Clinical Infrastructures to Support Proteomic Studies of Tissue and Fluids in Breast Cancer*

Henning T. Mouridsen‡ and Nils Brünner§¶

The Danish Breast Cancer Cooperative Group (DBCG) was established in 1977 with the aim to ensure optimal breast cancer diagnostics and therapeutic modalities on a nationwide basis. DBCG was organized in such a way so it represents a broad interdisciplinary collaboration with established clinical databases and biobanks. This review summarizes the infrastructures, such as those of the DBCG, that are required to facilitate translational research studies aiming at further diagnostic and therapeutic improvements through interactions directed at prevention, early diagnosis, and treatment of primary breast cancer. ***Molecular & Cellular Proteomics 3:302–310, 2004.***

Breast cancer is the most common malignancy among women in the western world and constitutes ~18% of all cancers in women (1). Although recent years have seen an improvement of the prognosis of breast cancer, the disease still carries a significant health problem and it is our belief that future improvements will be based on results obtained by basic research. The natural history of breast cancer and the potential methods of interaction with the disease are briefly presented in Fig. 1.

CLINICAL INFRASTRUCTURES

Translational research requires an organization with a close collaboration between different clinical disciplines and basic research. It requires current registration and report of demographic, histopathological, therapeutical, and clinical patient data together with an establishment of biobanks.

The Danish Breast Cancer Cooporative Group (DBCG)[1] represents such an organization (2). The DBCG was established in 1977 on an initiative by the Danish Surgical Society with the aim of ensuring optimal diagnosis and treatment of operable primary breast cancer on a nationwide basis. To achieve this purpose, the DBCG has worked out uniform guidelines for the whole country, and these guidelines are currently being amended according to requirements for evidence-based medicine.

A clinical database of invasive and *in situ* breast cancer was established in 1977, and since then the involved departments of surgery, histopathology, radiotherapy, and medical oncology have submitted systematic reports by means of standardized data forms, and data on diagnosis, treatment, and follow-up of patients. About 3,500 new incident cases of invasive primary breast cancer are reported every year, and by January 1, 2003 the database included ~70,000 patients. In addition since 1999, a database of women examined for hereditary disposition of breast cancer has been established.

The organizational structure of DBCG consists of a council, an executive committee, county committees, scientific committees, and a secretariat (Fig. 2). The council is the highest authority of the entire organization, consisting of representatives from the participating departments, and all members of the county committees, the scientific committees, and the executive committee.

The executive committee, which consists of elected members among the participating departments and subcommittees, coordinates and promotes decisions made by the council and the various committees. Furthermore, the executive committee will handle matters concerning the practical implementation of DBCG projects, coordinate the tasks of the scientific committees, and serve as advisor to the county committees and the different departments as well as to public health authorities. Furthermore, the executive committee will take the initiative to prepare evidence-based guidelines for diagnosis and treatment in agreement with official international guidelines. The executive committee consists of three surgeons (including one plastic surgeon), two histopathologists, four oncologists, one clinical physiologist, one diagnostic radiologist, one tumor biologist, and one clinical geneticist. These members are appointed by their respective scientific societies. In addition, the executive committee includes the director of the secretariat and DBCG statisticians. Among its members, the executive committee elects a chairman for a period of 2 years with the possibility of reelection.

The county committees in the respective counties have the responsibility for organizing clinical assessment, treatment strategies, and follow-up control in accordance with the protocols.

The purpose of the scientific committees is to ensure a scientifically optimal accomplishment of the clinical treatment

From the ‡Danish Breast Cancer Cooperative Group, Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; and §Institute of Pharmacology and Pathobiology, Royal Veterinary and Agricultural University, Ridebanevej 3, 1870 Frederiksberg, Denmark

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[1] The abbreviation used is: DBCG, Danish Breast Cancer Cooperative Group.

