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Molecular classification of high risk breast cancer as predictor of benefit from dose intensification of adjuvant chemotherapy: Results of randomized WSG AM-01 trial.

Dissertation

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1. Introduction

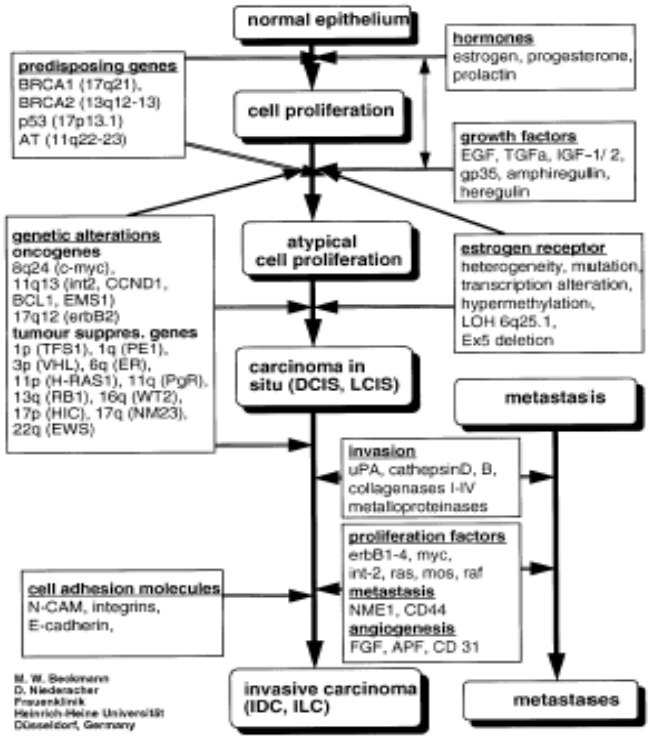
1.1. Overview

Breast cancer (BC) is the most common female cancer. More than 1 mio. women per year are affected worldwide by this diagnosis and 400.000 patients per year die from it. In Germany there are about 47.000 new cases/year with annual mortality rate of about 17.000[1]. However the mortality and incidence in developed countries has decreased in the past 5 years due to implementation of screening, improvement of adjuvant systemic treatments and decreasing use of hormone replacement therapy (HRT)[2], but the incidence of breast cancer worldwide is still increasing.

Multiple factors, such as family history, genetic mutation (e.g. BRCA 1 or 2), early menarche (<12 years old) or late menopause (>54 years old), late first pregnancy (after 40 years old), exogenous (e.g. hormone replacement therapy) or endogenous (body mass index >25 in the menopause) female hormones, benign breast diseases (e.g. epithelial hyperplasia) radiation or environmental factors are playing a crucial role in the development of the BC[3]. Most breast cancers have invasive ductal histology and have their origin in ducts of the mammary gland. Other subtypes are invasive lobular, medullary, papillary and others..

Although there are many well known preneoplastic lesions, which are described in breast cancer, like ductal or lobular carcinoma in situ (DCIS or LCIS), atypical ductal atypia (equal to low-grade DCIS) and others [4], There is still no widely established model of carcinogenesis for breast cancer and discussion about stem cell or stochastic development of invasive breast cancer is still ongoing. Figure 1. displays only one of many possibilities of breast cancer appearance, as proposed by Beckmann et al.[5]

Fig. 1. Carcinogenesis of breast cancer



Prognosis of breast cancer patients is dependent on several clinical-pathological prognostic markers, such as tumor size, number of involved lymph nodes, nuclear grade, age. Molecular features, such as UPA-1/PAI, hormone and Her-2/neu receptor status are critical determinants of long term survival. They provide classification of breast cancer in three prognosis groups (low, intermediate and high risk), which guide adjuvant therapy in Europe[6] (St. Gallen Criteria).

1.2. Prognosis of patients with high risk breast cancer

Patients with multiple positive lymph nodes have a very poor prognosis. The average annual mortality rates in patients with ≥10 involved lymph nodes (LN) are five times higher than for N0 patients[7].

A retrospective study from two academic institutions have identified the 15-year disease-free survival (DFS) without application of adjuvant therapy for the subgroup with >10 positive lymph nodes as 17%, this result could be improved by adjuvant athracycline-based chemotherapy and tamoxifen by 10%[8]. Similar data could be obtained from the Natural History Database, this contains information on 1199 T1/T2 tumors with >10 metastatic lymph nodes treated only by surgery. The 5-year DFS and OS rates were estimated by 29% and 44% respectively[9]. A second study investigated the prognosis of 1401 patients with similar characetristics, who did not receive any systemic therapy. 5-

year OS rates were presented for 3 groups by the number of affected lymph nodes: (11-15 LN) 55%, (16-20) 48% and (>21) 39%, respectively[10].

Generally an adjuvant poly-chemotherapy improves breast cancer outcome in terms of both relapse by 23% and mortality by 17%, [11], but subgroup analysis from randomized trials shows that in this high-risk subgroup few standard anthracycline-based regimens achieve 5-year EFS exceeding 40-52%[12-14].

Taxane-based combinations may improve 5-year DFS by further 5% and OS by 3%, as shown by a review of 13 randomized trials[15]. Although no groups with more or less benefit could be identified by the meta-analysis, the impact from taxanes in the HRBC subgroup remains unclear on the basis of published retrospective, unplanned subgroup analyses from trials evaluating third-generation taxane combinations. Both positive for overall survival (OS) trials have found no benefit for sequential or concomitant taxane-based therapy in these patients[16, 17] compared with the most pronounced advantage in the group with 1-3 positive LN. Only one phase II trial (n=61 patients) investigated the impact of taxane based ET-CMF chemotherapy in 61 patients with >10 LN and found 5-year DFS of 60%[18].

1.3. Principles of adjuvant chemotherapy

The relapse-free survival (RFS) of adjuvant treated breast cancer (BC) is determined by (1) the size of subclinical residual tumor burden (RTB) and (2) growth curve trajectory for the residual tumor in that patient. The RTB in by adjuvant therapy treated patients depends further at RTB after surgery (it would mean, that patients with more involved LN's have also subsequently more micrometastasis), dose and schedule of treatment and sensitivity of tumor cells to treatment[19].

Preclinical experiments have identified, that anticancer effect of chemotherapy is dependent on a dose-response relationship. It would mean, that greater tumor burden also requires higher doses of chemotherapy due to log-kill hypothesis investigating in murine leukemia cells (L1210) by Skipper and Slabel, which builds a basis for modern principles of adjuvant chemotherapy in cancer[20].

This hypothesis has been supported in breast cancer by clinical data of Peters et al, published 1993[21]., At the same time several clinical studies have shown, that this dose-response curve is not linear. It means, that clinical benefit doesn't rise consistently and proportionately in response to escalated doses, but is dependent on the growth fraction of tumor, which is not so linear, as proposed by Skipper and Slabel, but rather it follows

Gomperthezian growth function, describing a more rapid growth in smaller tumors and slower in greater tumor size, reaching a plateau (but in most cases after lethal excess), as described by Norton and Simon in 1986[22].
In conclusion it could be stated, that dose size (to reduce number of residual tumor burden, possible resistant to doses of chemotherapy administrated with normal ranges) and dose dense (to reduce the time for tumor re-growth between cycles of chemotherapy and subsequently to profit from relatively faster tumor re-growth of smaller number of tumor cells, which is the working point of chemotherapy) are both critical determinates of clinical chemotherapeutical effect.

1.4. Results from trials with high-dose and dose-dense regimens in HRBC.

Dose size and density are critical variables of chemotherapy, that is based on log-kill hypothesis by Skipper and Slabel, [20]where dose-response relationship is described as greater tumor burden needing higher doses of chemotherapy. This hypothesis has been supported by Peters et al. data who have presented very impressive short-time event free survival (EFS) of 72% after 2,5 years of median follow up after high-dose chemotherapy (HD) with autologous bone marrow transplantation[21] in patients with HRBC, so that a number of randomized trials investigating a HD in HRBC as single shot (consolidations principle like in leukemia) have been planned in mid 1990-s. It will be intensively discussed, whether linear or Gomperthezian growth curve of tumor cells and related therapeutical efficacy are playing the most important role in outcome of patients[22].

Up to now, findings from the trials investigating the effects of HD with stem cell or autologous bone marrow support in this high-risk group remain controversial. Designs of investigated approaches and patient's selection criteria are different in these trials.
Some studies investigated a classical "phase III design" comparing a single HD with some kind of actual standard. Rodenhuis et al compared 5 cycles of FEC followed by single course of HD (CTCb) or one further cycle of FEC in 885 patients with more than 4 positive lymph nodes and have shown a non-significant trend to better efficacy of HD in terms of DFS and OS. The prospectively predefined subgroup of patients with more than 10 lymph nodes had a significant benefit from HD[13]. Roche et al compared 4xFEC followed by single HD (CMA) or no further treatment in 314 patients with >7 involved LN. This study has shown a significant improvement of DFS for HD group after 3,25 years of median follow up[23]. The second German group headed by Axel Zander compared 4xEC

followed by one cycle of HD or 3 cycles of CMF in patients with more than 10 positive LN with a non-significant advantage for HD group in terms of both DFS and OS after 6 years of follow up[24]. 6 cycles of CEF followed by single STAMP V cycle or no treatment were compared by Tallmann et al. in 511 HRBC patients[14]. This study, marked by a very high protocol violation rate of 23%, displayed non-significant better EFS in the HD arm and comparable OS rates after 6 years of median follow up. Further two studies of Leonard et al. (4xE-8xCMF vs. 4xE-1 cycle HD (C-CT) in 605 patients with more than 4 LN)[25] and of Coombes et al. (6xCEF vs. 3xCEF-1 cycle of CTCb in 281 HRBC patients with >4 positive LN)[26] have shown similar DFS and OS rates in both study arms.

Second group of trials compared HD regimens with dose-intensive standards. The Scandinavian trial compared after a standard anthracycline induction a single STAMP V HD course versus individually (according to WBC count) escalated FEC in 525 HRBC patients with 4,3 times higher single doses of anthracyclines in the control arm. DFS is in favour of the control arm and OS rates are comparable after 5 years of median follow up[27]. An Intergroup study leaded by Peters investigated two dose levels of an identical combination the one given as an dose intensified standard the other given with stem cell support in 785 HRBC patients after an identical induction. A very high therapy related lethality of 9% makes therapeutical considerations very difficult. EFS rates after 7,3 years of median follow up are in favor of HD arm, OS rates are comparable[28].

Other trials like our and IBCSG used a multicycle high dose approach in HRBC patients compared with antracycline-based standard dose or dose dense (Dd) therapy. Basser et al. investigated conventional 4xEC followed by 3xCMF vs. 3 cycles of HD (HD-EC) in 344 HRBC patients. They have shown a significant benefit in DFS for patients with more than 10 LN after 5,8 years of median follow up[29]. Up to now, our previously reported WSG-AM-01 is the only study demonstrating that the tandem HD arm q3w after 2 DD cycles EC 2qw compared to DD EC/CMF q2w therapy was significantly associated with reduced risk of both relapse and death[30] after 48,5 months of median follow up in 403 patients with >9 positive LN. This trial incorporated both early dose-intensification and dose-dense concepts of adjuvant chemotherapy within the experimental arm.

Recently presented meta-analysis of 15 trials in 6210 patients after 6.2 years of median follow up indicates only a little non-significant benefit of HD in term of overall survival ($p=0.12$), breast cancer specific survival ($p=0.1$) and a modest but significant benefit in RFS ($p<0.001$)[31].

At the same time several trials have shown no additional benefit for simple dose intensification of single agents. So NSBAP B-22 and B-25 have shown no statistically significant additional benefit from dose intensification of cyclophosphamide beyond 600 mg/m^2 (1200 and 2400 mg/m^2)[32, 33]. Cancer and Leukemia Group B 8541 trial have previously reported no additional clinical effect of increasing anthracycline dose by 50% (from 60 mg/m^2 to 90 mg/m^2)[34]. But the dose of 60 mg/m^2 has been shown as superior to 30 and 40 mg/m^2 in CALGB B 8541[35]. However numerous trials indicated the dose of anthracyclines of 100 mg/m^2 to be superior to lower doses of 50 and 60 mg/m^2 respectively[36, 37], particularly in patients with more than 4 positive lymph nodes[36].
In reference to the DD chemotherapy there are several published trials, which indicate benefit for DD concept in early breast cancer. So, CALGB 9741 has shown a significant benefit for 2-weeks schedule (EC– Taxol) over 3-weeks in terms of both DFS and OS in women with node-positive BC[38]. Moebus et al. reported a significant benefit for dose-dense (q2w) and -intensive ETC vs. conventional EC-Taxol q3w in patients with >4 LN for both DFS and OS after 5 years of median follow up[39]. Venturini et al. have compared 6xCEF every 3 weeks versus the same regimen administrated every 2 weeks in node-positive or high risk node-negative BC. After median follow up of 10.4 years there is a non-significant trend to better both DFS and OS within the dose-dense arm, where unfortunately a low dose 60 mg/m^2 epirubicine was used[40]. Very interesting, recently published first results of MA 21 trial provide evidence, that dose-intensive FEC is similarly effective as dose-dense EC-Taxol after 30,4 months of median follow up in 2011 patients with node-positive or high risk node negative BC. Both regimens were significantly more effective as conventional EC-Taxol every 3 weeks[41].

1.5. Predictive factors

However EBCTG analysis has addressed the most impact of adjuvant chemotherapy to younger patients with HR negative disease (the 5-year gains from CT would be about twice as large for HR-poor disease as they are for tamoxifen-treated HR-positive disease)[11]. Berry et al. reported a meta-analysis from three CALGB trials and showed 55% reduction of recurrence and death rate in node-positive, HR-negative patients for 2-week EC-T schedule, compared with low-dose CAF, used in the first 8541 study. For similar HR positive tumors, the risk reduction was only 26%. Older patients seem to have the same benefit from adjuvant CT in the later published analysis from the same database[42].

In the HD setting the discussion about predictive markers seems to be more complex. Published trial designs are very heterogeneous in terms of regimens, HD strategy, control arms, and patient selection criteria. To date, the predictive value of established prognostic factors such as grade, estrogen receptor (ER), tumor size, Her-2/neu or age is still under debate. All subgroup analysis from the trials are of retrospective and unplanned nature. Younger age of patients [13, 24, 28, 30, 43], HR positive[13, 29, 44] or negative[30] disease, Her-2/neu negative status[45], p-53 overexperession[45, 46] are possible markers of particular benefit from HD, but there is no consensus about their value.
Considering the heterogeneity of the published data, facts of scientific manipulations in this field, costs and toxicity of this therapy, the aim should now be to identify promising HD strategies and biological tumor subtypes deriving maximum benefit from HD.

1.6. Molecular basis of disease

There is increasing evidence that breast cancer is a heterogeneous disease and will be classified in distinct biological subgroups implicating significantly different prognosis.
Gene expression studies based on DNA-microarray analysis have enabled molecular subtyping of breast tumors with distinct patterns of proliferation, apoptosis, and DNA-repair as well as with distinct prognostic implications[47]. Two major subtypes of breast cancer were noted on the expression levels of 496 cDNA's (an intrinsic gene subset). The first subtype contained tumors that were clinically described as ER positive, and the second by tumors that were mostly ER negative. The ER positive tumors were distinguished by the relatively high expression of genes of breast luminal cells, whereas ER negative were characterized by the gene expression of basal/myoepithelial cells. The latter group was subdivided into three different groups, basal-like, erbB2 positive and normal breast-like.
Luminal A, hormone receptor high expressing tumors have better prognosis than luminal B tumors. Her-2/neu and triple-negative (ER-/PR-/Her-2/neu-) /basal-like subtypes have worse outcome, compared with both luminal subtypes. Follow up studies have shown these subtypes to be conserved across diverse patient collectives and array or protein expression platforms. In the same time diverse supervised gene expression based predictors of survival, as supervised variable where developed[48, 49] with only single genes as overlap between signatures. Recently published study by Fan et al. identified similar pathways in all used signatures and has shown a significant agreement between molecular classification and gene prognostic tools in predicting patients outcome. Basal-

like and Her-2/neu subtypes where presented by high risk genomic signatures in both 70 gene Amsterdam signature and 21 Recurrence Score[50].

Among the subtypes basal-like/triple negative breast cancer has drawn particular attention, because of possible absent treatment options beside of chemotherapy, what makes the management of triple-negative tumors a clinical challenge, due to limited therapeutic options in this subtype. Approximately 15%-25% of breast cancer are basal-like and are associated with poor survival[51, 52]. A recent published study has shown that this subtype is more frequent among African American women, which may contribute to the poor outcome in this collective. Hereditary BRCA-1 breast cancers also resemble sporadic basal-like tumors[53, 54] and are mitotically active high-grade tumors, expressing cytokeratines 5 /17 and EGFR, and associated with younger patient age[55]. The triple-negative breast cancer are associated with higher risk of early distant recurrence[56], particularly visceral metastasis[37, 57]. There are controversial data on the loco regional relapse risk in triple-negative BC[57, 58].

But the optimal distribution of triple-negative (characterized by ER/PR/Ner-2/neu negative phenotype) and basal-like (based on microarray data or multiple protein expression sets) remains unclear.

Recent studies suggest higher chemosensitivity of this subgroup to neoadjuvant chemotherapy[59, 60], associated with pCR rates of 27%[60] to 45%[59] to standard anthracycline/taxane based neoadjuvant chemotherapy compared with only 6% in luminal tumors.

However those triple-negative breast cancers, which did not have complete response, had the highest rate of relapse. In the recent study from the MD Anderson Cancer Center 255 patients treated by neoadjuvant chemotherapy had higher pCR rates (22% vs 11%) than 863 patients with non-TN tumors, but significantly decreased DFS and OS rates. If pCR could be achieved there were no survival differences between both subgroups[58, 60].

Currently, there is only limited experience with regard to the response of molecular subtypes to different cytotoxic therapies[59], but ER and growth factor such as Her-2/neu and EGFR play a key role in both molecular classification of breast cancer[61] and in prediction of chemotherapy effects[11, 42, 62].

The aim of our study was to evaluate the benefits from HD in clinically relevant subgroups, particularly triple negative patients, utilizing tumor samples from a

retrospective, central-pathological blinded review with updated follow-up of the randomized prospective WSG AM 01 trial in HRBC.

