author	Marker	Method	Patients	Positives
Saito et al. [474]	prepro-GRP	RT-PCR	ED= 19	58%
			LD= 11	38%
Perey et al. [475]	N-CAM	RT-PCR	ED= 14	43%
		ICC	LD= 15	27%
Bessho et al. [476]	NMB-R	RT-PCR	ED= 21	43%
			LD= 23	19%
Peck et al. [312]	CK-19	RT-PCR	ED=10	33%
			LD=5	20%
Brugger et al. [96]	CK/HEA	ICC	ED= 10	20%
			LD= 7	0%

Prepro-GRP = Prepro-Gastrin-Releasing Peptide , ED = Extensive stage Disease, LD= Limited stage Disease, N-CAM= Neural-Cell Adhesion Molecule, NMB-R= Neuromedin B Receptor, CK-19= Cytokeratin 19, HEA = Human Epithelial Antigen

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13 Abbreviations

ACS	American Cancer Society
ADM	Automated Digital Microscopy
AFP	Alfa-Fetoprotein
B2M	Beta 2-Microglobulin
BCSS	Breast Cancer-Specific Survival
B-HCG	Beta chain of the Human Chorio Gonadotropin
BM	Bone Marrow
BRCA	Breast Cancer Susceptibility gene
CCD	Charged-Couple Device
CEA	Carcinoembryonic Antigen
Cfn	False-Negative Cost
Cfp	False-Positive Cost
CGH	Comparative Genomic Hybridization
CK	Cytokeratin
CMV	Cytomegalovirus
CP	Crossing Point
CR	Complete Response
CR	Cost Ratio
CTC	Circulating Tumor Cell
CUP	Carcinomas of Unknown Primary
DALYs	Disability-Adjusted Life Years'
DAPI	4',6-diamidino-2-phenylindole
DCC	Deleted-in-Colorectal-Cancer gene
DCIS	Ductal Carcinoma In Situ
DDFS	Distant Disease-Free Survival
DFS	Disease-Free Survival
DG	Density Gradient
DT	Decision Tree
DTC	Disseminated Tumor Cell
ED	Extensive stage Disease
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	Epithelial Membrane Antigen
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
EU	European Union
EUROCARE	European Concerted Action on survival and Care of Cancer Patients

FACS	Fluorescent- Activated Cell Sorter
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein Isothiocyanate
GD2	disialoganglioside GD2
GFP	Green Fluorescent Protein
HEA	Human Epithelial Antigen
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
ICC	Immunocytochemistry
IF	Intermediate Filament
IME	Immunomagnetic Enrichment
ISET	Isolation by Size of Epithelial Tumor Cells
IVVM	Intra-Vital Video Microscopy
LD	Limited stage Disease
LN	Lymph Node
LOH	Loss of Heterozygosity
M/F	Male/Female
MAGE	Melanoma Antigen Gene
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
MNC	Mononuclear Cell
MoAb	Monoclonal Antibody
MUC-1	Mucine-1 gene
NCAM	Neural Cell Adhesion Molecule
NCI	National Cancer Institute
NE	Non-Enriched
NIH	National Institute of Health
NMB-R	Neuromedin B Receptor
NPGP	Non-Penetrating Glycoprotein
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
PB	Peripheral Blood
PBGD	Porphorynbilinogen Deaminase
PBS	Phosphate-Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PMSA	Prostate-Membrane Specific Antigen
pp65	CMV lower matrix phosphoprotein
Prepro-GRP	Prepro-Gastrin-Releasing Peptide
PSA	Prostate-Specific Antigen
PTH	Parathyroid Hormone
qRT-PCR	quantitative RT-PCR

RBC	Red Blood Cell
REIS	Rare Event Imaging System
ROC	Receiver Operating Characteristic
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase-PCR
SCLC	Small Cell Lung Cancer
SEER	Surveillance, Epidemiology and End Results
SES	Socioeconomic Status
SV40-T	SV40 large tumor antigen
TAG-12	Tumor-Associated Glycoprotein-12.
TIMP2	Tissue Inhibitor of Metalloproteinase 2
uPA	urokinase Plasminogen A
WBC	White Blood Cell
WHO	World Health Organization
YLD	Years Lived with Disability
YLL	Years of Life Lost

14 Publications

14.1 Publications related to this thesis

Ladanyi A, Sher AC, Herlitz A, Bergsrud DE, Kraeft SK, Kepros J, McDaid G, Ferguson D, Landry ML, Chen LB. Automated detection of immunofluorescently labeled cytomegalovirus-infected cells in isolated peripheral blood leukocytes using decision tree analysis. Cytometry A. 2004 Apr;58(2):147-56.

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14.2 Publications not related to this thesis

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