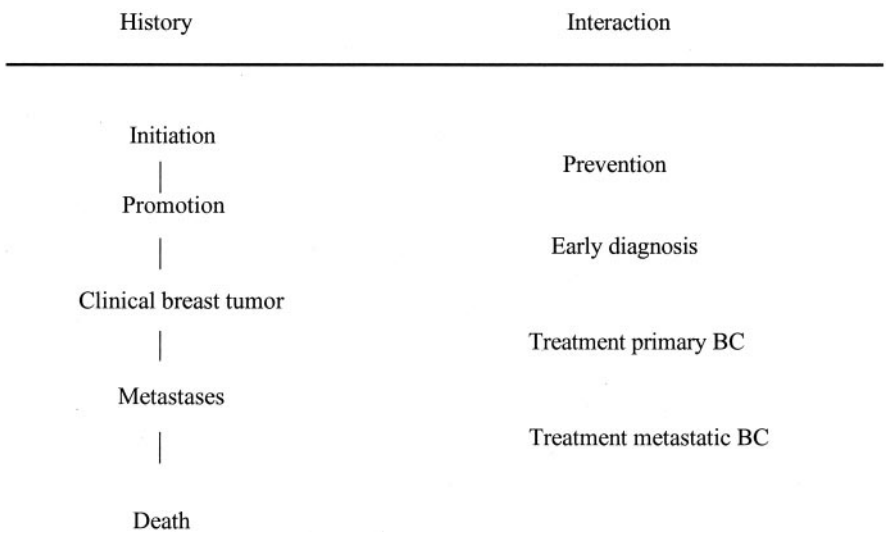

FIG. 1. **Natural history of breast cancer (BC) and methods of interaction.**

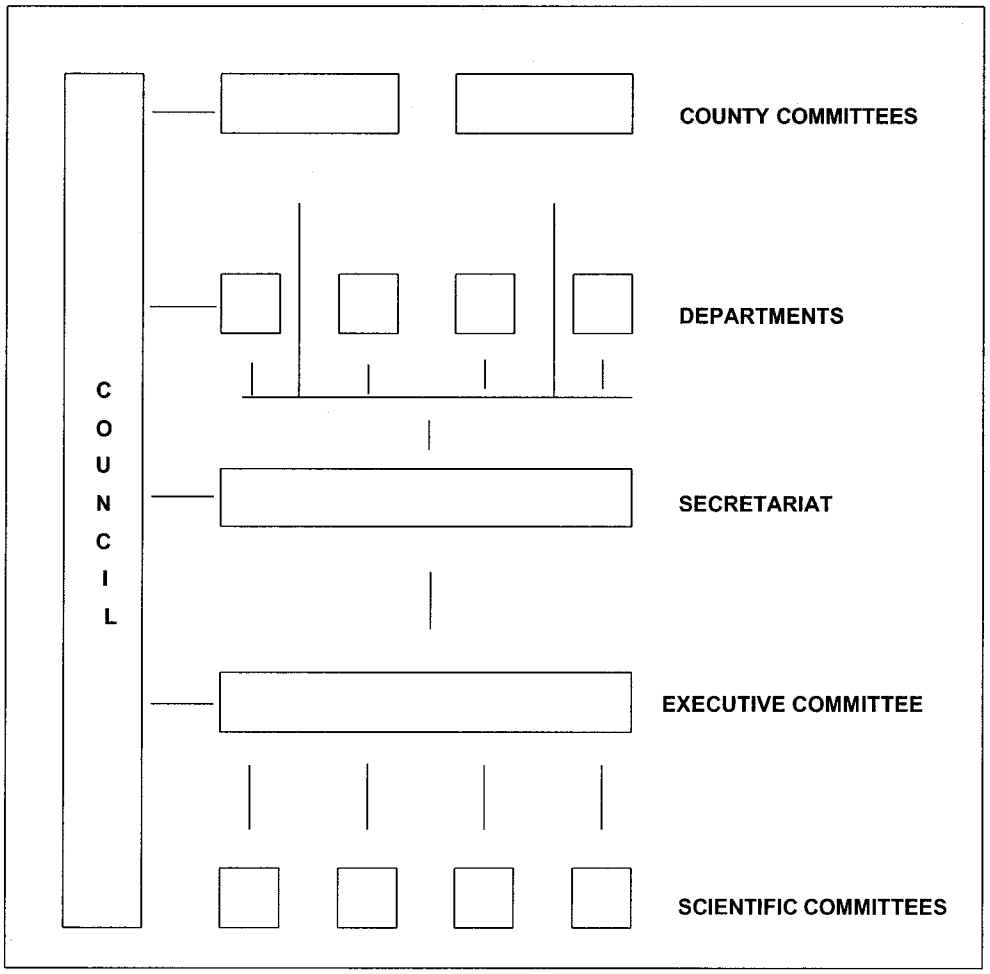

FIG. 2. **Structure and organization of DBCG.**

programs and to initiate concurrent scientific projects. A scientific committee may be formed on the initiative of the executive committee and will in such cases preferably include representation from the executive committee, or by initiative from persons with a special expertise or interest in relation to the work of DBCG. The scientific committees may be formed as standing committees or ad hoc committees. The committees will draw up their own guidelines for the time period of