2. Methods

2.1. Patients

Patients included in our analysis participated in the randomized multicenter WSG-AM01 trial, which compared tandem HD versus DD conventional chemotherapy. Eligible patients were 18 to 60 years old with Eastern Cooperative Oncology Group performance status less than 2, had histologically proven breast cancer and ≥9 positive axillary LN. Breast-conserving surgery or mastectomy, both with free margins, and resection of at least ten axillary lymph nodes was required before randomisation. Absence of distant metastases was verified by normal findings in chest X-ray, liver ultrasonography, and bone scan. An inadequate psychological cardiac, pulmonary, hepatic, renal and haematopietic functions, serum bilirubin concentration more than 34·2 mmol/L, serum creatinine concentration out the normal range (as defined by each institution), leucocyte count less than 3·5_109/L, and platelet count than 100_109/L were exclusion criteria, as well as a history of previous cancer other than cervical carcinoma-in-situ or basal-cell carcinoma or other serious disease or uncontrolled infection.

2.2. Treatment

Patients were assigned either two cycles of epirubicin and cyclophosphamide followed by tandem epirubicin and cyclophosphamide plus thiotepa with autologous peripheral-blood-progenitor support (high dose chemotherapy) or four courses of epirubicin and cyclophosphamide, followed by three courses of cyclophosphamide, methotrexate, and fluorouracil given at intervals of 2 weeks with growth-factor support (dose dense conventional chemotherapy).

For high-dose chemotherapy, patients received an induction regimen with two courses of epirubicin and cyclophosphamide. Epirubicin was given as a 1 h infusion at a dose of 90 mg/m2 followed by cyclophosphamide (1–2 h infusion) at a dose of 600 mg/m2. Intravenous mesna at 20% of the cylophosphamide dose was given at the same time as the cyclophosphamide infusions then 4 h and 8 h later. The therapy was repeated after 2 weeks with growth-factor support (Filgrastim 5 g/kg body weight) from day 5 to day 12. After the second course, Filgrastim was given at a dose of 5 µg/kg from day 2 onwards. Peripheral-blood stem cells were collected around day 8–10, depending on peripheral

CD34-cell counts. Around the collection time, the Filgrastim dose was adapted individually, depending on peripheral CD34-cell counts. Different study sites had different strategies of Figrastim dosing and administration before stem-cell collection. For all patients, a minimum of 2x106 CD34- positive cells per kg bodyweight for each transplant and an identical back-up sample were stored. 4 weeks after the start of chemotherapy, patients were scheduled to receive the first cycle of high-dose chemotherapy, which was repeated 3 weeks later. Treatment delay was at the discretion of the investigating clinician. Complete haematological reconstitution was not mandatory for the start of the second course.

For each cycle of high-dose chemotherapy, patients received via central venous access epirubicin 90 mg/m2 on day –5 (1 h infusion) followed by cyclophosphamide 1000 mg/m2 on days –5 to –3 before transplantation (2–3 h infusion, total dose 3000 mg/m2), followed by thiotepa 133 mg/m2 on days –5 to –3 (2–3 h infusion, total dose 400 mg/m2). Intravenous mesna at 20% of the cyclophosphamide dose was given with the cyclo-phosphamide infusions then 4 h and 8 hours later. Peripheral-blood stem cells were given on day 0 and Filgrastim treatment started on day 1 after transplantation. According to the protocol, total doses of 360 mg/m2 epirubicin, 7200 mg/m2 cyclophosphamide, and 800 mg/m2 thiotepa were planned over 11 weeks.

Dose-dense conventional chemotherapy consisted of four courses of epirubicin and cyclophosphamide, followed by three courses of cyclophosphamide, methotrexate, and fluorouracil. Epirubicin was given as a 1 h infusion at 90 mg/m2, followed by cyclo-phosphamide (1–2 h infusion) at 600 mg/m2. The second regimen consisted of cyclo-phosphamide (1–2 h infusion) at 600 mg/m2, methotrexate at 40 mg/m2 as a short infusion, and .fluorouracil (1 h infusion) at 600 mg/m2 with conventional mesna support. All courses were given with intervals of 2 weeks with Filgrastim support (5 _g/kg) from day 5 to day 12. The total planned doses were 360 mg/m2 epirubicin, 4200 mg/m2 cyclo-phosphamide, 120 mg/m2 methotrexate, and 1800 mg/m2 .fluorouracil over 14 weeks.

4 weeks after completion of chemotherapy, all patients underwent radiotherapy of the chest wall, breast, and infra-clavicular and supraclavicular fossa at conventional doses (50 Gy and 50 Gy plus 10 Gy boost for the breast) and fractions (2 Gy/day). Tamoxifen (20 mg daily for 5 years) was started in all patients with hormone-receptor positive disease or unknown hormone-receptor status after the completion of chemotherapy.

Patients were reassessed every 3 months during the first year, every 6 months during the second year, and thereafter every 12 months. At each visit, a history was taken and

complete clinical examination and blood chemical studies were done. After breast-conserving surgery, mammography was done at 6 months and 12 months and then once a year. Chest radiography and liver ultrasonography were done every 6 months during the first 2 years and then every 12 months. Bone scans and further radiological examinations were done only if there were clinical symptoms. Treatment-related mortality and morbidity were assessed at the time of the first follow-up.

2.3. Prognostic factors and histopathological analysis

At time of diagnosis, the following baseline characteristics were determined: age, histopathology, tumor size, grade, number of examined and positive lymph nodes, and ER/PR status.

2.3.1. Tumor samples

Paraffin embedded tumor blocks were requested from all 403 patients participating in the trial. Representative sections from 252 of these (63%) were received. Sufficient primary tumor tissue was available in 236 (59%) tumor samples; these sections were reviewed and analyzed at our central laboratory for specific morphologic features, including grade (based on the criteria of Elston and Ellis)[63] and vascular invasion.

2.3.2. Tissue microarrays and antibodies for immunohistochemistry

Breast cancer TMA were prepared as follows: representative tissue blocks were selected as donor blocks for the TMA. Sections were cut from each donor block and stained with hematoxylin and eosin for detecting of a representative area. Using these slides, one morphologically representative region was chosen from each of the 236 tumor samples. One cylindrical core tissue specimen per tumor block (diameter = 2.0 mm) was punched from these regions and precisely arrayed into a new recipient paraffin block (20·35 mm) using a custom-built precision instrument (Beecher Instruments, Silver Spring, Md., USA). Distance between single core specimens was 0.8 mm. Nine tissue array blocks were prepared, seven containing 30 and two containing 16 tumor sample cores, respectively. Each block was subsequently stained with hemotoxylin and eosin to verify presence of tumor within each 2.0 mm tissue core. All sample cores had definitive places in the recipient block, and a plan of cores noted by study patient number was entered in the Excel table (Microsoft Office 2000).

2.3.3. Immunohistochemistry (IHC)

For clustering analyses, we used a large panel of 34 protein markers (table 1). Most of the proteins selected, play a well-established role in breast carcinogenesis[47, 64]. Furthermore, the gene transcripts of these proteins have been reported to be important candidate discriminator genes in stratifying breast cancer into distinct groups based on previous cDNA microarray and protein expression studies[47, 65, 66].

Table 1. Proteins stained on tissue micro arrays.

Proteins	Antibodies	Origin	Clone	Dilution	Staining pattern
ABCA3	Rabbit			1:800	Cytoplasmic
BCL2*	Mouse monoclonal	DAKO	124	1:50	Cytoplasmic
BCRP*	Mouse monoclonal	Chemicon	BXP-21	1:100	Cytoplasmic/membranous
β-Catenin*	Mouse monoclonal	BD Trans-duction	14	1:200	Membranous
C-kit *	Rabbit polyclonal	DAKO	Code A4502	1:200	Cytoplasmic/membranous
CK 5*	Mouse monoclonal	Novocastra	XM26	1:600	Cytoplasmic
CK 8*	Mouse monoclonal	BioGenex	C-51	1:5000	Cytoplasmic
CK 17*	Mouse monoclonal	DAKO	E3	1:20	Cytoplasmic
COX-2	Mouse monoclonal	Cayman		1:400	Cytoplasmic
CXCR4*	Mouse monoclonal	Zymed	12G5	1:100.	Cytoplasmic Nuclear
Cyclin D1	Rabbit monoclonal	DCS	SP4	1:50	Nuclear
Cyclin E	Mouse monoclonal	Novocastra	13A3	1:50	Nuclear
E-Cadherin*	Mouse monoclonal	Novocastra	36B5	1:50	Membranous
EGFR*	Mouse monoclonal	Merck	E30	1:100	Cytoplasmic/membranous

ER*	Rabbit monoclonal	DCS	SP1	1:800	Nuclear
Erk1/Erk2*	Rabbit monoclonal	Cell Signaling	p44/42 MAPK (20G11)	1:400	Cytoplasmic/nuclear
ET-1*	Mouse monoclonal	Alexis	Antiendothelin-1	1:200	Cytoplasmic
ETR-α	Sheep polyclonal	Alexis	ET-$_{\alpha}$-R Antiserum	1:200	Cytoplasmic
ETR-β*	Sheep polyclonal	Alexis	ET-$_{B}$-R-Antiserum	1:200	Cytoplasmic
FHIT	Rabbit polyclonal	Zymed	ZR44	1:150	Cytoplasmic
HER-2*	Rabbit polyclonal	DAKO	c-erbB-2	1:500	Membranous
MGMT	Mouse monoclonal	Neomarkers	MT3.1	1:250	Cytoplasmic/nuclear
MIB1/Ki-67*	Mouse monoclonal	DAKO	MIB1	1:1000	Nuclear
MUC-1	Mouse monoclonal	Novocastra	Ma695	1:100	Membranous/cytoplasmic
p16*	Mouse monoclonal	Neomarkers	16P07	1:50	Nuclear
p27	Mouse monoclonal	Novocastra	1B4	1:50	Nuclear
p53*	Mouse monoclonal	Oncogene	DO-1	1:500	Nuclear
p63*	Mouse monoclonal	BD Pharmingen	4A4	1:200	Nuclear
PR*	Rabbit monoclonal	DCS	SP2	1:800	Nuclear
pTEN*	Mouse monoclonal	Santa Cruz	A2B1	1:50	Cytoplasmic
Synaptophysin	Rabbit polyclonal	DAKO	A0010	1:200	Cytoplasmic

S6*	Rabbit polyclonal	Cell Signaling	S6 ribosomal protein antibody	1:400.	Cytoplasmic
Topo-IIα*	Rabbit polyclonal	Novocastra		1:200	Nuclear
Vimentin*	Rabbit monclonal	Biogenex	V9	1:20.000	Cytoplasmic

* used for clustering analysis

Related pathways are displayed in table 2.

Table 2. Pathways of examined markers.

proliferation/differentiation status of the mammary gland and tumor cell	cellular origin	oncogenes or tumorsuppressors	markers for metastasis	multidrug resistance markers
ER-α, PR, Synapto-physin, MUC1, BCL2, Ki67/ MIB1, Cyclin D1, Cyclin E, p27, topo-IIα, COX-II, ERK1/ ERK2, S6	CK5, CK8, CK17, Vimentin	HER-2, EGFR, p53, p63, E-cadherin, FHIT, pTEN, ß-Catenin, p16, c-kit	Endothelin-1 [ET-1], Endothelin-α [ETR-α], and β-receptor [ETR-β], CXCR4	BCRP, ABCA3, MGMT

Immunohistochemical staining was performed on 3 µm paraffin sections. Pretreatment for antigen retrieval was mainly done by pressure cooker except for EGFR and S6, where pronase and autoclaving was used, respectively. Dilutions of the antibodies are listed in table 1. After blockage of biotin (by avidin-biotin) and peroxidase (by H_2O_2), immunohistochemical staining was performed on an automated immunostainer (Biogenex, i6000, San Ramon, California) using a standard labeled streptavidin-biotin method (UltraTek Reagent Detection Kit, Scy Tek, Logan, Utah) followed by 3,3 V-diaminobenzidine enzymatic development. Sections were counterstained blue with hematoxylin. Omission of the primary antibody as well as antiisotype antibodies served as negative control.

2.3.5. Immunohistochemistry scoring

Staining results were assessed by one pathologist and were reevaluated randomly by a second pathologist without any knowledge about clinical follow-up. A good correlation (96%) was found between the two observers. Any discrepancies were resolved with a multihead microscope by discussion. The immunostaining of protein markers was compared in 15 cases to approve the correlation between protein expression in the 2 mm core biopsy and whole tumor block among each other. There was concordance among the triplicate scores in 97.3% and concordance between mean score of triplicates and main block of 93,3%, which indicates, that core specimens are representative for the whole tumor.

The scoring for the single marker evaluation was performed according to the literature as follows:

- Bcl-2 (0:none cytoplasmatic staining, 1+: <10% of cells, 2+: 10-50% of cells, 3+:>50% of cells. 1-3 scores were summarized as positive for statistical analysis),
- c-kit (0: none or <10% of tumor cells, 1+: low or moderate level cytoplasm staining in >10% of tumor cells, 2+: strong staining in >10% of tumor cells; 1+ and 2+ were stained as positive),
- COX-2 (0: none cytoplasm staining, 1+: low, 2+: moderate and 3+: strong staining; 2+ and 3+ were stained as positive),
- Cyclin E (in at least 10 fields of view: 0= staining of 0-5% of nuclei, 1+=5-33% of nuclei, 2+: 34-66%, 3+: 67-100%: 1+-3* were stained as positive),
- CXCR4 (determination between nuclear (<30% vs. >30%) and cytoplasmatic staining (moderate or strong staining as positive and low as negative),
- ET-1, ETR-α, ETR- β (0 (none) or 1+ (low cytoplasm staining) as negative and 2+ (moderate) and 3+ (strong) as positive) ,
- FHIT (none (0) staining as negative and 1+ (low/moderate) and 2+ (strong) as positive), p16 (The percentage of positive nuclei was scored as: 0, no positivity; 1, up to 25% positive; 2, 26–50% positive; 3, 51–75% positive 4, 76-100% positive. Intensity was scored as 1+ weak, 2+ moderate and 3+ strong positive. The two scores were added to obtain a total score. Total score of 0 and 1 was scored as negative and 2-7 as positive) and p27 (0= none expression, 1+ in 1-10% of tumor cells, 2+: 10-49% of tumor cells, 3+: 50-100% of tumor cells. Intensity of staining was scored as follows: 1: weak, 2: moderate, 3: strong. The two scores were

multiplied, cases with product 0 and 1 were scored as negative, and >2 as positive) were scored by assigning a proportion score and an intensity score.

- ER and PR were also scored by a proportion and intensity score according to (25), respectively: Intensity of the staining: 1=weakly positive; 2=moderately positive; 3=strongly positive. Proportion of the stained tumor cells: <10%=1, 10-50%=2, 50-80%=3, >80%=4. The intensity score and the proportion score were multiplied to give a final score ranging from 0 to 12, designated as negative or positive as follows: score of 0–3, negative; score of 4–12, positive.
- ABCA3, BCRP, ß-Catenin, Cyclin D1, MGMT, MIB1, MUC-1, p53, p63 and Topo-IIα were considered positive when >10% of the cancer cells unequivocally showed positive staining.
- CK5, CK8, CK17, E-cadherin, EGFR, pTEN, Vimentin, Synaptophysin and S6 were scored positive if any specific staining in the carcinoma cells was observed.
- HER-2, only membranous staining was scored using the HercepTest protocol and a score of 3+ was recorded as a positive result. Cases with 2+ scores were further evaluated by fluorescence in situ hybridization (FISH) to evaluate HER-2/neu gene amplification. HER-2 gene amplification was analyzed by FISH in those cases that had an immuno-histochemical HER-2 score of 2+. Hybridization was performed on 4µm-thick paraffin sections using the Oncor®/ Ventana® INFORM ®HER-2/neu Gene Detection System (Ventana Medical Systems, Frankfurt, Germany).
- ERK1/ERK2 staining was considered positive if a moderate or strong nuclear staining was observed in breast cancer cells. Cases with single or few, focally positive tumors cells were assessed negative.

2.4. Statistical analysis

The primary endpoint of the study was event-free survival, which was defined as survival without recurrence or therapy-related mortality. Overall survival and treatment related toxicity/mortality were secondary endpoints. Statistical analysis was done by both per protocol and intention-to-treat methods.

On the basis of preliminary results from historically controlled trials on adjuvant high-dose chemotherapy of breast cancer, the WSG AM 01 study was originally designed to detect an improvement in event-free survival after 3 years from 50% after dose-dense conventional therapy to 70% in the tandem high-dose group. When the less favorable preliminary results from randomised trials published in 1999 (Bergh) became available, a protocol amendment was effected so that the study would be able to detect a difference of

60% compared with 70% in event-free survival after 3 years. The amendment was effected without any knowledge of the outcome data from the study. 400 patients had to be recruited over a period of 5 years to achieve 80% power to identify an improvement of this size at 5% significance (one-sided because a finding of better event-free survival in the conventional-treatment group would have the same practical consequences as a finding of no superiority of high-dose chemotherapy). A recruitment phase of 5 years was planned and minimum follow-up of 3 years, corresponding to a 5-year median observation time, was required for analysis of event-free survival.

Survival analysis was performed using the Kaplan–Meier method including the log-rank test group comparisons. Uni- and multivariate analyses of EFS and OS used proportional hazard Cox regression models. Multivariate analyses (including treatment interaction analyses) were performed by backward elimination using the Wald test; hazard ratios for borderline significant factors and interactions are included; 95% confidence intervals are reported. All data were analyzed using SPSS for Windows; SPSS, Chicago, IL.

Bivariate correlations of individual markers were measured by Pearson's correlation. Association of markers with triple negativity was assessed by Fisher's exact test.

Cluster analysis (K-means)

K-means analysis was performed to cluster the patients into subgroups according to their protein expression profiles. Clustering was based on the initial scores reflecting the full dynamic range of the data. Five patients were excluded from further analysis because too many scores were missing for technical reasons (loss of tissue cores from the TMA). We applied K-means clustering with K=5 and Manhattan Distance as similarity measure with 25 iterations as implemented in the Genesis Software Package.

3. Results

3.1. Patient population

Between May 1995 and June 2002, 403 patients were randomized to tandem HD (201 patients) or DD conventional chemotherapy (202 patients). From 236 patients (59%), paraffin embedded tumor blocks with primary tumor tissue were retrieved for central pathological review and further immunohistochemical investigations (116 from HD and 120 from DD). Patient and tumor characteristics are detailed in Table 2. The subpopulation characteristics were the same as for the original study population[30].

Histologic breast cancer subtypes, according to the World Health Organization histology typing, included invasive ductal carcinomas (79%), lobular (17%), tubular (3%) and other types (1%). Tumor samples included 15 (6.4%) well differentiated, 127 (53.8%) moderately differentiated and 94 (39.8%) poorly differentiated breast carcinomas.