function and for electing their own chairman and secretary. Of special interest in this context are the committees of pathology, tumor biology, radiotherapy, and systemic therapy.

The main secretariat conducts the centralized data registration and data processing and will ensure the contact with the various departments and committees. In addition, it keeps records of the biobanks of paraffin-embedded and fresh frozen tissues and blood samples. The secretariat functions as a national database in most of the international studies, typically including randomization, and as a contact to other data centers. The secretariat consists of a director of the secretariat, three statisticians, and 4.5 data secretaries. The DBCG secretariat is housed at Rigshospitalet, Copenhagen. The secretariat is financed by the hospital municipalities paying a certain amount per patient from their county, who is registered for the first time in the DBCG database. Any external funding covering scientific research activities is administered by Rigshospitalet.

The participating departments are the surgical departments responsible for the primary diagnosis and surgical treatment as well as the subsequent control of low-risk patients who do not receive adjuvant treatment; the departments of histopathology being responsible for the final diagnosis and grading as well as more specified markers, *e.g.* estrogen and progesterone receptor immunohistochemistry, c-erbB 2 determination; and the oncology departments, which are responsible for radiotherapy, systemic treatment, and follow-up. In addition, there are the departments taking care of a variety of other activities (basic research, clinical biochemistry, imaging diagnostics, clinical physiology/nuclear medicine, and genetics).

DBCG has close cooperation with several cooperative groups working with similar items, and the clinical trials of DBCG are increasingly performed through international cooperation. Since 1994, some trials have been conducted within the frames of the Scandinavian Breast Cancer Group, and DBCG is also affiliated with the Breast International Group, established in 1996 with the purpose of coordinating clinical and translational studies between cooperative breast cancer groups. More recently, the DBCG initiated close collaboration with the Danish Centre for Translational Breast Cancer Research (3).

The DBCG registration includes almost all women (~95%) in Denmark with operable noninvasive and invasive primary breast cancer. After recommendation by DBCG, the Danish Data Protection Agency has laid down regulations for the DBCG database according to the Public Authorities' Registers Act. Data are sent to DBCG from all the participating departments on forms produced and distributed by the DBCG secretariat (Fig. 3). Data are fed into a database by double entry, and checking for logic consistency and completeness constitutes data validation. The database is updated and corrected daily. Reminder lists are sent out for patients whose information is missing, *e.g.* missing Breast form or Pathology form, or missing reports concerning follow-up according to the guidelines of the protocol. When the Breast and Pathology forms of a patient are received, the patient is allocated to the recommended protocol (= adjuvant treatment). If the patient enters a randomized trial, DBCG performs the randomization upon receipt of a registration form from the department.

The primary research task is the analysis of DBCG prospective treatment protocols, in particular survival analyses in order to compare treatments and evaluate prognostic and predictive factors. In addition, annual national figures of the dataset are produced for the DBCG Newsletter, which is issued annually following each council meeting. Moreover, participating departments are entitled to their own datasets by request to the database. Provided an approval by the DBCG executive committee and the Registers Act, data may be combined with other databases (*e.g.* The National Patient Register, The Danish Cancer Register, the Hospital Discharge Register) with the purpose of data validation and control or in connection with scientific projects. After approval by the DBCG executive committee, extracts may be produced from the database for special scientific projects, as well as extracts for use in major international meta-analyses.

Since the mid-1980s, DBCG has facilitated collection and storage of leftover tissue or cytosols and nuclear pellets. In addition, DBCG has recently initiated a prospective collection of serum and plasma obtained preoperatively from patients scheduled for breast surgery for benign or malignant disease. The DBCG secretariat is responsible for the corresponding databases for these collections. It has been the policy of DBCG that collected samples should be stored at the place of collection if the necessary facilities were available, otherwise at a centralized facility. DBCG keeps track on all samples, but before getting access to the samples any outside investigator should obtain acceptance from the Executive Committee and the local department where the samples were collected.