Table 2. Patient's and tumor characteristics

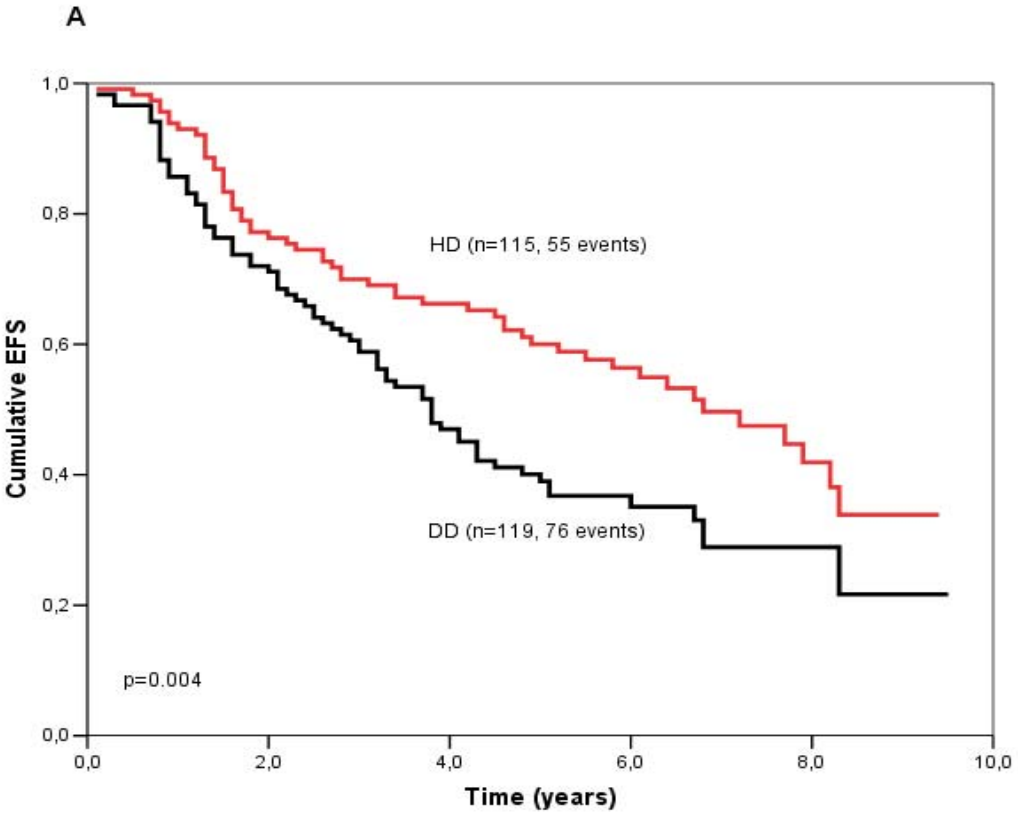
	Tandem HDC		DD-conventional	
Characteristics	N=116	%	N= 120	%
Age (years) • median ± SD	50.1 ± 9.0		48.8 ± 8.5	
Menopausal status • pre • post • unknown	63 48 5	54% 41% 5%	59 58 3	49% 48% 3%
Surgery • breast conserving therapy	39	34%	47	39%
Tumor size (cm) • mean ± SD • median	3.5 ± 2.1 2.8		3.4 ± 2.1 3.0	
Positive nodes • mean ± SD • median	17.3 ± 7.5 15.0		16.9 ±6.9 15.0	
Receptor status	51	44%	49	41%

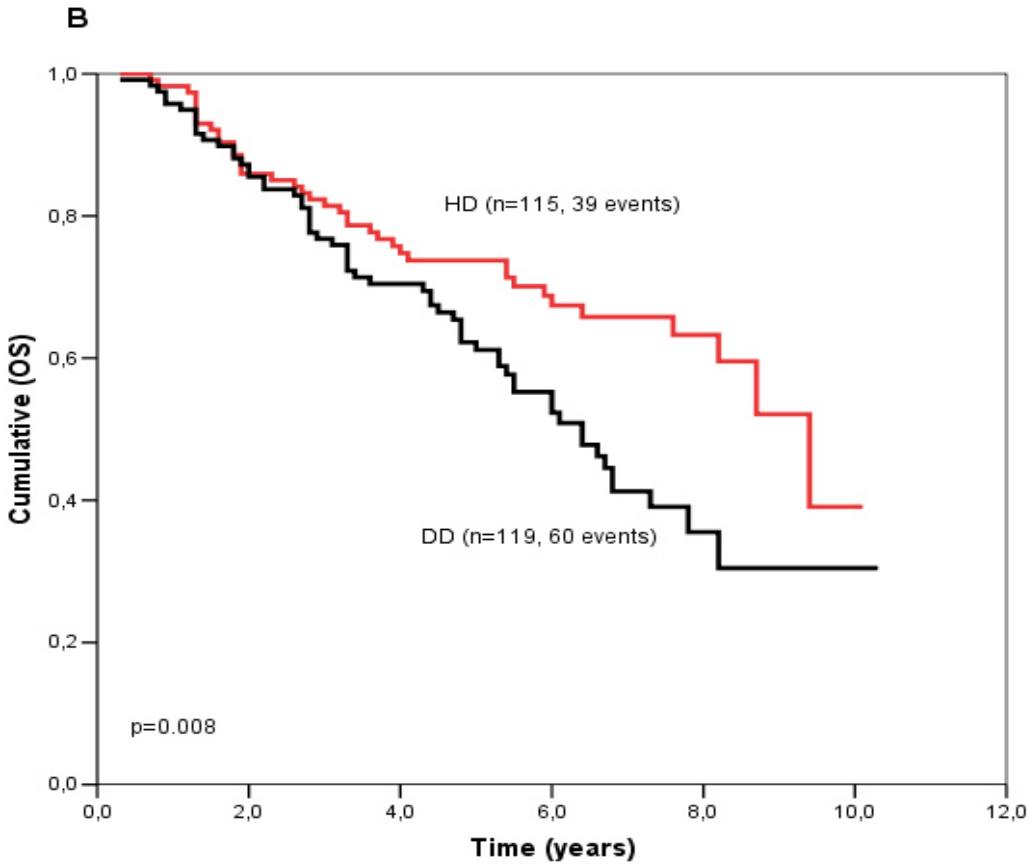
• ER+ • PR+ • ER+ and/or PR+ • Unknown	40 63 2	35% 54% 2%	46 62 4	38% 52% 3%
Grading • G1 • G2 • G3	 4 61 51	 3% 53% 44%	 11 66 43	 9% 55% 36%
Her-2/neu Staining • Positive • Negative • Unknown	 24 88 3	 21% 76% 2%	 19 96 5	 16% 80% 4%
Triple negative	30	26%	36	30%
EGFR Staining • Positive • Negative • Unknown	 12 103 1	 10% 89% 1%	 19 96 5	 16% 80% 4%
MIB-1 Staining • Positive • Negative • Unknown	 69 42 5	 60% 36% 4%	 62 53 5	 52% 44% 4%

3.2. Patient outcome according to study arm

236 patients (116 HD and 120 DD) were followed up until November 2005, leading to a median follow up time of 61.7 months in patients still alive at the time of analysis with a range of 4.2-121.9 months (HD: 67.9 months, DD: 56.3 months). 56 relapses were reported in the HD and 76 in the DD group. Estimated 5-year EFS rate was 62% for the HD group and 41% for the DD; (HR 0.60, 95%CI: 0.43-0.85, p=0.004). A total of 99 deaths were reported (39 in HD; 60 in DD); 5-year OS was 76% in the HD arm and 61% in the DD arm; (HR 0.58, 95% CI: 0.39-0.87, p=0.007) (Fig 2).

Figure 2. Event free (A) and overall (B) survival


A
Cumulative EFS
1,0
0,8
0,6
0,4
0,2
0,0
HD (n=115, 55 events)
DD (n=119, 76 events)
p=0.004
0,0
2,0
4,0
6,0
8,0
10,0
Time (years)


B
Cumulative (OS)
1,0
0,8
0,6
0,4
0,2
0,0
HD (n=115, 39 events)
DD (n=119, 60 events)
p=0.008
0,0
2,0
4,0
6,0
8,0
10,0
12,0
Time (years)

3.3. Correlation of prognostic factors

HR negative tumors as a whole were significantly associated with G3 (60% vs. 23.2, p=.001), as well as over-expression of MIB-1 (67% vs. 52%, p=0.028), Her-2/neu (31% vs. 9.6, p<0.001) and EGFR (28% vs. 4%, p<0.001).

With regard to the clinically interesting triple-negative (TN) subgroup, 66 (28%) patients had triple negative tumors i.e. that stained negative for ER, PR, and HER2. Triple negative tumors were significantly associated with a younger age (median: 45.2 years +/- 9.0 vs. 51.2 +/- 8.1; p<0.001), G3 (57% vs. 31%; p=0.001), and EGFR overexpression (34% vs. 7%; p<0.001).

3.4. Protein expression analysis

236 primary tumors were stained for a set of 34 protein markers by immuno-chemistry on TMA. A total of 7437 (92.7%) of a maximum possible number of 8024 tissue core sections produced interpretable immunostaining. The number of positive cases of the interpretable cases is seen in table 2. Missing immunostaing data was usually caused by loss of a core from the section, less commonly by exhaustion of tumor material or necrosis in the core on deeper sections.

Table 3. Results of proteins immunhistochemical expression.

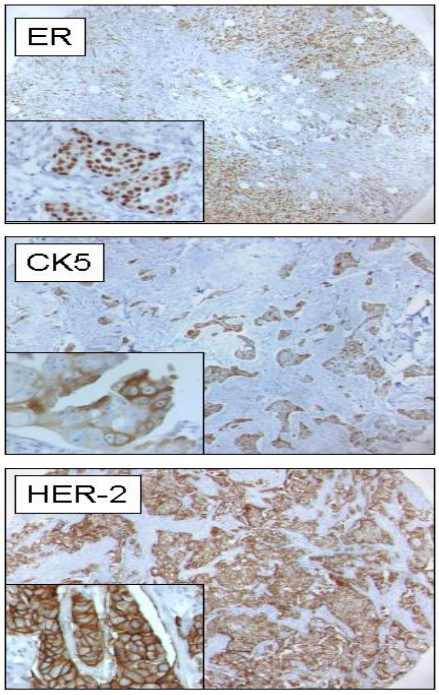
No.	Proteins	N (evaluable)	positive	%
1	ABCA3	201	21	10.4
2	BCL2*	213	67	31.5
3	BCRP*	212	85	40.1
4	β-Catenin*	217	133	61.3
5	C-kit *	219	27	12.3
6	CK 5*	221	20	9.0
7	CK 8*	216	201	93.1
8	CK 17*	220	20	9.1
9	COX-2	215	84	39.0
10	CXCR4*cytoplasmatic	189	108	57.1
	Nuclear	189	22	11.6
11	Cyclin D1	223	116	52
12	Cyclin E	218	23	10.6
13	E-Cadherin*	216	166	76.9

14	EGFR*	224	33	14.7
15	ER*	222	110	49.5
16	Erk1/Erk2*	204	80	39.2
17	ET-1*	202	69	34.2
18	ETR-α	201	102	50.7
19	ETR-β*	197	138	70.0
20	FHIT	215	170	79.1
21	HER-2*	205	40	19.5
22	MGMT	213	131	61.5
23	MIB1/Ki-67*	222	128	57.7
24	MUC-1	225	174	77.3
25	p16*	215	78	36.3
26	p27	217	96	44.2
27	p53*	224	67	29.9
28	p63*	218	4	1.8
29	PR*	218	83	38.0
30	pTEN*	220	43	19.5
31	Synaptophysin	221	8	3.6
32	S6*	197	169	85.5
33	Topo-IIα*	205	65	31.7
34	Vimentin*	209	26	12.4

*markers used for k-clustering analysis

Representative staining of 3 biomarkers is shown in fig. 3 (ER, Her-2/neu, CK 5). For statistical cluster analysis, 5 patients were excluded from the study due to either technical reasons (lack of staining) or lack of representative cancer tissue. In summary, breast cancer samples from 231 patients were analyzed in this study.

Figure 3. Representative staining of ER, Her-2/neu, CK5



3.5. Identification of protein clusters

The protein expression patterns of 231 tumor samples were analysed by k-means clustering and using Manhattan distance as a similarity measure. A clear separation of cases into distinct groups with adequate linkage distances was achieved by calculating different numbers and sets of the protein markers. Finally the most stable subtypes were built using 24 proteins (labeled by * in table 2).

5 subtypes according to the microarray analysis were identified: luminal-A (27%), luminal-B (12%), HER-2 (21%), basal-like (13%) type, and a this far unknown group (27%), which we called the ‘‘multiple marker negative’’ (MMN) group characterized mainly by the strong expression of luminal marker CK8, a lower expression of ER and PR, and the absence of any further specifying markers. Distribution of protein expression in the subtypes is shown in the fig. 4.

Fig. 4. K-mean clustering results. Different shades of red code for increasing levels of immunhistochemical staining intensity.

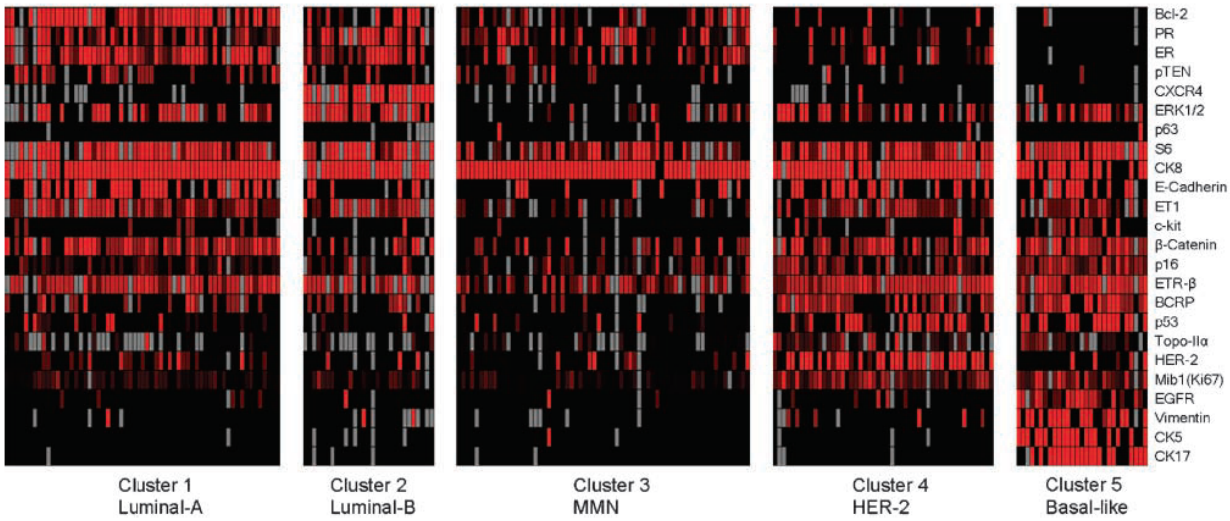
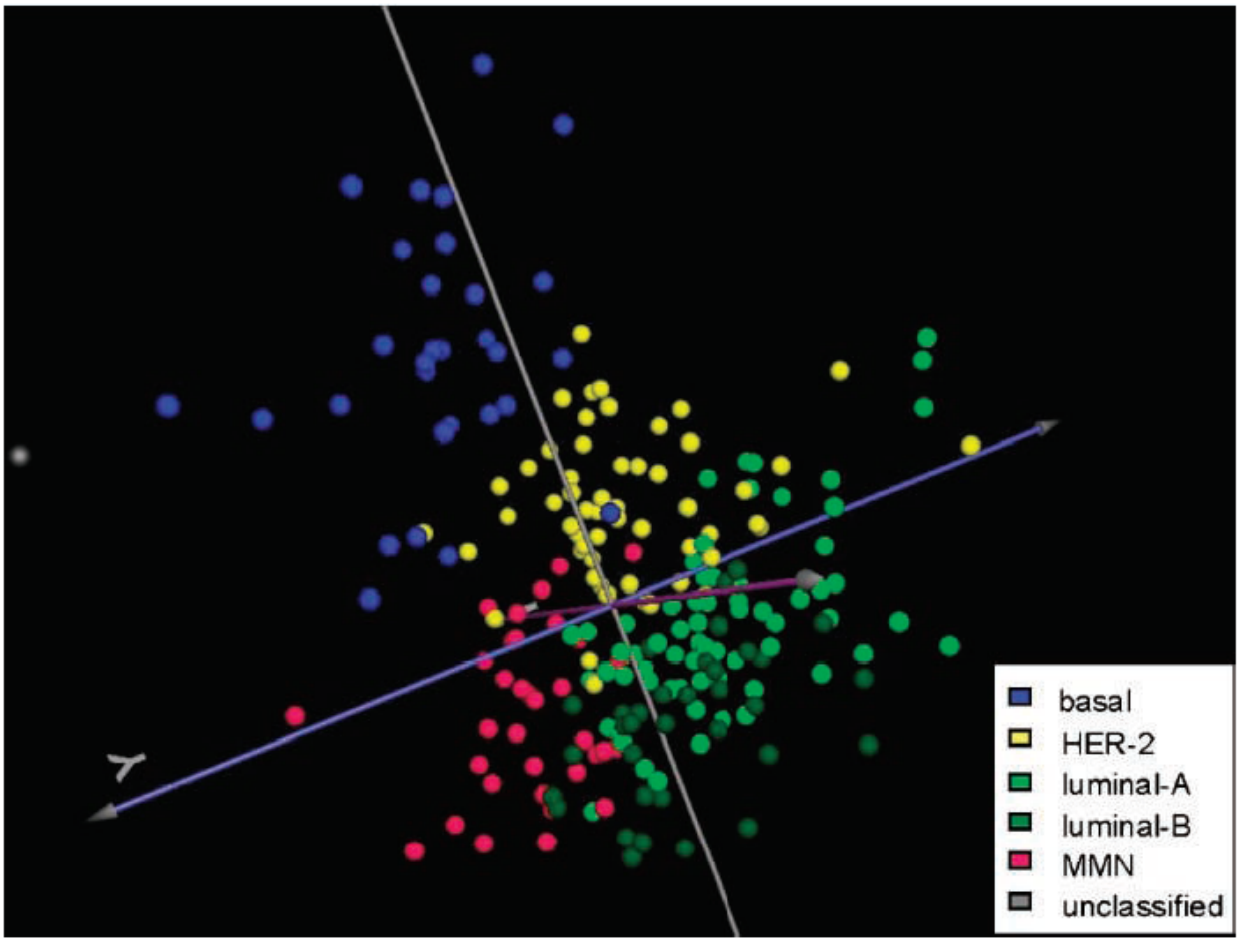


Fig. 5 shows three-dimensional representation of the results from the principal component analysis. The closer two samples appear in the three-dimensional space, the more similar are their respective expression patterns.