Below we will describe selected features related to clinical infrastructures allowing proteomic research aiming at the identification and validation of new markers to be used to select women for prevention studies, for early diagnosis, and for studies on adjuvant treatment.

PREVENTION

A combination of environmental factors (exposure to carcinogens, lifestyle, and hormonal factors) and genetic factors play a role in the development of breast cancer and may also affect mortality rates. Prevention is the best cure, but increased understanding of the mechanism of malignant transformation and subsequent metastatic spread, as well as the identification of potential markers of increased risk of developing breast cancer, are prerequisites to develop chemo prevention as a potentially useful early interaction. Estrogens are involved in the multiple stages of the malignant transformation (4). This knowledge has lead to four prevention studies using antiestrogens (tamoxifen) *versus* placebo in healthy women; the Royal Marsden Pilot study (5), the Italian Tamoxifen Pre-

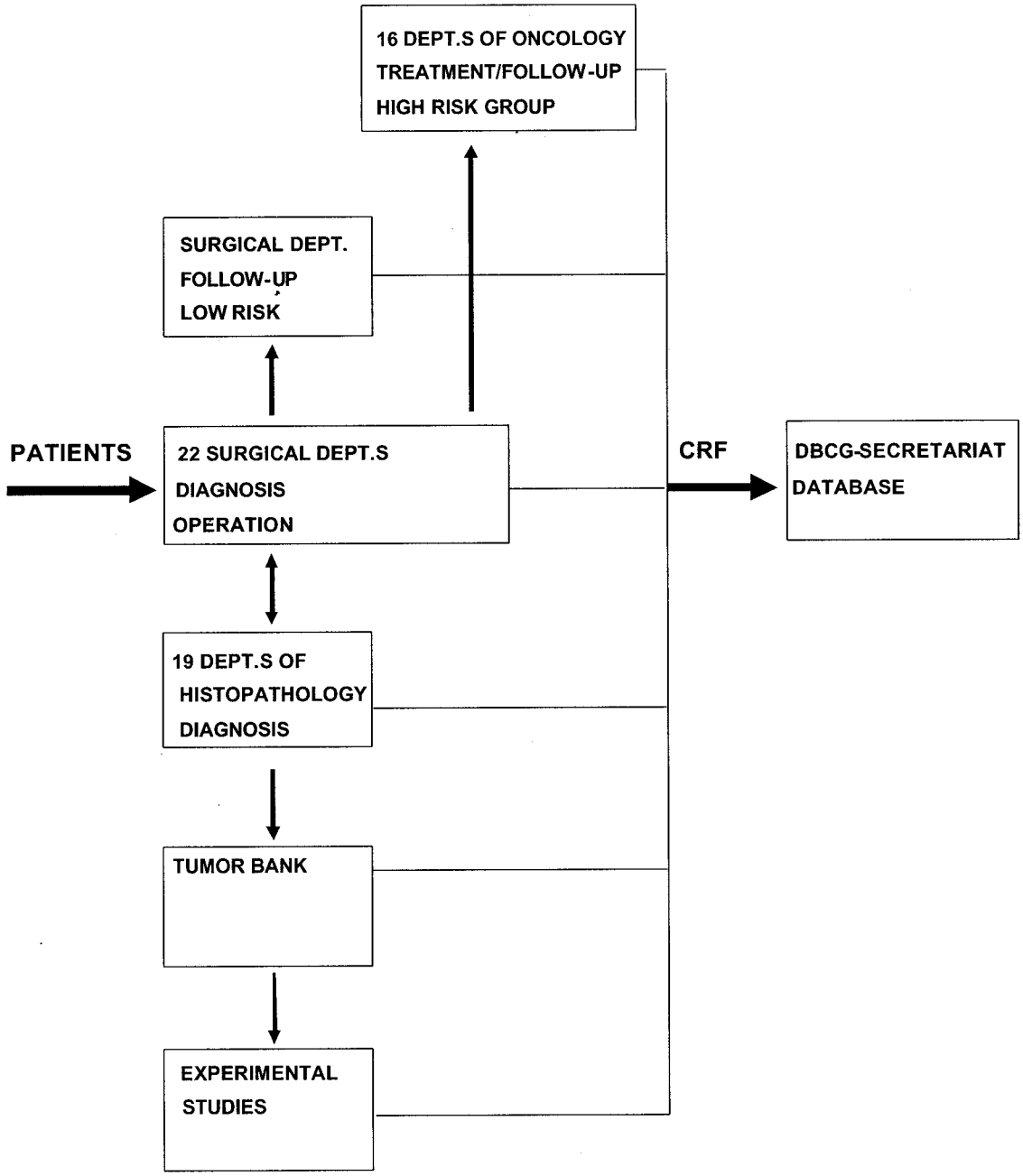

FIG. 3. **Patient flow and data registration.**

vention study (6), the National Surgical Adjuvant Breast and Bowel Project (NSABP) study (7), and the International Breast Intervention Study (IBIS) (8). Three of the trials included women at increased risk of developing breast cancer (5, 7, 8). The risk increase in these studies was 4.0-, 1.7-, and 2–3-fold, respectively. The Italian study (6) included low-risk women only.

The results of the studies have been somewhat conflicting, maybe due to the difference in power of the studies and different criteria of eligibility to enter the trials. Two of the high-risk trials (7, 8) observed a significant reduction in risk of developing breast cancer following tamoxifen treatment. In the largest trial with longest median follow-up (~5.5 years), 86 fever-invasive breast cancers were observed in the tamoxifen arm, corresponding to a 49% risk reduction. However, this was achieved at the expense of increased risk of thromboembolic complications and endometrial cancer (7). No risk reduction was observed in the third high-risk study (6). However, this study may be underpowered and recruited, in contrast to the other high-risk trials, mainly premenopausal women. The Italian trial demonstrated no significant benefit with tamoxifen. This trial included a low-risk population of relatively young women, and there was a high drop-out rate.

Another antiestrogen, raloxifen, has been investigated in relation to its role in preventing osteoporosis in postmenopausal women. In this study (the Multiple Outcomes of Raloxifene Evaluation, or MORE), the incidence of primary breast cancers in the two groups was also recorded. The study showed that treatment with raloxifene was associated with a 58% reduction in the risk of developing primary breast cancer (9).

These results stimulated the design of another major prevention trial, the Study of Tamoxifen and Raloxifen (STAR) comparing the toxicity, risks, and benefits of raloxifene with those of tamoxifen (4). A next generation of prevention trials with aromatase inhibitors are now being launched (10).

One of the critical issues of the present prevention trials is the significant over-treatment, an issue that is important seen in the light of the potential side effects of these prevention drugs. Using the data from the NSABP trial, which used 5 years of tamoxifen (7), it appeared that avoidance of one case of invasive breast cancer would need the treatment of ~200–300 women for 1 year. It is therefore of explicit importance in addition to the model used most widely today (11), which estimates the risk according to the woman's age, age at menarche, number of first-degree relatives with breast cancer, multiparity or age at first birth, number of breast biopsies, and presence or absence of atypical hyperplasia, to develop improved methods to estimate the breast cancer risk of an individual.

With the discovery of the BRACA 1 and BRACA 2 mutations (12), the first steps toward molecular risk estimation were taken. With the clinical infrastructures of the DBCG, the basis for future genetic and proteomic studies are secured. For example, by combining the DBCG data files with the Danish Cancer Registry, familiar accumulation of breast cancer cases can be identified and these families can be included in future genomic and proteomic studies. In addition, with our prospective blood sample collections, where we obtain samples from women with benign and premalignant (*in situ*) breast lesions, screening for differences in protein fingerprints between these two groups may identify new protein-based risk factors. Finally, like in many other countries, we have in Denmark performed large-scale health studies where the individuals are called for a first clinical examination, blood sampling, as well as a questionnaire. Among the women being included, a fare percentage will develop breast cancer and therefore such retrospective samples with clinical information are of immense value for the identification of new markers to be used in prevention studies.