Fig. 5. three-dimensional representation of the results from the principal component analysis



There is the widest distance between luminal A and basal-like tumors on the X-axis, reflecting their biological differences, as shown in the fig. 5. Overlaps between luminal A/B and basal-like/Her-2/neu subtypes in single protein expression were observed.

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3.6. Distribution of molecular subtypes

Luminal A (27%) and B (12%) groups

Both groups were characterized by a strong expression of luminal markers such as the two hormone receptors (ER/PR) and cytokeratin 8. Endothelin-1 and Endothelin receptor α expression was also associated with luminal subtype. A stronger expression of bcl-2 and β-catenin in the A subtype and nuclear CXCR4 expression in the B subgroup should be highlighted as differences between luminal A and B subtypes. Both groups were completely negative for basal markers, e.g. CK 5 and 17, EGFR and vimentin.

Multiple marker negative subtype (27%)

This subgroup was positive for most luminal markers (e.g. Ck 8), but was less positive for hormone receptors and bcl-2, that would be led to the place between luminal and basal subtypes. Furthermore this type was completely negative for basal markers (Ck 5 and 17, EGFR) and had a lower proliferation rate than basal/Her-2/neu subtypes.

Her-2/neu subtype (21%) and basal-like (13%)

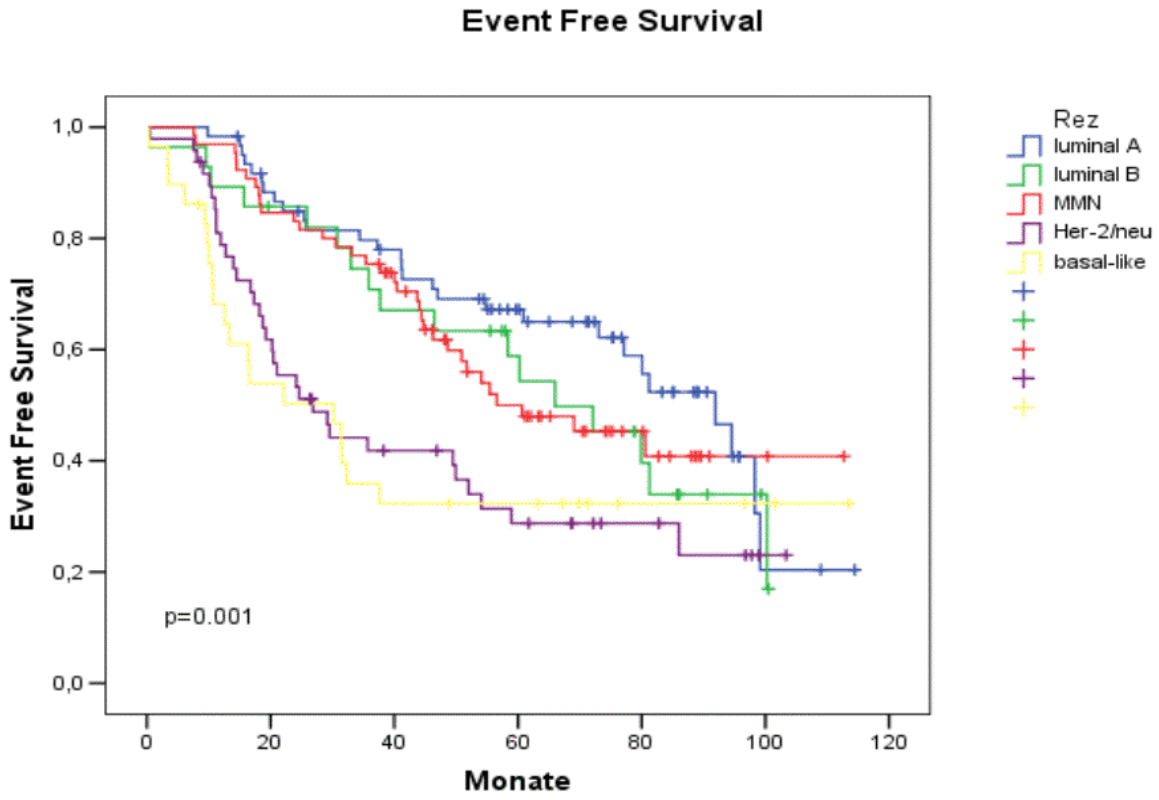
Both subtypes had similar expression patterns, were negative for hormone receptors and CK 8, except for the basal proteins (CK 5 and 17, vimentin, c-kit) and Her-2/neu, which are either negative or positive in corresponding groups. Both groups were characterized by frequent and stronger expression of p53, p16, BCRP, and high proliferation rate MIB-1. Both subtypes were significantly associated with G3 tumors (62,5% in Her-2/neu and 79% in basal-like subtypes vs. 30% in other subtypes), furthermore patients with basal-like tumors were younger than in other subgroups (48,3% <40 years old vs. 15% in other subgroups and there was a non-significant trend to higher mean number of LN in basal-like tumors (similar as MMN group) compared with other subtypes. Basal-like subtype was significantly associated with local recurrence within the study population (p=0,009).

3.7. Survival analysis according to molecular classification

The luminal A subtype had the best EFS compared with other subtypes (5 year EFS 69%), followed by non-significant trend to worse EFS In luminal B [HR for EFS luminal B

vs. A=1,326, p=0,326] and MMN subtypes [HR for EFS MMN vs. luminal A=1.343, p=0.256]. The basal-like and Her-2/neu subtypes had a similar poor prognosis vs. luminal A subtype [HR for EFS basal-like or Her/2-neu vs. luminal A=2.4, p<0,001]. Details see Fig.5

Fig. 5. Event free survival in protein cluster subgroups



3.8. Correlation of basal-like subtype and triple-negative status of tumors.

However in regard to the determination of triple-negative phenotype to protein clusters a significant association of basal-like breast cancer with triple negative phenotype could be confirmed (p<0,001), but only 33% of triple-negative tumors were defined as basal-like, although 80% of basal-like tumors are triple-negative. Surprisingly 8 (12%) of triple-negative cases were classified as luminal A or B tumors. Details are given in the table 3. From the whole cohort of the measured protein markers there is only a significant positive correlation between triple-negative tumors and typical basal-like markers (e.g. CK 5 and 17, EGFR, vimentin, c-kit, p16, BCRP).

Triple-negative phenotype was associated with a non-significant trend of a higher incidence of visceral metastases (p=0.065), but not local relapse.

Table 3. Distribution of triple negative breast cancer between protein clusters.

		Protein cluster					
		Luminal A	luminal B	MMN	Her-2/neu	Basal-like	Total
triple-negative	No	52	24	44	39	6	165
	Yes	8	5	20	10	23	66
	Total	60	29	64	49	29	231

3.9. Patient outcome according to conventional prognostic factors and molecular subtypes

Tumors from 211 patients with available staining results for all protein markers and protein classification were included in univariate and multivariate analysis.

In the univariate analysis, the following factors were significantly associated with better EFS: HD, positive PR status, tumor size (>5 and respectively 2-5 cm), G1/2, and negative status of Her-2/neu and of MIB-1 as well luminal or MMN protein cluster.

The same factors as well as positive ER status and negative EGFR were significantly associated with longer OS.

The multivariate analysis was performed, including the factors therapy arm, tumor size, ER, PR, grade, HER-2/ neu, MIB-1, and EGFR and protein cluster.

After inclusion of protein cluster (basal-like or Her-2/neu vs. other subtypes: HR=2.43, p<0.001) in the multivariate analysis only tumor size ≥2 and ≥5 cm (as two step analysis) (HR=1.51, p=0.012) and HD therapy (HR=0.50, p=0.001) remained as independent prognostic factors.

For OS, again HD therapy (HR=0.48, p<0.001) and positive PR status (HR=0.43, p=0.001) were favourable, whereas in addition to protein cluster (HR=2.30, p<0.001) and larger tumor size >2 and >5 cm (HR=1.46, p=0.04) were associated with an increased risk of death. Detailed results for the univariate and multivariate analysis are shown in Table 5.

Table 5. Multivariate analysis for event free and overall survival

		Event Free Survival			Overall Survival		
		univariate	multivariate		univariate	multivariate	
	Comparison	P*	p	Hazard ratio§ [95%-CI]	P*	p	Hazard ratio§ [95%-CI]
Therapy	HD vs. DD	0.004	0.001	0.50 [0.34-0.74]	0.008	<0.001	0.48 [0.31– 0.75]
Tumor size	≥5 cm vs. ≥2-5 cm vs. <2 cm	0.01	0.012	1.51 [1.09-2.07]	0.02	0.04	1.46 [1.02-2.09]
ER	Pos. vs. neg.	0.14			0.004		
PR	Pos. vs. neg.	0.05			< 0.001	0.001	0.44 [0.26 – 0.74]
Grade	G 3 vs. G1/2	0.07			0.003		
HER-2/ neu	Pos. vs. neg.	0.048			0.005		
MIB-1	Pos. vs. neg.	0.01			0.005		
EGFR	Pos. vs. neg.	0.12			0.005		
Protein cluster	Basal-like/Her-2/neu vs. other	0.001	<0.001	2.43 [1.66-3.56]	0.001	<0.001	2.30 [1.45-3.64]
Age	≤50 years vs.<50	0.45			0.69		

* Log rank test

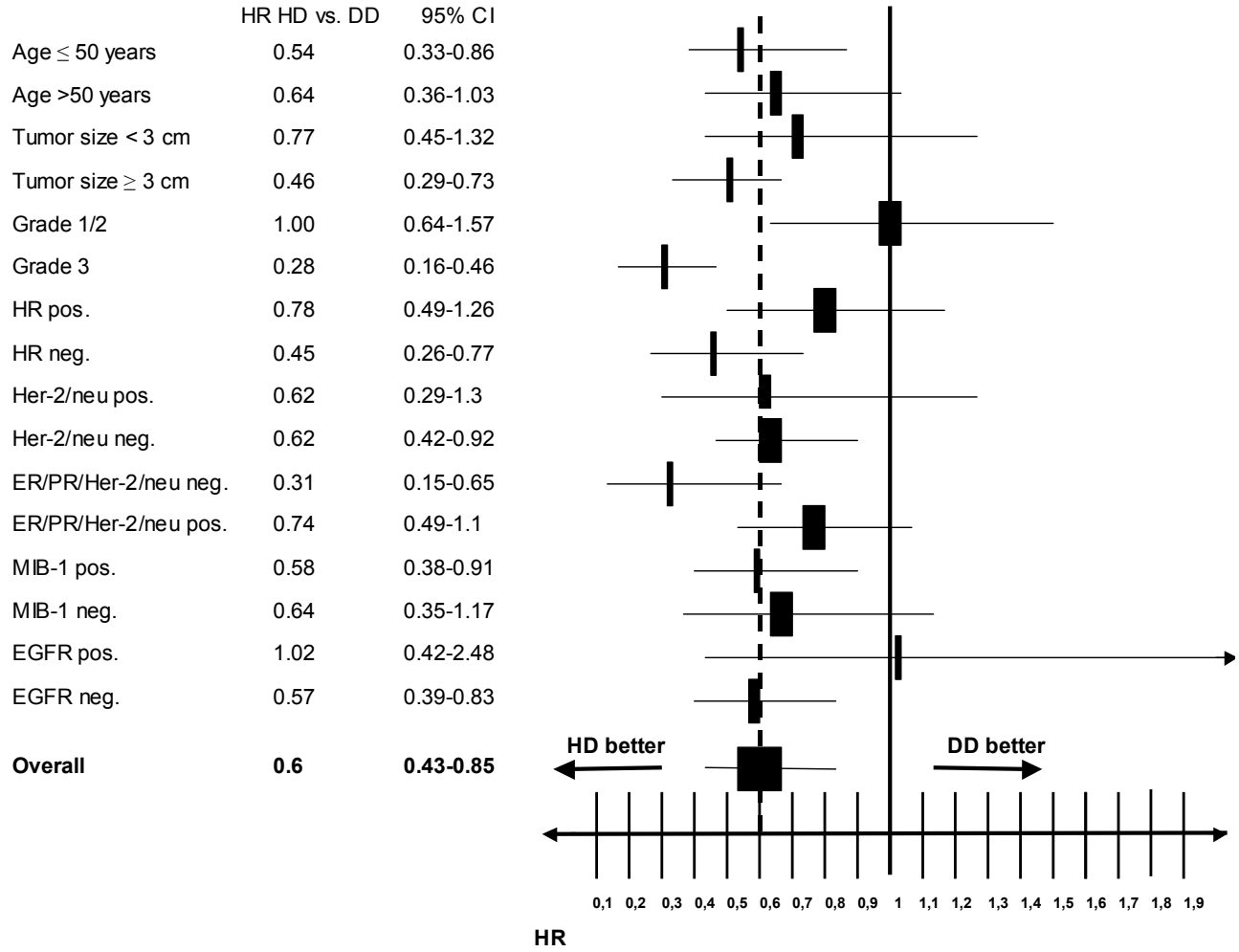
§ Cox proportional hazards model adjusted for age

3.9.1. Therapy effect in subgroups by conventional markers

Therapy efficacy was evaluated in clinically relevant subgroups defined according to the clinico-pathological criteria as well as protein markers. Hazard ratios for EFS between the two treatment groups are displayed in Figure 7. The following parameters were significantly associated with better outcome after HD vs. DD: age ≤50 years; tumor size > 3 cm (was chosen as cut off as median tumor size); G3; negative HR, Her-2/neu and

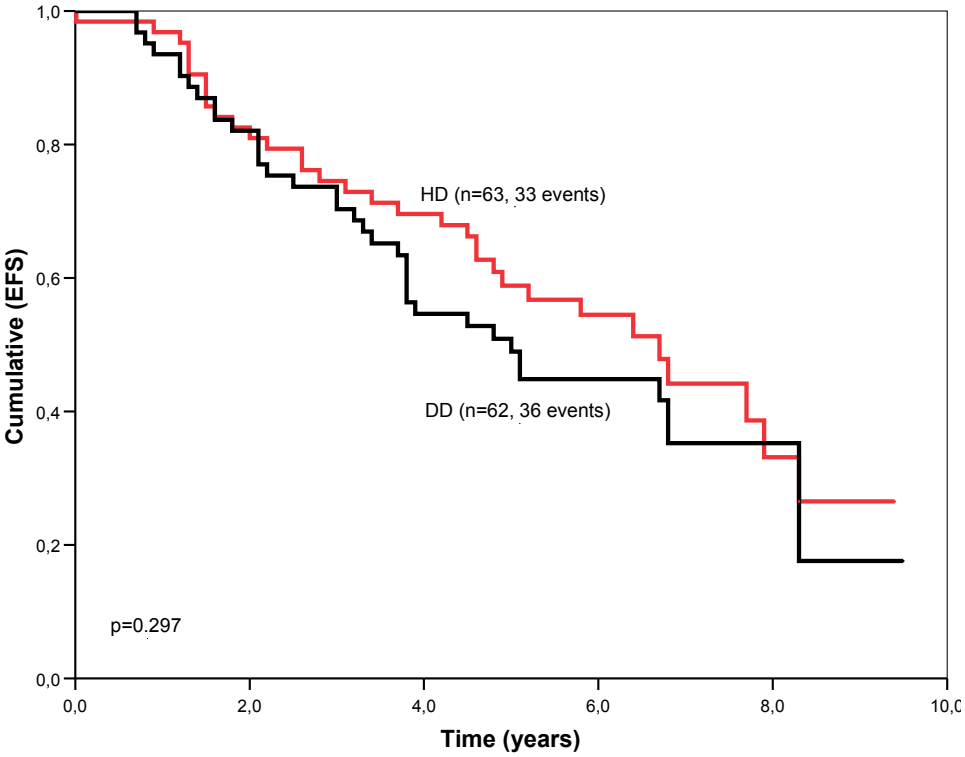
EGFR status; triple negative tumors (ER/PR/Her-2/neu-), as well as positive MIB-1 staining as conventional prognostic markers.

Figure 7. EFS hazard ratios (HD vs. DD) and 95% CI's in patient subgroups



As noted regarding Figure 7, significant benefits from HD vs. DD was seen in the HR negative but not in the HR positive subgroup (see Figure 8). Within the HD arm, HR status did not have a significant impact on EFS (p=0.592), whereas within the DD arm, HR positive tumors had significantly better EFS (p=0.017). Her/2-neu negative tumors were significantly associated with better EFS in both therapy subgroups (Fig 9).

Fig. 8. Kaplan-Meier plot of EFS in the HD vs. DD arms in patients with HR positive and negative tumors.