EARLY DIAGNOSIS

The mortality rate of breast cancer increases with clinical and pathological stage (13). Hazard rates of mortality show that there is an initial peak in hazard rates of several years followed by a gradual decline over subsequent years (14). In patients with more advanced stages (III and IV), most relapses and deaths occur within the first 3–5 years. In contrast, in patients diagnosed with stages I-II disease, the events are less frequent and occur later. Thus, the survival curves of patients with more advanced or higher-risk breast cancer start to parallel the survival curves of the general population earlier and at a lower level (at ~10–15 years) than the survival curves of the earlier stages or low-risk breast cancer (15–20 years after diagnoses). Consequently, earlier diagnosis is associated with superior prognosis, and indeed mammographically diagnosed early breast cancer (stages 0-I) is associated with excellent survival rates exceeding 90% at 20 years following local therapy of the breast cancer (15).

Generally, patients dying of breast cancer suffer from disseminated disease. The dissemination of the disease starts to occur during the promotion phase and in the majority of patients before the diagnosis is assessed clinically. With the hypothesis that this dissemination is less likely to occur the earlier during the promotion phase systematic studies of breast cancer screening using mammography are started, ~40 years ago in New York and since then large randomized trials were initiated in Edinburgh, Canada, and in several counties in Sweden. For women of all ages combined, all but one trial demonstrated some degree of benefit, although in almost all trials the confidence intervals included the value 1.0 and thus the benefit was not statistically significant (16).

The first combined analysis from the Swedish mammography trials and a later meta-analysis of the existing mammography trial data demonstrated a reduction in mortality of 25–30% in women aged 50–69 years as a result of mammography screening (17, 18). These results generated wide debates on the efficacy of screening programs, on the methods used, and on the interpretation of the data, but an updated overview of the Swedish randomized controlled studies confirmed that the benefit of screening in terms of reduced breast cancer mortality persisted after a long-term follow-up (19).

However, the presently used screening model is not optimal. The mortality reduction is achieved at the expense of significant socio-economic costs and it causes side effects, especially in terms of false-positive results leading to other noninvasive or invasive procedures. To prevent one death from breast cancer at least a 500 women need to be screened for ~10 years. Therefore, new innovations are needed, especially innovations aiming at better selection for imaging studies, thus improving the cost-effectiveness of screening.

A protein-based screening method requires access to body fluids such as blood, urine, or saliva. The method should present with a high sensitivity (the proportion of positives that are correctly identified by the test) and a high specificity (the proportion of negatives that are correctly identified by the test). Such requirements are difficult to meet and need in addition extensive internal and external quality assurance of the assay in question. The requirements for sensitivity and specificity vary according to the cancer disease being studied. A breast cancer protein-based screening test could be

applicable even though it has a low specificity as long as the sensitivity is high because individuals testing positive, whatever being a true- or false-positive test result, could subsequently be referred to mammography. We would now have reduced the number of women who need mammography without losing those patients with breast cancer, because the sensitivity of the protein based test was high. Such a test could be classified as a pre-mammography test.

The DBCG prospective blood sample collection including both women with benign and with malignant breast diseases represents a very valuable source of material for studies aiming at the identification of new screening markers. However, it should be emphasized that very strict standard operative procedures for blood sampling and storage have to be in place to limit the influence of factors not related to the main question of disease/no disease. For example, what would be the effect of the menstrual cycle, age, food intake, drug intake, exercise, etc. on the blood level of the particular protein of interest? We also need to know the potential effects on the protein of freeze/thawing cycles and storage time at 4 °C, −20 °C, or −80 °C.

Clinical validation studies aiming at obtaining the needed evidence for the use of a screening marker for breast cancer could also be administered by DBCG, because DBCG has the direct contact with the Danish breast cancer screening centers where mammography is being offered to Danish women age >50 on a biannual schedule.

ADJUVANT TREATMENT OF PRIMARY BREAST CANCER

Nearly 100 years ago Halsted raised the hypothesis that breast cancer could spread first by direct extension into contiguous tissue and then subsequently through the lymphatics to the rest of the body (20). Therefore, extensive locoregional treatment was considered to be necessary to achieve cure, catching all the cells before they could infiltrate locally or break through the nodal filter. In the 1960s, studies by Fischer and coworkers demonstrated that lymph vessels and blood vessels were interrelated and both systems could serve as the route of neoplastic dissemination (21). It also became apparent that in many patients micrometastases already existed at the time of diagnosis (22, 23) and that extensive locoregional treatment could possibly achieve cure only in the minority of women not harboring such micrometatases. In addition, by that time the responsiveness to cytotoxic and endocrine agents had been observed in clinically overt metastatic breast cancer, and in experimental studies an inverse relationship between the size of the tumor and its curative response to cytotoxic agents was documented (24).