1,0
0,8
0,6
0,4
0,2
0,0
Cumulative (EFS)
HD (n=63, 33 events)
DD (n=62, 36 events)
p=0.297
0,0
2,0
4,0
6,0
8,0
10,0
Time (years)

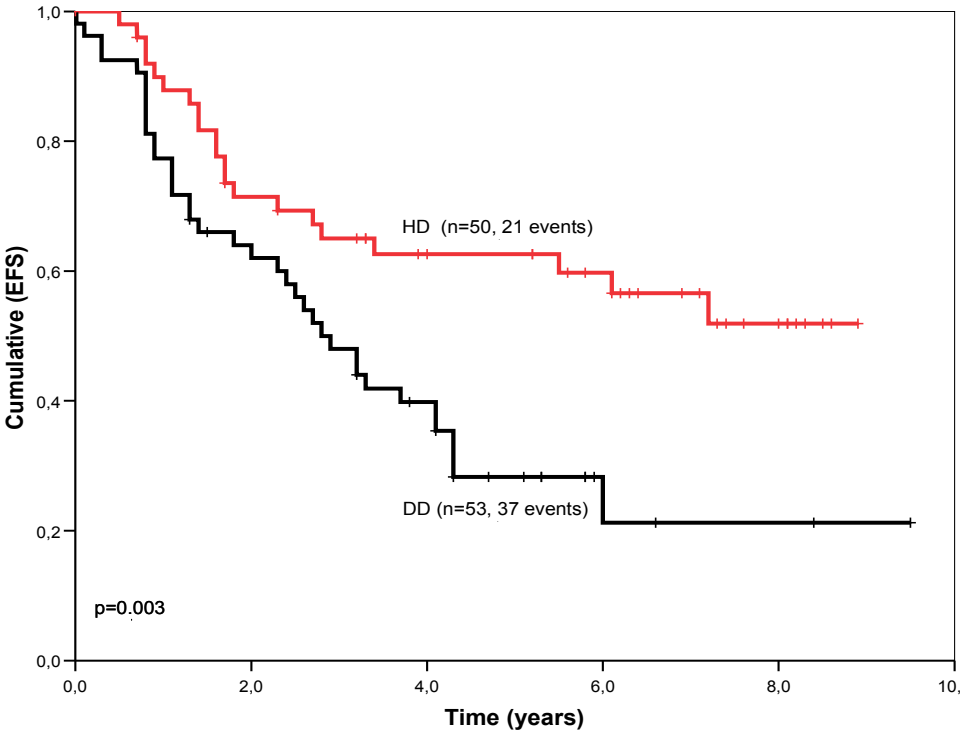
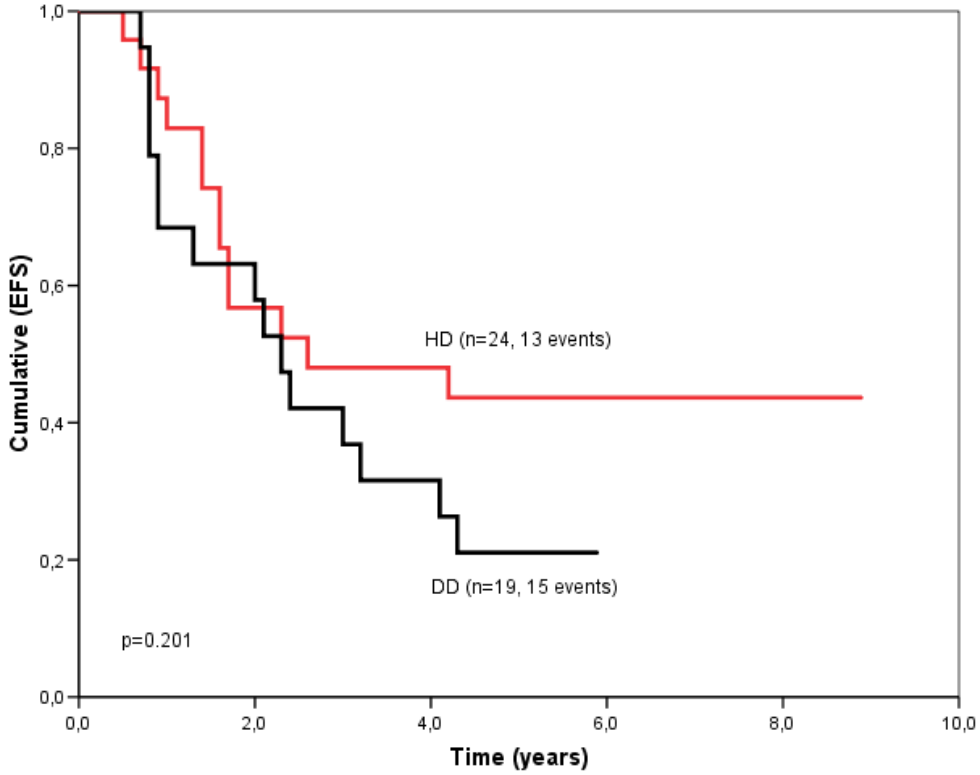
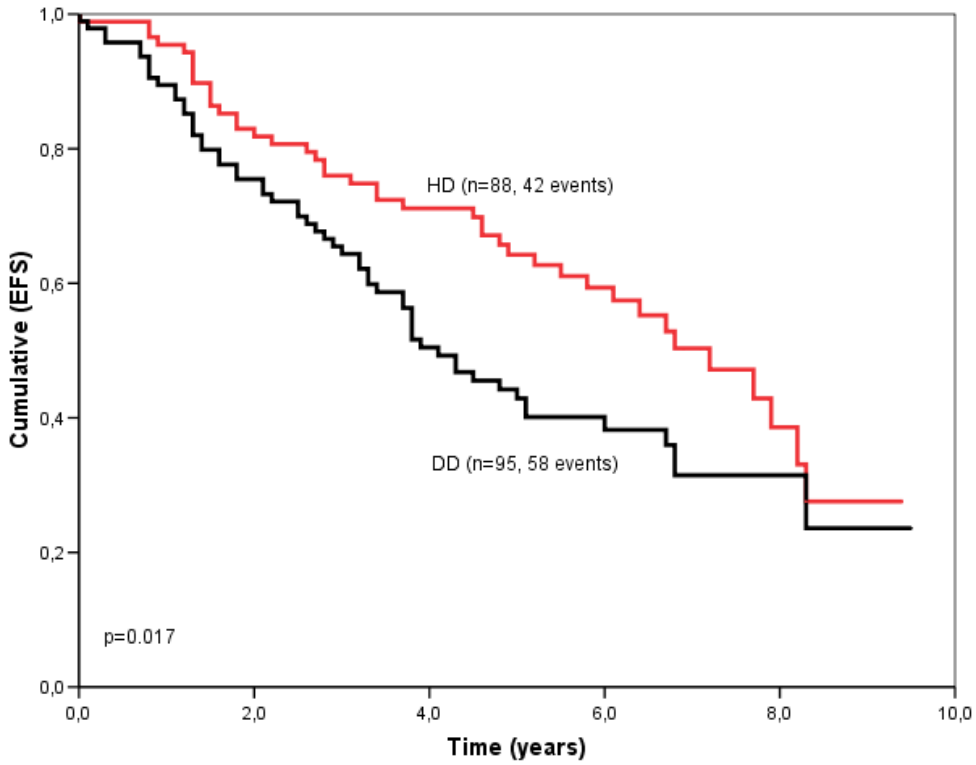

1,0
0,8
0,6
0,4
0,2
0,0
Cumulative (EFS)
HD (n=50, 21 events)
DD (n=53, 37 events)
p=0.003
0,0
2,0
4,0
6,0
8,0
10,
Time (years)

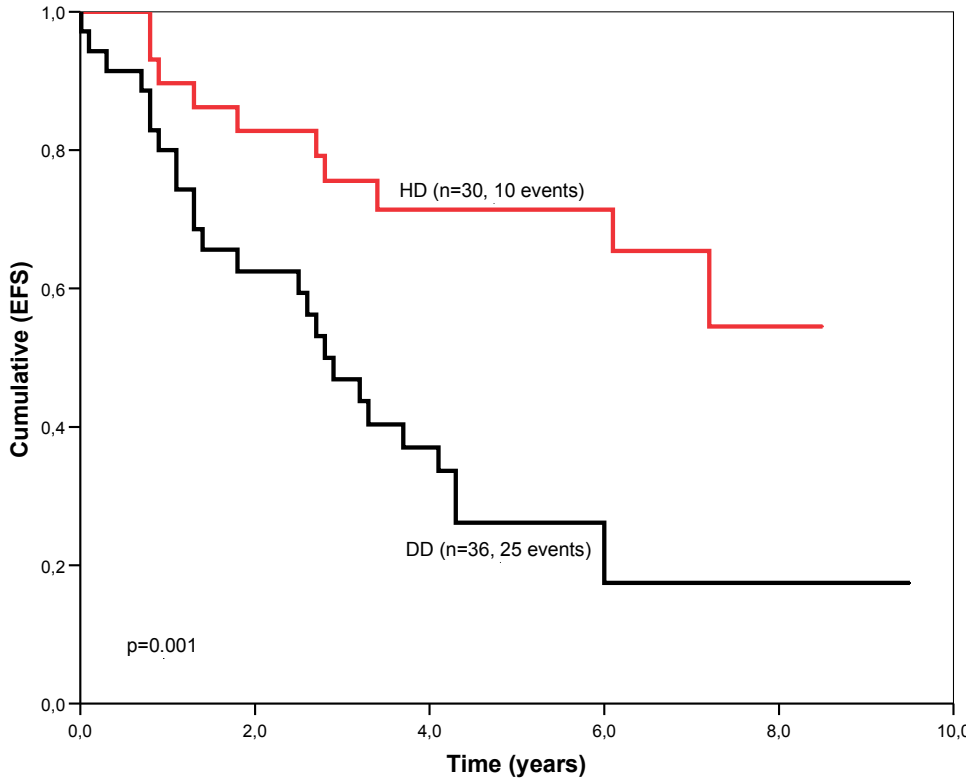
Fig. 9. Kaplan-Meier plot of EFS in the HD vs. DD arms in patients with Her-2/neu positive and negative tumors.

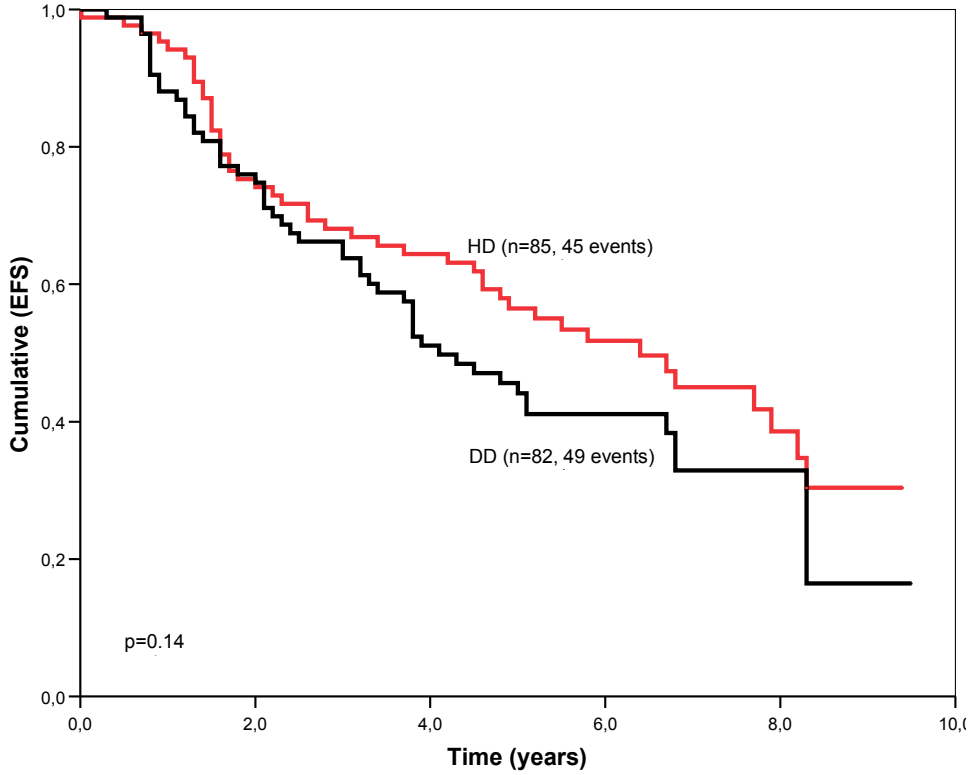




In TN tumors, the most pronounced effect of HD was observed. In this subgroup, median EFS was not reached in the HD arm whereas it was only 32.3 months in the DD arm. This translates into an estimated 5-year EFS of 71 % in the TN cohort treated by HD compared to only 26 % in the DD arm. There was no significant outcome difference by therapy arm associated with the remaining tumors (see Figure 10).

Fig. 10. Kaplan-Meier plot of EFS in the HD vs. DD arms in patients with triple-negative (ER/PR/Her-2/neu) and non-triple-negative tumors.

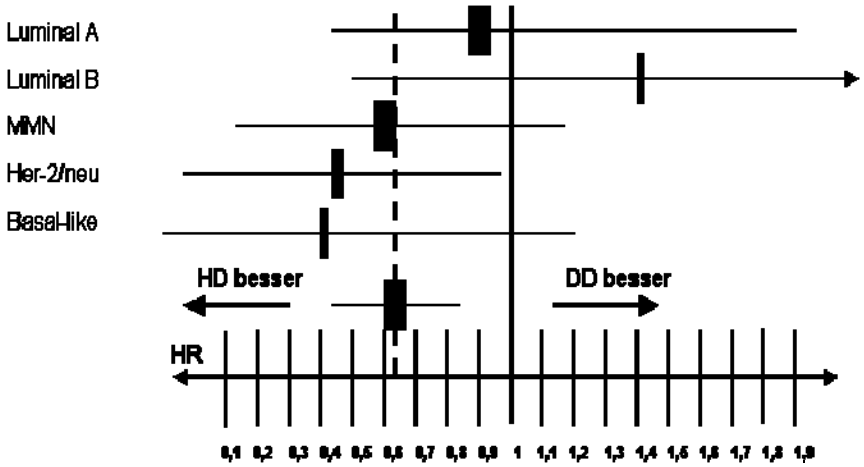




3.9.2. Therapy efficacy in molecular subtypes

Figure 10 shows the results of the univariate analysis for EFS (Cox model) concerning the five cluster subtypes in relation to the treatment. For a quantification of treatment effects on EFS and OS the hazard ratios with 95% CI are displayed. A non-significant trend to better efficacy of HD has been shown in basal-like and MMN subtypes. Only in the Her-2/neu subtype has the level of significance been reached.

Figure 11. Hazard ratios HD vs. DD with 95% CI within different molecular subtypes.



3.10. Adjuvant therapy interactions in multivariate analysis

An additional perspective on predictive significance of factors for optimal individualized adjuvant therapy is provided by multivariate interaction analysis: Each marker that was significant in univariate Cox analysis was considered both as a potential "main effect" and as a potential interaction with adjuvant therapy (i.e., HD vs. DD) in a proportional hazards model for EFS, the primary endpoint of this randomized prospective trial. (Interaction analysis for EFS provides predictive information about adjuvant therapy efficacy that is not confounded by possibly differing palliative therapy strategies after relapse.)

The resulting model is given in Table 6, which includes the β coefficients of the underlying Cox model to aid in the interpretation. We first note that, according to the first prognostic model, tumor size, PR, and MIB-1 are purely prognostic, whereas grade are both prognostic and predictive, and triple negative status is purely predictive. HD benefit (compared to DD) is entirely attributable to interactions in this model. Basal-like subtype had no independent significant both prognostic and predictive effect in this multivariate model.

Controlling for status of tumor size, PR and MIB-1, HD is associated with relative benefit in TN tumors (HR=0.31, 95% CI: 0.13-0.77, p=0.03) and in G3 tumors (HR=0.35, 95% CI: 0.18-0.64, p=0.001), whereas it is associated with relative risk in EGFR positive tumors (HR=6.38, 95% CI: 1.69-24.1, p=0.006). More precisely, for an individual patient, the composite HD hazard ratio (a number less than unity if there is relative benefit) can be calculated from the model according to the formula

$$HR(HD\ vs.\ DD) = \exp(\sum_{j=1,3} \beta_j x_j)\ ,$$

where j refers to the binary factor (triple negativity, G3, or EGFR positivity), x_j is 1 if the factor j is positive (e.g., a triple negative tumor) and zero otherwise, and β_j is the appropriate entry from the last three rows of Table 6.

For example, according to the model, a G3 patient who is EGFR negative and not triple negative would have a hazard ratio of about 2.88 compared to a G1 or G2 patient with the same tumor size class, PR status, and MIB-1 status if both receive DD. If both receive HD, then the G1 or G2 patient would not benefit, whereas the G3 patient would have a benefit corresponding to a HR of about 0.20 compared to the same patient with DD and would even have a favorable hazard ratio of exp(1.06-1.60) or about 0.581 compared to the G1 / G2 patient (with either therapy).

If the patient is triple negative, then the model implies a benefit from HD for any grade if the patient is EGFR negative (the usual). If the patient is EGFR positive, the model formally implies that the benefit of HD (to triple negative patients) would occur only for grade G3, i.e., not for G1 or G2; however, this is a rare occurrence: all but one of the triple negative & EGFR positive patients were G3.

Table 6. Multivariate adjuvant therapy interaction analysis for event free survival.

	Comparison	p	β (log HR)	Hazard ratio[§] [95%-CI]
Therapy	HD vs. DD	n.s.		
Tumor size	≥5 cm vs. ≥2-5 cm vs. <2 cm	0.06	0.29	1.33 [0.98 – 1.81]
ER	Pos. Vs. Neg.	n.s.		
PR	Pos. Vs. Neg.	0.02	-0.51	0.60 [0.40 – 0.91]
Grade	G 3 vs. G1/2	<0.001	1.06	2.36 [1.41-3.94]
HER-2/ neu	Pos. Vs. Neg.	n.s.		
MIB-1	Pos. Vs. Neg.	0.02	0.50	1.65 [1.08-2.52]
EGFR	Pos. Vs. Neg.	n.s.		
Age	≤50 years vs.<50	n.s.		
triple negative status	triple negative vs others	n.s.		
Basal-like	Basal-like vs. non-basal-like	n.s.		
interaction: grade * therapy	G3 and HD vs. all others	0.001	-1.05	0.35 [0.18 – 0.64]
interaction: EGFR* therapy	Pos. and HD vs. all others	0.006	1.85	6.38 [1.69-24.1]
interaction: triple negative status *therapy	triple and HD vs. all others	0.01	-1.16	0.31 [0.13-0.77]

4. Discussion

4.1. Dose and schedule of chemotherapy in HRBC

WSG-AM-01 was the first trial to report significant OS benefit in patients with ≥9 involved LN after tandem high dose chemotherapy (HD) with stem cell support (2 cycles EC q2w followed by 2 cycles of HD cyclophosphamide, thiotepa and epirubicin) compared to a dose-dense(DD) anthracycline based sequential regimen (4 cycles of EC followed by 3 cycles CMF q2w with growth factor support)[30]. For a representative subgroup of 236 patients from this trial, paraffin embedded tumor tissue was available for central pathologic review and immunohistochemical analysis of a panel of molecular markers. The tumor characteristics of this subgroup and survival rates were comparable with that for the whole study population. Our study presents unique results in the adjuvant treated patient collective with highest risk of relapse, defined by median number of involved lymph nodes of 15. 132 relapses (56%) and 99 deaths (42%) were recorded during the 5 year median follow up period. At 61.7 months, the EFS and OS rates significantly favor HD. A relative risk reduction of 40% for relapse and 42% for death was associated with HD treatment compared with standard DD arm.