These were the rationales for the introduction in the early 1970s of the adjuvant systemic therapy offered to certain risk groups following the primary locoregional treatment (surgery ± radiotherapy) of breast cancer. The majority of the first generation adjuvant studies used either chemotherapy with CMF (cyclophosfamide, metotrexate, and fluorouracil) given for approximately 1 year or endocrine therapy with the antiestrogen tamoxifen continued for 1–2 years. Meta-analyses demonstrated a 17% reduction in the risk of death with chemotherapy, ranging from 25% in the patients less than 50 years to 10% in patients over the age of 60 (25). Tamoxifen, used primarily in postmenopausal patients, reduced the risk of death by ~11–18% with 1 and 2 years treatment duration, respectively (26). The next generation of trials introduced antracyclines as a cytotoxic agent, which led to another 10% reduction in the risk of death (27), as did a prolongation of the duration of tamoxifen to 5 years (28).

During the past ~5 years, a large number of third generation trials have been initiated using taxanes and the third generation aromatase inhibitors. The interpretation of two studies of sequential AC (doxorubicin, cyclophosphamide) + paclitaxel has been made difficult by the confounding of duration, receptor status, and concomitant administration of tamoxifen. However, TAC (docetaxel, doxorubicin, and cyclophosphamide) has proved to be superior to FAC (fluorouracil, doxorubicin, and cyclophosphamide) but at the expense of significantly increased toxicity. The taxanes are definitely active in the adjuvant situation but whether the taxanes should replace or be added to anthracyclines still remains controversial (29). With aromatase inhibitors, 5 years of anastrozole proved superior to 5 years of tamoxifen in a 17% reduction in the risk of recurrence (30), and a recent study demonstrated a 41% reduction in the risk of recurrence at 2.5 years when patients following adjuvant tamoxifen for 5 years were randomized to another 5 years of the aromatase inhibitor letrozole *versus* placebo (31). No survival data are yet available from these two studies.

Today, adjuvant systemic therapy (chemotherapy and/or endocrine therapy) is offered to patients at different risks of recurrence and death, *i.e.* to a prognostically heterogeneous group with risks ranging from 10 to 80%. This group is characterized according to classical prognostic factors (nodal status (positive), and/or size of the primary tumor (>20 mm), and/or malignancy grade (II-III), and/or steroid receptor status (negative), and/or age (<35 years)) (32) and constitutes about 70% of all new breast cancer patients. As described above, 30–40% of the expected deaths can be avoided if adjuvant systemic therapy is offered to this patient group. However, in absolute terms the mortality reduction amounts to only a few percent (*i.e.* from 5 to 4%) in the low-risk group and to ~25% in the high-risk group (*i.e.* from 80 to 55%). Thus, although adjuvant systemic chemotherapy has led to a significant improvement in the survival of the breast cancer population, it also carries the significant adverse effect of over-treatment.

Ideally, the different types of systemic therapy should be offered based on predictive factors, *i.e.* factors that will predict the response to a specific treatment. However, in the adjuvant situation so far only the estrogen and/or progesterone receptor status is a recognized useful predictor of response to endocrine therapy. As a result, patients with recep-