Results from HD trials in HRBC are controversial, some reporting significant improvement in terms of EFS[23] or non-significant trends to improved RFS [28, 29, 44, 67] or overall survival[29, 67] , and others reporting no survival differences [14, 25, 27]. Designs and patient inclusion criteria differ substantially among these trials. Definitive evaluation of the role of HD from this large body of heterogeneous data is impossible, but further analysis of these studies could suggest successful strategies and/or subsets of patients with maximum benefit. The overview of inclusion criteria, therapy regimens and results of randomized phase III trials is given in the table 7.

The recent meta-analysis performed in 6210 patients from 15 randomized trails shows a significant longer DFS (HR 0,87, 0.87; 95%CI 0.81-0.94; p=0.0001), but not breast cancer specific survival (BCSS) (HR 0.93; CI 0.85-1.02; p=0.10) or OS (HR 0.95; CI 0.87-1.02; p=0.16). After adjusting as well for HR in the subset for which it was available, HD significantly prolonged DFS (HR 0.83; CI 0.77-0.90; p<0.0001) and had a modest and marginally significant benefit on BCSS (HR 0.88; CI 0.79-0.97; p=0.013) and OS (HR 0.89; CI 0.82-0.98; p=0.016)[68]. However for the patients with available HR und Her-2/neu status the significant improvement of DFS was reported only for the group with triple-negative tumors.

In view of extensive toxicity and costs of HD the subgroup with the most toxicity remains to be defined.

Table 7. Overview of randomized phase III HD trials.

Overview about HD trials										
Author	**n**	**Inclusion criteria**	**Follow up (years)**	**Control**	**HD**	**Outcome**	**Control (5y)**	**HD(5y)**	**Significancy**	**Comments**
I. Classical phase III trial design										
Hortobagyj	78	>10 positive LN or >4 LN after 4 cycles of neoadjuvant CT	12	8xFAC	8xFAC+2xHD Cyclo/ Etoposid/Cisplatin	RFS OS	51% 68%	45% 54%	0.11 0.13	too few patients
Tokuda	97	<56 years and >10 positive LN	5	6xFAC	6xFAC+1xHD Cyclo/Thiotepa	RFS OS	37% 62%	52% 63%	0.17 0.78	31% did not receive HD, too few patients
Schrama	81	extensive lymph involvement	6,90	4xFE(120)C	3xFEC-1xHD Cyclo/ Carboplatin/Thiotepa	RFS OS	48% 62%	49% 61%	0.37 0.85	too few patients
Roche	314	>7 positive LN	3,25	4xFEC	3xFEC-1xHD Cyclo/ Mitoxantron/Mephalphan	EFS (3Y) OS (3Y)	55% 84%	77% 86%	0.002 0.33	too short follow up
Zander	307	>10 positive LN	6,1	4xEC-3xCMF	4xEC-1xHD Cyclo/ Thiotepa/Mitoxantron	EFS OS	42% 62%	49% 64%	0.15 0.33	trend to benefit of HD in G3, ER+, premenopausal, significant in p53 positive
Rodenhuis	885	<55 years old and >4 positive LN	7	5xFEC	4xFEC-1HD Cyclo/Carboplatin/Thiotepa	RFS OS	59% 70%	64% 73%	0.076 0.22	significant in >10 LN, Her2-, G1 and <40 years old
Tallmann	511	>10 positive LN	6	6xFEC	6xFEC-1HD Cyclo/Thiotepa	RFS OS	48% 62%	55% 58%	0.12 0.32	23% had protocol violations, significant in per protocol treatment
Lenard	605	>4 positive LN	6	4xE-8xCMF	4xE-1 immediate Cyclo-1HD Cyclo/Thiotepa	RFS OS	54% 64%	57% 62%	0.38 0.78	
Coombes	281	>4 positive LN	6,5	6xFEC	3xFEC-1HD Cyclo/Carboplatin/Thiotepa	RFS OS	59% 67%	57% 66%	0.76 0.4	trend to better efficacy in >10 positive LN
II. Comparison of HD with dose/dense or intensive standard										
Bergh	525	expected 5 year RFS < 30%	5	9xtailored FEC	3xFEC-1 HD Cyclo/ Carboplatin/Thiotepa	RFS OS (HR)	72% 0.866	62% 1	0.02 0.287	more dose-intensity and treatment-related deaths (AML/MDS) in control arm, benefit only in Her-2 positive
Peters	785	>10 positive LN	7,3	4xFEC-1x intermediate Cyclo/ Cisplatin/Carmustin	4xFEC-1xHD Cyclo/Cispaltin/Carmustin	EFS OS	58% 71%	61% 71%	0.24 0.75	RFS 48% vs. 58%, 9,3 treatment mortality in HD arm, HD significant in younger patients
Moore	536	>4 positive LN	5,8	DD/intensive 3x Doxorubicin-3x Taxol-3xCyclo q2w	4xAC (A-intensive)-1xSTAMP I (Cyclo-Cisplatin-Carmustin) or STAMP V (Cyclo/Carboplatin/Thiotepa)	DFS OS	80% 88%	75% 84%	0.35 0.40	Planned sample 1000 patients, standard arm had higher doses than HD
III. Comparison of multicycle HD with any standard										
Basser	344	>9 positive LN or >4 positive LN and/or T3 and ER negative	5,8	4xEC-3xCMF	3xHD Epribucin/Cyclo	DFS OS	43% 61%	52% 70%	0.07 0.17	HD significant in ER positive, tumor size 2-5 cm and +10 LN (p=0.06)
Nitz	403	>9 positive LN	4	4xEC-3xCMF q2w	2xECq2w-2xHD Cyclo/Thiotepa/Epirubicin	EFS OS	42% 60%	54% 72%	<0.01 0.02	too short follow up, HD significant in younger patients, G3, HR negative
Gianni	398	>4 positive LN	11,5	3xE-6xCMF	HD:1xCyclo-1xMethotrexat-2xEpirubicin-1xThiotepa/ Mephalphan	PFS (12y) OS (12y)	44% 51%	52% 60%	n.s. n.s.	trend to better efficacy of HD in younger patients (<35 y.o.) and 4-9 LN

Management of HRBC remains a clinical challenge. There are only a few data regarding to prognosis of patients with more than 10 involved LN outside the strong selection criteria of the HD trials. Natural history data base reports 5-year DFS of 20-30% for patients with >10 LN treated only by surgery without adjuvant therapy[8]. In prospective randomized adjuvant chemotherapy trials patients with >10 LN make up for 5%-10% of the whole population, so that data refer for small subgroups. Schmoor et al. have shown 5 year EFS of 22% and OS of 39% in 141 patients with >10 positive LN from two German Breast Cancer group trials treated by CMF[69]. The 5 EFS rates in athracycline-treated patients vary from 39% to 47%[12, 70]. Comparable 5 year EFS rates of 37%-48% in patients with >10 involved LN were reported also for control arms of HD trails, where standard anthracycline-based combinations were given[19].

The impact of third-generation taxane-based adjuvant chemotherapy remains unclear. Unplanned retrospective subgroup analysis from the early phase III trials fail to demonstrate a significant survival benefit. Only the Spanish GEICAM study showed more pronounced effects on DFS of remarkably dose-dense 8 cycles of Taxol weekly therapy after 4 cycles of FEC compared with 6 cycles of FEC in patients with more than 4 positive LN vs. group 1-3 LN[71]. In contrast to these results stands recently published results of randomized BIG 02-98 study found no survival difference for concurrent E/Doctaxel-CMF vs. conventional EC-CMF in node-positive BC.

The second important point of discussion is the question of dose intensity and density in adjuvant therapy of HRBC. Although the NSBAP B-22 and B-25 as well as CALGB 9344 revealed no benefit for dose escalation of cyclophopsphamide beyond 600 mg/m^2 and doxorubicin beyond 60 mg/m^2 in unselected cohorts of patients[32, 33, 35], it is remarkable that the best survival rates in control arms in HRBC studies were reached particularly by dose-intensive/dense regimens[27, 28, 72]. The recently presented results of AGO Intergroup study comparing dose-intensive and dense 3xEpirubicin(150 mg/m^2)/3xCyclophosphamide(2500 mg/m^2)/3xTaxol(225 mg/m^2) q2w vs. conventional 4xEC-4xTaxol q3w in patients with >4 positive LN confirm these observation. Impressive 5 year RFS rate of 70% and OS rate of 82% have been estimated in 1284 patients with median number of 8 positive LN[39]. Similar significant superior results in favor of dose-dense concept are shown by Citron et al. (CALGB 9741) who compared dose-dense EC-Taxol q2w vs. every 3 weeks administration in node positive BC[38]. Although the third dose-dense study conducted by Venturini et al. comparing standard 6 cycles of FEC every three weeks vs. every two weeks in node-positive and high-risk node-negative BC

has failed the value of significance after 12 years of median follow up (HR for RFS 0,88, 95% CI 0.71-1.08; HR for death 0.87, 95% CI 0.67-1.13), the risk reductions were similar to those in CALGB 9741 study, by better than expected survival in control arm, possibly due to lower risk profile of patients and insufficient dose of anthracyclines applied within the study[40].

On the other hand recently published INT 0137 study comparing sequential dose intensive and dense 4xDoxorubicin (91 mg/m^2) followed by 3xCyclophopshamide (2400 mg/m^2) q2w vs. simultaneous dose-intensive 6 cycles EC(54/1200 mg/m^2) q3w in node-positive (1-3 positive LN) and node-negative BC revealed similar survival rates in both study arms (5 year DFS EC vs. E→C: 79% vs. 81%, p=0.2). But it should be remarked that survival rates in patients with node-positive BC are in favor of dose-dense/intensive arm (5 year DFS EC vs. E→C: 76% vs. 81%)[73]. The results of above mentioned trials are indirectly compared by e.g. study of Piccart et al., which highlighted a benefit for higher dose of epirubicin (100 mg/m^2) over lower dose particularly in patients with >4 positive LN[36]. The first results of NCIC MA 21 study indicate also a clear significant benefit for dose-intensive Canadian $FE_{120}C$ q3w or dose-dense and intensive 6 cycles of $E_{120}C$ followed by 4 cycles of Taxol q2w over conventional 4xEC-4xTaxol in high-risk node-negative and node-positive BC[41] after certainly short follow up of 30.6 months.

Until the subgroup with the most benefit is not defined, HD and dose-intensive chemotherapy in breast cancer is not recommended as a standard treatment option in unselected patient collectives by guidelines in Germany and other countries.

4.2. Prognostic and predictive factors

In our present study collective, we have analyzed prognostic value of established parameters such as age, tumor size, number of involved LN, grade as well as HR, Her-2/neu, MIB-1 and EGFR, which are important switching points of proliferation, apoptosis, DNA repair and related pathways. In the second step of the analysis we compared the prognostic impact of above mentioned factors with molecular subgroups determined by unsupervised clustering of expression using a panel of 24 protein markers.

4.2.1 Conventional prognostic markers

In multivariate analysis, the only established parameters correlating significantly with poor outcome were tumor size ≥5 cm (EFS, OS) and negative PR (OS).

Other phase II and III trials in HRBC also demonstrated an association of tumor size[74-77], negative HR[14, 23, 74-77], poor differentiation (G3) [75, 76] and higher proliferation[77] with poor outcome. In contrast, the recently published study of Kroger et al[17] did not show a prognostic effect of MIB-1. However, in view of the limited data on molecular markers in HRBC, further analyses of prognostic markers are still needed.

4.2.2. Molecular classification

Previous RNA[47] and cDNA[64] and later protein expression studies have shown the existence of several molecular subtypes of breast cancer, which are associated with a different course of disease in unselected breast cancer patients. The ER and ER related genes are reported to be the critical variables in the development of both normal breast gland and breast cancer.

Differences in expression profiles between ER-positive and negative BC have been reported using both unsupervised and supervised approaches. Gruvberger et al. showed that the differences in gene expression profiles between ER positive and negative tumors could only partly be explained by the activity of a functional ER pathway, suggesting that these differences are largely explained on the basis of different cell lineage[78].

Perou et al.[47] and than Sorlie et al. [64] were the first ones to identify and refine the molecular classification by expression analysis of RNA in 42 and than 496 cDNA (intrinsic set) in 78 T3/4 tumors, partly treated by neoadjuvant chemotherapy. Three groups characterized by low ER expression were identified: Basal-like marked by high expression of keratins 5 and 17 (Ck 5/17), c-kit, caveolin 1, with a high frequency of p53 mutations, mostly G3, where all hereditary breast cancer with BRCA1 mutation were failed[53], erbB2/Her-2/neu subtype with high expression of several genes of the erbB2 amplicon and the normal-like breast subgroup. Two further subgroups mostly ER positive were identified: luminal A and B tumors were mostly ER positive, but luminal B subtype was characterized by higher expression of proliferation genes, lower expression of bcl-2 and higher frequency of p53 mutations. Patients with luminal A and B subtypes have significantly better outcome than patients with Her-2/neu and basal-like subtypes. With regard to the luminal subtypes type A was noted to have a better outcome compared with luminal B subtype[64]. The molecular signature was validated in three independent data sets and revealed very similar results[53].

The existence of the molecular subtypes was also confirmed by protein expression studies in unselected cohorts of patients[79]. Although different statistical methods (unsupervised/supervised by survival or ER status as discrimination variable) and number of measured proteins were used and various number of subgroups (from two to six) were identified, similar survival differences have been shown for subgroups as defined by protein expression studies.

So six groups in 1076 patients on TMA using expression of 21 protein markers are identified by Abd El Rehim et al. Clustering was performed by multiple layer perceptron (MLP) artificial neural network (ANN). Group 1 and 2 (48%) were predominatly HR positive, with strong expression of luminal markers and MUC 1. Group 3 (21,7%) was characterized by lower expression of HR, higher expression of erbB2 and some basal markers, higher incidence of grade 3 tumors vs. groups ½ (60% vs. 30%). Group 4 had only 4 cases and was to small to be described. In the group 5 (17%) there was a strong expression of p53 protein and basal markers, and a reduced level of BRCA1 expression. 89% of these tumors were G3 and had a higher incidence of extensive lymph node involvement. Younger age and more medullar histology were also observed. The sixth group (13%) was predominantly erbB2 positive with negative HR phenotype and absent luminal markers. Most tumors (78%) in this group were poorly differentiated[79].

An another expression profile of 31 protein markers by unsupervised clustering in 438 unselected patients with 15,8 years of median follow up has been investigated by Makretsov et al. A set of 11 markers discriminates in three prognostic groups and predicts survival as good as lymph node status. Group 1 was frequently HR positive with lower expression of p53, basal and proliferative markers compared with group 2 (more Her-2/neu positive) or group 3 (more proliferative). These last groups were characterized by higher expression of CK5. Group 1 had a significantly better EFS and OS rates[65].

Jacqmeier et al. have applied both unsupervised and supervised clustering of 26 selected proteins from 552 consecutive patients with early breast cancer. The unsupervised clustering has revealed three subgroups (A1: mostly ER positive and less proliferative; A2, defined as basoluminal subtype characterized by lower ER expression and B: basal/proliferative subtype). The next step of supervised analysis has related B subtype to the 21 protein poor prognosis signature in both learning and validation set. The 5 year EFS was 90% in the good- prognosis and 66% in the poor prognosis class, which was significant in the multivariate analysis including nodal status, treatment, tumor size and MIB-1 status[66].

Based on the gene-expression studies Nielsen et al.[51] and later Carey[52] et al. have applied the so-called five marker immunhistochemical method to determine basal-like breast cancer, as non-expressing ER, PR, Her-2/neu (triple negative) and expressing either EGFR or CK 5/6. In the Canadian study by Nielsen et al. the basal-like subtype was identified by using of triple-negative phenotype and expression of either EGFR or CK5 and/or c-kit in 930 patients[51]. After mean follow up of 17,3 years the basal-like BC was correlated to shorter disease-specific survival. The basal-like subtype from gene expression array could be identified by immunhistochemical expression of the five markers with specifity of 100% and sensitivity of 76%.

The Carolina Breast Cancer Study evaluated BC subtypes in 496 participants. Luminal A was defined as ER+ and/or PR+ and Her-2/neu-, luminal B as the same combination but Her-2/neu positive[52]. The basal-like breast cancer subtype was more prevalent among premenopausal African American women (39%) compared with postmenopausal African American women (14%) and non–African American women (16%) of any age (p=0.001). Compared with luminal A, basal-like tumors had more TP53 mutations (44% vs. 15%, p<0.001), significantly higher mitotic index, higher combined grade and was associated with the shortest survival compared with luminal A. Both Her-2/neu and basal sub-types had significantly the shortest breast-specific survival.

In the high-risk collective, interesting results were provided by Laakso et al. They investigated the impact of cytokeratine expression (CK 5/14) in 506 primary breast cancer patients. By uniformity of expression and third cytokeratine (CK17) two groups of basal (uniform positive for CK 5/14) and basoluminal (partial positive) subtypes were identified. Basal tumors were associated with vimentin, c-kit and ki-67 expression in contrast Her-2/neu ampflication was observed only in basoluminal tumors. These results were evaluated within 382 patients from the Scandinavian HD study. 73 patients (19%) had CK 5/14 expression, but the whole basal subgroup had a comparable outcome to other patients. However basoluminal tumors, particularly with co-expressed Her-2/neu had significantly shorter RFS[80].

In our analysis 5 subtypes of molecular classification very similar to that in the gene expression and other immunhistochemical studies could be identified by unsupervised clustering (k=5). ER was found to be a critical variable between the subtypes. Luminal A (27%) BC was mostly ER positive and CK8 positive with strong expression of bcl-2, as induced by ER pathway[81]. The luminal B (12%) subtype was also ER/CK8 positive, but as characterized by lower expression of antiapoptotic protein bcl-2, stronger expression of

chemokine receptor CXCR4 and shorter EFS respectively compared with luminal A subtype. For the first time the described MMN (multiple marker negative) subtype could be partly addressed to the basoluminal subtype, as transformation form of luminal and basal BC, marked by lower expression of ER and luminal markers but absent expression of basal markers. Both Her-2/neu (21%) and basal-like (13%) subgroups were strongly associated with proliferative patterns (p53, p16, MIB-1, topo-II) and subsequently with poor differentiated G3 tumors. The difference between groups were only the different profiles of expression of Her-2/neu and basal-markers (c-kit, Ck 5/17, vimentin). The basal-like subtype was significantly correlated to younger age of patients and higher incidence of locoregional relapse.

In contrast to the other protein expression studies we have identified luminal B subtype, whereas only in one immunhistochemical study it was possible to determine it[79]. We didn't observe significant survival differences between luminal subtypes, only a trend to a worse survival rate has been seen in luminal B subtype. This prognostic determination between both luminal subtypes can also be marked by different response to therapy.