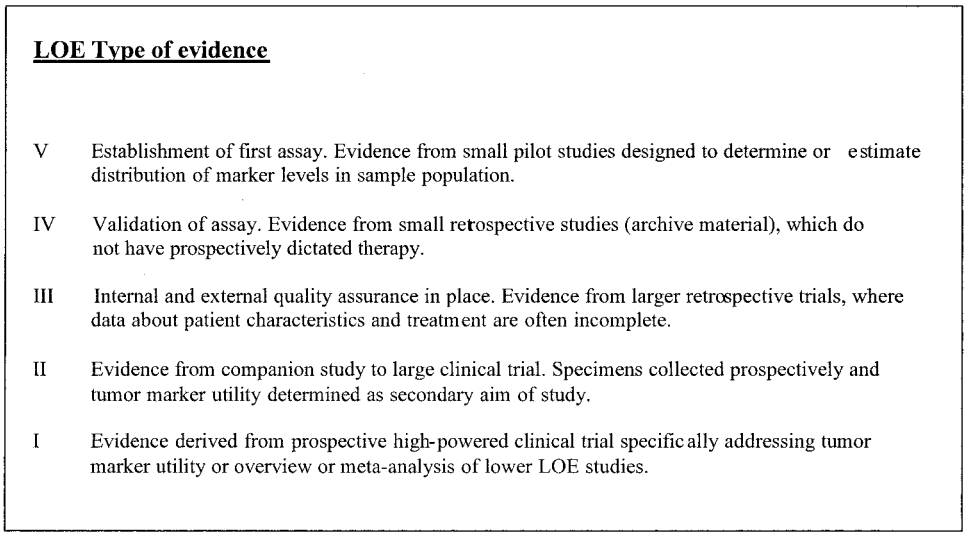

FIG. 4. **Classification of Level of Evidence (LOE) for tumor marker studies.**

tor-negative disease are offered chemotherapy only whereas patients with receptor-positive disease as a standard are offered endocrine therapy combined with chemotherapy in some high-risk subsets (32).

It is well known from the treatment of advanced breast cancer that patients nonresponsive to one specific type of chemotherapy, or endocrine therapy, may react positively to another type of one of the two modalities, indicating that response or lack of response to a specific treatment may relate to specific characteristics (predictive factors) of the tumor. Thus, there is a pressing need to develop new independent prognostic and predictive indicators or signatures in primary breast cancer to improve the selection of patients for specific, ideally tailored treatments (29, 33–38). Among potential markers to predict response to chemotherapy in advanced breast cancer, a recent study has suggested that topoisomerase IIα alterations predict benefit to epirubicin in the adjuvant situation (39).

An overview recently described the benefit of adjuvant radiotherapy in terms of reduction in loco-regional failure, disease-free survival, and breast cancer-specific survival (40). However, it is also apparent from the data that this benefit is achieved at the expense of significant over-treatment, and future studies should be directed at a better selection of patients to receive radiotherapy.

Proteomics may thus be useful in two settings, one being a prognostic indicator and the other being a predictive marker for therapy response. The former will be useful to select those patients in need of additional therapy after the primary surgery or on the other hand to select those patients who are already cured by the primary surgery and therefore do not need additional treatment. A prognostic marker gives the clinician a tool for estimating the risk of disease recurrence for an individual patient following the initial surgical treatment. This is different from a predictive marker, which will foretell how the patient is likely to respond to a given therapy.

Based on the presence of frozen or paraffin-embedded stored tumor material with full clinical follow-up on the individual samples, the DBCG tumor tissue bank has been widely used to evaluate new protein-based prognostic markers (41–43). We have recently shown that also protein determinations performed in preoperative collected blood samples may carry prognostic information (44). Because most of the DBCG patients have been treated and followed according to strict clinical protocols, retrospective validation of new predictive markers will also be possible.

QUALITY CONTROL OF NEW MARKERS

It should be emphasized that thorough validation of new markers are needed before clinical implementation. We have adapted the Tumor Marker Utility Grading System (TMUGS), with the addition of the Level of Evidence (LOE) scale introduced by Hayes and coworkers (45). Fig. 4 shows a slightly modified LOE scale, where we have added establishment and validation of reliable assay conditions taking pre-, peri-, and postanalytical variables into consideration (38, 46). Development and use of tumor marker assays should always meet requirements of Good Laboratory Practice and Good Clinical Practice.

CONCLUSIONS

In conclusion, the structure of the DBCG with close interdisciplinary collaboration between clinical and basic research covers the important elements necessary to conduct high-quality translational research studies. The clinical databases with long-term follow-up in connection with the biobanks allow for the identification of potential new diagnostic, prognostic, or predictive markers and subsequent validation and prospective confirmation studies.

Similar, although generally not nationwide, cooperative

groups have been established in many countries all over the world. In addition many of these groups have established intergroup collaboration. An example is the Breast International Group, which represents collaboration between ~5 national or international cooperative groups.

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¶ To whom correspondence should be addressed: Institute of Pharmacology and Pathobiology, Royal Veterinary and Agricultural University, Ridebanevej 3, 1870 Frederiksberg, Denmark. Tel.: 45-35283130; Fax: 45-3535314; E-mail: nbr@kvl.dk.

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