Very similarly to the most microarray and immunhistochemistry studies a significantly worse DFS (as primary endpoint of the study) in basal-like and Her-2/neu BC compared to luminal A/B subtypes was found by us. Luminal A subtype had the best survival followed by luminal B and MMN subtypes.

The molecular classification of breast cancer seems to be also very relevant in light of new data of prognostic gene signatures and theories of breast cancer development. The existence of molecular subtypes as different sub-diseases within the BC challenges the whole understanding of biology of primary disease and further development of metastasis. Previously it has been reported that tumor progression is characterized by changes from a luminal epithelial –like to mesenchymal like phenotype, or from well-differentiated to high grade disease[82], but that was not validated by in vivo findings. Van t'Veer et al, investigating whether the metastatic capability occur early in the malignant process and that gene expression profiles would already reflect prognosis at an early stage in breast cancer, selected 98 primary breast cancers, all untreated systemically, 34 who had distant metastasis within 5 years and 44, who remained disease free. 231 genes were identified to be significantly associated with metastasis. Using "leaving-one-out" cross validation 70 genes could be further selected that could predict a poor prognosis[48]. This gene signature was further validated in 295 node-positive and negative patients and was able to be the strongest predictor for metastasis-free survival. The further work of Weigelt and

co-workers confirmed stability of the gene signature through the metastatic process of disease. Remarkably, multiple metastases from one patient all display the same molecular breast cancer subtype independent of the organ in which they developed and still maintain the unique molecular identity of the primary that they arose from[83].

Using multi-gene reverse transcriptase (RT-PCR) assays performed in fixed paraffin embedded tissue, Genomic Health has identified 21 gene significantly associated with distant recurrence in breast cancer patients. This gene signature was validated in several randomized sets of systematically treated patients and was a very strong predictor of prognosis in node-negative [49] breast cancer patients. Only patients with high genomic risk had benefit from adjuvant CMF chemotherapy added to standard tamoxifen.

There is a old discussion about existence of breast cancer stem cell characterized by self-renewal like a haematopoietic malignancy and could responsible for development of early and late metastasis. Liu et al confirmed a prognostic significance of 186 gene signature from tumorgenic breast cancer cells (expressing CD 44+/CD 24-) in the same 295 patients, where 70 gene signature of Van t'Veer et al was validated[48] and 286 patients from the Erasmus cancer centre[84]. In the second step of analysis the same signature has been shown to have prognostic impact in patients with other cancers (Medullosblastoma, lung and prostate cancer)[85].

Interestingly Fan et al. recently published a very high concordance between both commercially available gene signatures (70 Gene and 21 Gene recurrence score) and molecular subtypes of BC in 295 tumors[50]. All basal-like cancers (as defined by intrinsic genotype[64]) were scored as high risk by 70 Gene Signature and by 21 Gene Recurrence Score. The same results have been obtained for Her-2/neu subtype. Subsequently several gene products of the basal-like cluster are also expressed in stem cells of various tissue types[86]. Collectively, the gene-expression profile of basal-like BC provides a myriad of candidate genes that might contribute to their aggressive phenotype and may suggest a less differentiated "stem/progenitor" cell origin for these tumours.

In regard to the subgroup of patients with multiple lymph node metastasis one study the signature found to predict lymph node invasion seemed to be different to that which predicted distant recurrence, suggesting that different biological processes are involved in both metastasis development[87].

4.2.3. Correlation of triple-negative/basal-like subtype

Most studies reported, that measurement of basal-marker expression can more exactly define basal-like subtype than triple-negative (TN) subtype defined by absent expression of ER, PR, Her-2/neu. In contrast to the basal-like subtype in the microarray analysis there is only limited information about characteristics of triple-negative breast cancer (TNBC) available.

4.2.3.1. Prevalence

The TN prevalence of 17% in 3744 unselected BC patients from the British Columbia Cancer Agency has been reported recently[88]. These results are in line with an another study assigned 281 of 1726 cases as TN (16,3%)[89]. In the randomized HRBC collectives only Hannemann et al. investigated the incidence of TN phenotype in the Dutch study and shown that 137 of 753 tumors were stained as TN (18%)[90]. 24% of tumors from a conservatively treated database of Yale University School of Medicine were classified as TN[57]. So far the highest incidence of 26,4% was reported by Carey et al in the Carolina Study[52]. This increase could be partly explained by more American-African participants, who have significantly more frequently TN BC. This finding was confirmed by a further population-based study from California[91]. The TN incidence of 23% in a prospectively collected data of BC patients treated by neoadjuvant chemotherapy at the MD Anderson Cancer Center has been reported by Liedtke et al. [58].

In our study 28% of tumors were defined as TN. This small difference to other studies can be partly explained by unique high risk patient set in our analysis and small sample size.

4.2.3.2. Correlation basal-like and triple-negative status

We have found that only 33% of TN tumors were clustered as basal-like, although 80% of basal-like tumors were TN. Even 12% of luminal tumors were clinically triple-negative. There are 29 TN cases (44%), which were completely negative for all measured basal markers (EGFR, Ck5 and 17, vimentin, c-kit) even though the positive correlation has been observed between TN phenotype and all basal markers. There is evidence, that TN phenotype is not an ideal correlate of basal-like BC. Two expression profiling studies where the expression of hormone receptors was analysed in tumours classified according to the “intrinsic gene list”, 5-45% of basal-like BC expressed ER[64, 92]. In addition, Rouzier et al. have demonstrated that 14% of basal-like subtype express Her-2/neu[92]. On the other hand, triple-negative tumours are not necessarily basal-like BC. Tan et al., found that 6/31 (19%) of the triple negative tumours were negative for both EGFR and

basal CKs, whilst 15/207 (7.3%) of non-triple negative tumours were positive for basal markers[93]. In the recently published comparison of triple negative and five marker method in identification of basal-like BC, 17% of tumor were stained as triple-negative and only 9% as basal-like by 5 biomarkers[88].

Similarly to other studies the positive correlation of TN status and younger age of patients[91], similarly as for basal-like BC[52] could be also confirmed by our study. A significantly higher risk for local relapse in basal-like subtype and borderline significance (p=0,06) for increase of visceral metastasis risk in TN cases have been shown.

Several studies reported positive significant association of TNBC with visceral and lower risk for bone metastasis[55] or distant relapse as whole[57]. Several current studies identify TN phenotype also as predispositinated to cerebral metastasis and interestingly correlated with shorter median survival of approximately only 4-5 months after their occur compared with 11-12 months in other subtypes[94]. Regarding to local relapse the risk remains unclear. Rodriguez-Penilla revealed increased risk of local relapse in basal-like BC (defined by TN phenotype and CK5 and/or EGFR expression) in 258 node-negative patients[55]. In contrast to these results a study from Yale University revealed similar local relapse rates in TN and non TN BC, and found a significant difference only in ipsilateral axillary node relapse in 482 patients (117 were TN) treated by breast conserving surgery[57]. Dent et al. reported similar rates of local relapse (13 % in TN and 12% in non-TN, p=0.77), but shorter time to occur of event in TN vs. non-TN BC (2.8 vs. 4.2. years, p=0.02). The significant association of likelihood of distant relapse and death was pronounced within the first 5 years after diagnosis, but not later[56].

The recently published randomized study from the Danish group in 1000 patients treated by mastectomy and radiotherapy demonstrates the higher risk for locoregional relapse in TN BC and no additional benefit for radiotherapy after radical surgery in this subtype compared with patients with HR positive disease[95]. An another study of Nguyen et al. reported HR of 7.1 for basal-like (defined as TN) tumors for local relapse after lumpectomy and radiation therapy compared with luminal A (ER/PR + and Her-2/neu -) subtype 732 patients after 70 months of median follow up. The highest incidence of visceral metastases was also found in TN (HR 2,3) and luminal B (defined as HR and Her-2/neu positive) subtype[96].

4.2.3. Prognosis

In regard to the molecular classification as prognostic tool in HRBC our data are the world's first reported in this heavily treated collective.

Of the evaluated molecular markers, protein cluster subgroups (high proliferative and G3 associated basal-like and Her-2/neu were summarized due to similar 5 year RFS rates by Kaplan-Meier analysis and small size of subgroups) have overweighed all other single markers as independent prognostic factors for increased risk of relapse or death.

Some studies revealed prognostic impact of TN (defined by absent expression of HR and Her-2/neu) in patients treated by adjuvant/neoadjuvant chemotherapy [57, 58, 60]. In the study of Hanemann et al. TN and Her-2/neu subtypes had significantly worse RFS and OS compared with HR positive subtype in HRBC patients[90]. Tan et al. reported also worse DFS and OS in 245 patients homogenously treated by anthracyclines [93].

The TN phenotype, which was the spotlight of our interest, had no independent prognostic impact within our study population. The controverse of absence of prognostic impact in TN phenotype and significantly worse prognosis in basal-like BC within our study collective could play a important role for clinical routine. Recently Nielsen et al. reported the most comprehensive study worldwide in 3744 patients, where 17,4 % were identified as triple negative and 9% as basal-like using previously reported 5 marker method (HR, Her-2/neu, EGFR and/or CK5/6)[88]. The authors hypothesized, that negative impact of TN phenotype is affected only by a subgroup of basal tumors within TN collective. Patients with tumors identified as basal-like had a significantly worse outcome compared with other triple-negative cases. Particularly in patients treated by adjuvant anthracycline-based chemotherapy the addition of basal markers allowed the identification of a subgroup with higher risk for relapse. These results are in line with the previously reported study of Rakha et al. where a worse prognosis was associated to basal subtype (positive for basal markers) within the TN cases particularly in node-negative patients has been found[89].

Although there several studies indicating the worse outcome of Her-2/neu and basal-like subtypes by univariate analysis, as discussed above, there are very limited data comparing prognostic value of multiple protein expression with single markers by multivariate analysis. To our best knowledge there is no published trial investigating molecular classification as prognostic tool. The significant prognostic impact by multivariate analysis including several single conventional markers (e.g. tumor size, grade, age, etc.) has been demonstrated only for gene expression signature (e.g. 70

gene[48] or genomic grade signature[97]), which are significantly associated with basal-like or Her-2/neu subtypes[50].

4.2.4. Prediction

4.2.4.1. Age

Within most of the above mentioned trials as well in the WSG AM-01 trial, young patient age was demonstrated as a predictive factor for benefit from dose intensification and/or dose dense chemotherapy[11, 13, 28, 40, 76, 98]. The updated analysis of EBCTG overview confirmed these observations and highlighted more pronounced effect of adjuvant chemotherapy in patients younger than50 years old. In this group the allocation of anthracycline-based polychemotherapy was associated with 38% reduction of risk of death compared with 20% in patients older than 50 years old [11]. In contrast Muss et al. have shown similar benefits of chemotherapy in all age groups treated within CALBG trials[99]. The different pattern could be due to the aggressive biological profile (e.g. higher incidence of basal-like subtype) in younger patients.

4.2.4.2. HR Status

Ovarian ablation due to HD has been extensively discussed as one potential mechanism of action in hormone sensitive disease. Three trials reported better survival trend for HD within the HR positive subgroup[29, 76, 90]. Nevertheless as reported for other “dose dense” trials, the strongest benefits from HD in our own trial were found in HR negative disease[30, 40, 42]. These effects are very similar to overall chemotherapy efficacy reported for conventional chemotherapy trials[11, 42]. Rates of chemotherapy-induced amenorrhea were high in both therapy arms of our study (78% vs. 97%). However, it appears that biological characteristics such as proliferation and drug resistance within the luminal molecular classification subtype may be the decisive mechanisms for this correlation. This hypothesis is supported by molecular classification, where several markers of chemoresistance, like bcl-2 were expressed in the luminal A/B subtype.

In a clinical decision framework (HD vs. DD) the presence of three therapy interactions as found here by multivariate interaction analysis would imply that the benefit or risk associated with HD depends on which of the eight possible combinations (some of which are rare due to correlations) of the binary variables for grade, EGFR status, and triple negative status is present.

4.2.4.3.Grade

Poor nuclear grade, which is frequently associated with high proliferation and negative HR, was the strongest predictor for benefit from HD in our study and in other trials[76]. The predictive impact of G3 has been shown here by multivariate interaction analysis for the first time. More precisely, G3 patients in HRBC appear to benefit from HD rather than DD. Exceptions were the patients EGFR positive tumors who are not triple negative, who would appear to fare better with DD, all other things being equal. In only one study have G1 and low mitotic activity (next to age under 40 years old) been reported to correlate with benefit from HD, although this trial randomized 885 patients[13].

Our results regarding HD are fully consistent with chemosensitivity profiles based on negative HR, G3 status, as reported in patients intensively monitored under neoadjuvant chemotherapy[62].

4.2.4.5. Her-2/neu

Her-2/neu status has been identified in several randomized studies as well in our study as a reliable prognostic factor for poorer outcome in HRBC[100, 101] as well as in unselected cohorts of patients. In our study, hazard ratios for therapy are comparable in both groups of Her-2/neu status with significant benefit of HD in Her-2/neu negative and non-significant in Her-2/neu positive disease. These results are in line with other trials which used similar anthracycline doses in both treatment arms.[46]

However, negative Her-2/neu status correlates significantly with benefit from HD, as reported by Rodenhuis et al.[67], who used a 25% lower cumulative anthracycline dose in the HD arm compared to the standard arm. The proposed explanation of this observation (as being due to associated Topo IIα amplification in Her-2/neu positive disease as a target for anthracycline efficacy) was not confirmed in the recently published study of Hannemann et al.[90] Only 22% of the tumors in the Dutch study had co-amplification of Topo IIα and Her-2/neu, and there were no survival differences between both therapy groups in relation to Topo IIα status[90]. In contrast to these results, patients with positive Her-2/neu status and associated Topo IIα amplification (37%) had the most prolonged benefit from tailored dose-escalated FEC compared with HD (CTCb) in the sub-study of Tanner et al. in 396 tumors (75%) from the Scandinavian high dose trial. In the control arm the median total anthracycline dose was 4.3 times higher than in the HD arm[100]. In our study we have measured the protein expression of topoisomerase Iiα and have found the significant correlation of its expression with the high proliferative subtypes (her-2/neu and basal-like). It remains controversial what can be the optimal way to measure

anthracycline sensitivity in regard to Topo II (ampfilication/deletion/abnormal protein expression)[102].

Several studies have identified positive Her-2/neu status as a global marker, particularly for dose/schedule-response anthracycline sensitivity[40, 103] and for resistance to alkylating compounds[104], particularly in HD regimens in phase II trials[77, 105, 106]. A recently published meta-analysis of 5099 patients supports the conclusion of significant benefit of anthracycline-based vs. non-anthracycline-based chemotherapy only in Her-2/neu positive BC[107].

The significant benefit of HD vs. DD in Her-2/neu molecular subtype in our study in contrast to rather negative impact of Her-2/neu as single marker confirms the complex network within the very chemosensitive Her-2/neu subtype[59]. It could be hypothesized that the significant correlation of Her-2/neu subtype with G3, which is a highly predictive factor of benefit from rapidly cycled tandem HD by interaction analysis overweighs the negative impact of Her-2/neu at the resistance to alkylating agents, used for dose intensification. A second argument could be a expression p53 protein within the whole Her-2/neu subtype.

Beyond trastuzumab, optimal chemotherapy regimens in Her-2/neu positive breast cancer and its combination with HD in HRBC[108] remain to be defined. In particular, further investigations of the impact of several cytotoxic agents and their interaction with Topo IIα are needed in Her-2/neu positive HRBC, and this issue should preferentially be addressed by randomized trials.

4.3. Role of triple negativity and basal-like subtype as predictive factors

Triple negative breast cancer is of particular clinical interest because the chemotherapy is the only treatment option in this subgroup of patients. In our study, young triple negative (defined as ER, PR and Her-2/neu negative) patients benefited most from rapidly cycled tandem HD. In the TN group 5 year EFS rates were estimated at 71% in the HD arm and at 26% in the DD arm of the study. This trend to better efficacy of HD in TNBC was confirmed at borderline significance by other research groups[90]. The similar result has been observed in the basal-like subtype in our study but didn’t reach statistical significance. This fact can reflect also the smaller size of basal-like cohort due to wide distribution of triple-negative cases between several subtypes (at first basal-like and MMN).

In regard to the molecular classification Her-2/neu and basal-like subtypes responded significantly better to the rapidly cycled tandem HD compared with luminal A and particularly B subtype, where no difference between both treatments (HD vs. DD) has been observed.

Basal-like and/or TN BC is identified as a highly chemosensitive phenotype of BC by several studies. In vitro studies revealed different response patterns to 5-fluoruracil and anthracycline chemotherapy in luminal and basal cell lines[109]. Rouzier et al. identified molecular classification by microarray analysis in 82 patients with stage I-III BC and have shown impressive pathological complete remission (pCR) rates to preoperative paclitaxel/FAC chemotherapy of 45% in basal-like and Her-2/neu subtypes compared to only 6% in luminal subtypes[59]. In the second study by Carey et al. similar high pCR rate of 27% in TN (defined by 3 antibodies) BC vs. 7% in luminal tumors after 4 cycles of AC have been seen in 107 patients with stage II-III BC (32% had TNBC) [52]. Liedtke et al. demonstrated recently that TNBC has significantly higher pCR rates if treated by neoadjuvant chemotherapy of 22% vs. 11% compared with non-TNBC [58]. In both trials the worse survival of TN patients was addressed mainly to patients with residual disease after neoadjuvant chemotherapy and was most pronounced within the first 3 years after diagnosis, but not later. This study from the MD Anderson Cancer Institute involved more than 1100 patients provides further very important information. Patients who experienced a pCR had an excellent survival by DFS rates of 98% and 94% (TN and non-TN respectively) after 5 years irrespectively of their biological subtype. However if pCR was not achieved the 5 year DFS rates were 68% vs. 88% in favor of patients with non-TNBC. This experience led to conduction of a phase III trial by the Spanish breast cancer group, which investigates Capecitabine as maintenance treatment in patients with TNBC following standard adjuvant chemotherapy consisting of a minimum of six cycles.

The pCR rate to neoadjuvant FEC chemotherapy of 17% has been reported in TNBC vs. only 4% in non-triple negative cases[110]. At the same time different other studies have shown negative impact of TN status on survival in conventionally “A-treated” cohorts of patients[93, 111]. There are limited data from randomized trials investigate impact of implementation of taxanes in the adjuvant setting. Hayes et al. confirmed a similar survival benefit for substitution of 4 cycles of paclitaxel to 4 EC in the whole HR negative (as well Her-2/neu negative as positive cohort)[112]. The French group of Jacquemier et al presented analysis from the PACS 01 trial compared 6 cycles of FEC to 3 cycles of FEC followed by 3 cycles of Docetaxel in node positive BC. This group has applied 33

markers to identify two molecular subtypes (one “basal” with 531 (48% of investigated 1100 tumors) and one “luminal” similarly to their first “prognostic” study). Basal subtype had worse survival compared with luminal but significantly better response to taxane-based therapy than a control group (HR=0.65; p=0.009)[113]. The first analysis from GEICAM 9906 trial shows, that 8 cycles of paclitaxel weekly (100 mg/m^2) after 4 cycles of FEC vs. 6 cycles of FEC are also effective mostly in the 243 triple-negative BC patients (HR=0.58 (0.35-0.94), p=0,02)[71]. The paclitaxel weekly data became more interesting after presentation of first results of the US Oncology trial. This group compared conventional 4xAC-4xPaclitaxel q3w vs. 4xA/Paclitaxel followed by 12xPaclitaxel weekly. Particularly in the triple negative subgroup (378 patients) the best 5 year OS data in favour of paclitaxel weekly arm have been obtained (87% vs. 79%, HR=0,59, p=0,037)[114]. There is also a small phase 2 study presented at the SABCS in 2007 which arose pCR rate of 67% in TNBC patients treated by paclitaxel weekly and carboplatin AUC6 q4w[115].

Rugo et al reported better PFS (4,1 months vs. 2,1 months) and ORR (27% vs. 9%) in TNBC patients resistant to anthracycline/taxane based chemotherapy for combination of Ixabepilone and Capecitabine compared with Capecitabine alone, which is the current standard in this situation[116]. Taken together with results from a neoadjuvant trial from the same group, where Ixabepilone was associated with a 26% pCR rate in TNBC as a single agent, that is leading to design of PACS 08 trial, which investigates Ixabepilone in adjuvant setting in TNBC.

What can be an explanation for this paradox?

Several proliferative mechanisms like mitogen activated protein kinase (MAPK) and protein kinase components of the ERK pathway are beside of growth factors like EGFR and c-kit or p53 mutation critical points of development and therapy response in TNBC[117]. MAPK immunhistochemical overexpression has been recently shown to be associated with resistance against anthracyclines and shorter relapse-free survival in 109 unselected TN BC patients[118]. Ivanov et al. reported that small heat-shock protein Alpha B crystalline was commonly expressed in TNBC, inducing epidermal growth factor independent growth, cell migration and invasion and constitutively activated MAPK and ERK pathway and decreased response of tumors to preoperative chemotherapy (AC or AC followed by Taxol)[119]. Even Caveolin-1, which is frequently expressed by triple-negative phenotype[120] and acts as receptor for transcytosis for transportation of some drugs like ABI-007 (Abraxane) from blood to tumor. Abraxane is a novel albumin-bound

paclitaxel particle, that has shown greater efficacy and a favorable toxicity profile than conventional paclitaxel[121]. In light of fact, that there is a growing incidence for better efficacy of dose-dense (e.g. paclitaxel weekly) regimens in TNBC it could be beneficial to make use of the biological pathways to increase efficacy of chemotherapy.

Based on several proliferative pathways within the TNBC the another important point to overcome resistance could be dose and density of applied chemotherapy. Our actual data support this hypothesis in a impressive wise. Analysis of Le Tourneau et al. revealed an enhance of response (pCR rate) to a neoadjuvant chemotherapy from 13% by 4 cycles of conventional FEC to 47% in patients treated by intensified FEC (EC 70/700 mg /m^2, d1+8, 5-FU d1-5)[122].

Several studies have also identified triple-negative/basal-like as a typical phenotype of BRCA1 associated tumors[123]. Significant associations of BRCA1 mutated cases with EGFR expression (BRCA1 mutated vs. non-mutated controls: 67% vs. 21%, p<0,001), ER negativity (90,4% vs. 33%, p<0,001), Her-2/neu negativity (97% vs. 85%, p=0,018), Ck 5/6 overexpression (58% vs. 7%, p<0,001), p 53 mutations (66% vs. 33%, p=0,05)[124, 125] have been shown in numerous investigations.

In the recently presented study of Kandel et al. this correlation was particularly pronounced in younger patients or in patients with familiy history of breast cancer[126], which are frequently associated with p53 mutations. 11,3% patients had a BRCA1 mutation in an unselected cohort of 177 TN patients. In the <40 years old group there were 23,5% BRCA1 mutated compared with 0% in the group >60 years old. In the group <50 years old with familiar history the incidence of 29% has been reached compared to no patient older than 50 years old not related to family history.

In the study of Sorlie et al.[53] all BRCA1 associated cases are failed into the basal-like subtype, where other sporadic TN cases have been found. It seems to be evidence, that frequent cytogenetic aberrations, e.g. deletion of 5q chromosome, which are typical for BRCA1 mutated cases are similarly frequent in the sporadic triple-negative controls[127, 128]. A second point of discussion should be the significant association with p53 mutation, although the spectrum of mutations is distinct from the from that occurring in sporadic TN tumors[124]. One actual study published in Oncogene reports, that ataxia-telangiectasia-mutated (ATM) kinase, which is a key transducer of DNA damage signals within genome and a tumor suppressor, is aberrantly reduced in a very similar way in both BRCA1 (33%) or 2 (30%) mutant familiar breast cancer or in sporadic TNBC (20%), vs. only 10% in other cases[129].

There are controversial reports about methylation status of BRCA1 and its action in basal-like BC. There is one report indicate the lower expression of BRCA1 mRNA and higher activity of negative regulators of BRCA in sporadic basal-like BC compared with controls matched for age and grade, but the mechanisms of hypomethylation remain be unclear[130].

BRCA1 plays a central role in the repair of double-stranded DNA breaks by homologous recombination. A lack of BRCA1 results in genomic instability and therefore cancer disposition. Loss or inactivation of BRCA1 function is thought to be associated with sensitivity to DNA-damaging (e.g. alkylating) chemotherapy, which was used for dose intensification within our study[131], especially to agents resulting into cross links, such as Mitomycin C and platinum drugs[131]. The sensitivity of BRCA1 mutated cells to microtubule-agents, like taxanes or vinca-alkaloids is controversial. A recent preclinical study demonstrated that overexpression of p63 (a p53 related transcription factor) and p73 (similarly p53 associated) is commonly found in the TNBC and is associated with sensitivity to cisplatin, supporting the hypothesis, that TNBC should have similar sensitivity like BRCA deficient tumors[132]. In a study from the Dana Faber Cancer Institute preoperative treatment with 4 cycles of cisplatin as single agent led to pCR rate of 22% in 28 patients with TNBC[133]. The European Institue of Oncology used a polychemotherapy approach and applied preoperative chemotherapy with epirubicin, cisplatin and fluoruracil followed by paclitaxel in 30 patients with TNBC. This treatment led to the pCR rate of 40%[134] and Spanish group evaluates a neoadjuvant docetaxel-containing chemotherapy vs. carboplatin-containing chemotherapy in TNBC.

There are only few studies investigated effects of chemotherapy in BRCA1 deficient BC. A retrospective study from Israel investigated the effect of Epirubicin/Cyclophosphamide neoadjuvant chemotherapy in Ashkenazi Jews. There are impressive 92% pCR rate in 10 from 11 patients with hereditary BC (BRCA1 or 2) compared with 30% in 38 sporadic controls[135]. In contrast a recent study presented at the last ASCO Meeting investigated the effect of neadjuvant CEF (100 mg/m) in 393 patients 55 form them were TN and 14 pateints in this group had confirmed BRCA1 mutation. There were 44% pCR in TNBC as whole and only 17% in BRCA1 deficient tumors[136]. Goffin et al. have shown worse prognosis of BRCA1 mutated cases if not treated by chemotherapy compared with controls with the same patient characteristics[137], but an another recent study didn't confirm these observations[138]. All studies in BRCA1 mutant breast cancer are of retrospective nature, which makes comparisons very difficult to interpret.

There are several ongoing studies in patients with e.g. metastatic TNBC where cis- or carboplatin are used as chemotherapy drugs. The National Cancer Research Institute Triple Negative Breast cancer Trial (TNT) randomly assigns patients in 1:1 ratio to carboplatin or docetaxel, on progression patients cross over to the altenate regimen, An another phase II trail on cisplatin is investigating p63/73 as biomarkers to potentially predict response to cisplatin[139].

Another approach involves the poly (ADP-ribose) polymerase (PARP) inhibitors. Single-stranded breaks are usually repaired by the gene by the base excision repair pathway, of which PARP1 is one of the central components. Ion the absence of this pathway single-stranded breaks degenerate to doble-stranded breaks, which are not repaired in BRCA1 null cells.There is some preclinical evidence, that BRCA1 null cells are sensitive to PARP1 inhibitors[140].

Basal-like breast cancer has been shown to be strongly associated with EGFR expression. In EGFR negative patients, either G3 or triple negativity imply a benefit from HD. In EGFR positive patients, who had poor outcome by multivariate analysis, a benefit from HD is apparent only for tumors (the large majority) that are both triple negative and G3. However, in view of the small numbers in this subgroup and the methodological difficulties regarding EGFR testing (e.g. determination activated/total expression)[141], our results need to be substantiated before definite conclusions are possible.

Yet, our preliminary results suggest the need for additional targeted therapies, e.g. the use of EGFR inhibitors particularly in combination with adequate chemotherapeutical options in this subgroup. Based on the hypothesis of better efficacy of Platinum-drugs in BRCA1 deficient BC and preliminary results of e.g. enhance of radiosensitivity by use of Cetuximab Carey and colleagues presented initial results from TBCRC 001, a phase 2 study that randomized patients with metastatic TNBC to either Cetuximab or combination with weekly Carboplatin[142]. This report described the results from an interim analysis of the monotherapy arm only. In the analysis, 21 patents were accrued; out of this group, 1 patient (4%) experienced a prolonged partial response, 4 patients (19%) had stable disease for at least 8 weeks, and the rest developed disease progression. Upon progression, 19 patients were crossed over to the combination therapy arm, wherein 4 (28%) had a partial response and 4 more had stable disease. Due to the low observed response rate with monotherapy, that arm was closed to further accrual; results from the combination arm are awaited. O'Shaughnessy et al. also presented data on the use of Cetuximab in a phase 2 study of weekly Irinotecan and Carboplatin, with or without

Cetuximab. In the overall intention-to-treat study population, response rate improved only marginally from 28% to 33% with the addition of Cetuximab. Approximately half of the study population (78 patients) had triple-negative tumors. In that subgroup, Cetuximab was associated with a response rate of 49% compared with 30% when chemotherapy was used alone. No significant differences in PFS or overall survival were observed in any subgroup, and significant toxicity led to dose reductions in starting doses of the chemotherapy agents[143]. These results confirm the previously reported study of Corkey et al investigated impact of several EGR inhibitors in TNBC alone (erlotinib, gefitinib, Cetuximab) or in combination with Docetaxel or Carboplatin. This study revealed only limited efficacy of single substances in TNBC in vitro, but enhance in response to CT as combination.

Two recent reports from in-vitro studies support the use of small molecule multi-tyrosine kinase inhibitor Dasatinib, currently approved for treatment of bcr-abl mutated chronic myeloid leukemia resistant to Imatinib, particularly in this subgroup of BC, where diverse tyrosine kinase receptors like stem cell factor receptor (c-kit) are over-expressed and/or mutated [144, 145]. A second candidate drug for improvement of treatment results in TNBC could be sunitinib malate, an oral multitargeted tyrosine kinase inhibitor, that inhibits vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor, c-kit and colony-stimulating factor-1 receptor. In a recently published phase II study activity of monotherapy in metastatic disease resistant to anthracyclines and taxanes has been shown particularly in TNBC and Her-2/positive patients[146].

There are numerous ongoing phase II trials investigating effects of EGFR and VEGF inhibitors as well drugs targeting DNA and microtubulis in TNBC, which results are highly expected and will be immediately important for clinical management of TNBC where limited treatment options are available[139].

An another important point of basal-like BC is the possible prevention and an early diagnosis of disease. Large phase III prevention trials have shown that therapy by tamoxifen could reduce the risk of HR positive BC by approximately a half, that underlines again the distinct biology of HR positive and negative disease. There is no option of therapeutical intervention to prevent basal-like BC, but epidemiological studies confirm that e.g. longer breastfeeding and more breastfeed children and normal weight reduce the risk of development of basal-like BC, but not of luminal A or B disease.

Unfortunately only a few studies revealed other mammographic patterns of TNBC, to be associated less frequently with microcalcifications and peritumoral DCIS [147], which

makes other diagnostic tools necessary, as well for TNBC and high-grade DCIS, as precursor of TNBC[148].

4.4. Conclusions

HD provided an independent relative benefit in the triple negative phenotype (as long as EGFR is negative) not attributable to the association of grade with triple negativity. The finding of a predictive impact of triple negativity is also remarkable considering the absence of a prognostic impact. This association could be one hypothetical explanation for our results.

In our study molecular classification has been identified as a very strong prognostic parameter, particularly in conventionally treated HRBC. Basal-like and Her-2/neu subtypes determined by k-clustering were strong predictors for poorer outcome, in line with other studies in HRBC[90]. HD efficacy was also more pronounced in these subtypes. No significant interaction between molecular subtypes and dose intensification in HRBC has yet been reported, possibly due to small subgroups.

In contrast, both triple negativity and G3 status as assessed by standard clinical methodology were highly predictive for HD efficacy in HRBC, as shown in our study by interaction analysis. This effect exceeds the predictive value of molecular subtypes for selection of patients for whom dose intensification could be warranted. One possible explanation could be the heterogeneous biology within TNBC. The another point is the distinct biology within the triple-negative tumors, including basal-like and non-basal-like tumors.

Following conclusions can be drown from my work:

- For the first time molecular classification could be identified in the HRBC by protein expression profile and k-clustering
- Basal-like and Her-2/neu subgroups have worse survival outcome compared with luminal subtypes
- Tumor size and HR status are valid prognostic factors within HRBC like unselected cohorts of patients for both EFS and OS
- Molecular subtypes are independent prognostic factors by multivariate analysis within randomized HRBC collective
- Age, tumor size, grade, HR, Her-2/neu and EGFR status were predictive for effects of HD.

- HD is mostly effective within triple-negative breast cancer and within Her-2/neu and basal-like subgroups identified by protein expression analysis
- Interactions multivariate analysis reveals particularly triple-negative and not basal-like BC, grading and EGFR status as predictive markers of sensitivity to rapidly cycled tandem HD
- Diverse proliferative pathways and BRCA1 dysfunction within triple-negative/basal-like are discussed as cause of worse outcome and higher sensitivity to chemotherapy in breast cancer
- Additional molecular options need further investigations in this poor prognosis subtype of BC within randomized trials

Before robust and standardized subtyping using gene arrays is available, the immunhistochemical determination of TN status in addition to of routine tumor grade seems to be feasible for patient selection for dose intense/dense regimens in clinical routine.

In view of the clinical therapeutic challenges in management of tumors with a triple negative phenotype up to now, the finding of a predictive impact of triple negativity by multivariate interaction analysis with adjuvant HD vs. DD therapy could have very important clinical consequences. The HD approach is intended to overcome some chemoresistance mechanisms and to target rapidly proliferating tumor cells within a distinct molecular chemo-sensitive subgroup and would provide further information for defining optimal chemotherapy regimens within triple-negative tumors. All of the observed benefit from HD can be attributed to patients with triple negative and/or G3 tumors, as demonstrated by interaction analysis. Regrettably these results are revealed by retrospective unplanned analysis and are about a small subgroup of triple.negatve tumors. But in my opinion our results support design of randomized trials within a defined biological chemosensitive subtype, such as triple negative breast cancer, and prospective implementation of molecular biological markers for HRBC.

5. References

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7. Curriculum vitae

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Education

Middle School Lugansk/Ukraine	1988-1996
Emigration to Germany	09/1996
Secondary School Kahla/Thueringia	1997-2000
Heinrich Heine University School of Medicine Dusseldorf, Germany	2001-2007

State examination 2007: Note: excellent (1,5)

Residency (Gynecology) City Hospital of Krefeld	2006-2007
Breast Cancer and Gynecology Fellowship, Breast Centre Niederrhein (Academic hospital of the University of Aachen)	06/2007-present
Scientific assistance at the West German Study Group	01/2002-present

Honors and Awards

Student's Surgical Oncology Fellowship (National Cancer Institute of Ukraine, Kiev) 2005
Top Ten Presentation Award of the Meeting of the Translational Research Group (TRAFO) 18.05.2007
Scientific Award of the Annual Meeting Rhine-Westphalia Society of Gynaecology and Obstetrics 23.04.2008

Grant of ASCO-EORTC-NCI for 2008 Annual Meeting on Molecular Markers in Cancer: From Hypothesis to Product: Diagnostic Development Tutorial, Hollywood, Florida, 30.10.-1.11.2008

Professional Society Memberships

American Society of Clinical Oncology 2006 to present
German Society of Senology 2006 to present
EORTC Pathobiology group 2008 to present

Publications

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Oleg Gluz

8. Summary

Purpose: This work evaluates the prognostic and predictive impact of protein expression of various molecular markers in addition to conventional prognostic factors in high-risk breast cancer (HRBC) patients from a randomized trial. Patients received different chemotherapy dose-intensification strategies. The molecular classification of breast cancer should be evaluated in this selected population.

Methods: 403 patients were randomly assigned to dose-dense conventional chemotherapy with 4 cycles of $E_{90}C_{600}$ followed by 3 cycles of $C_{600}M_{40}F_{600}$q2w (DD) or a rapidly cycled tandem high-dose regimen with 2 cycles $E_{90}C_{600}$q2w followed by 2 cycles of $E_{90}C_{3000}Thiotepa_{400}$q3w (HD). Paraffin-embedded tumors from 236 patients were available for retrospective central pathology review (116 HD /120 DD). Expression of 34 molecular markers was evaluated immunohistochemically using tissue microarrays. Cluster groups were analyzed by unsupervised k-clustering (k=5). Results were correlated with follow-up data and treatment effects by proportional hazard Cox regression models (including interaction analysis).

Results: After a median follow-up of 61.7 months, 5-year event-free (EFS) as well as overall survival (OS) rates for the 236 patients were significantly better in the HD arm: EFS: 62% vs. 41%; OS: 76 % vs. 61%. 5 stable molecular subgroups could be identified by cluster analysis using 24 proteins: luminal A (27%) and B (12%), multiple marker negative (27%), Her-2/neu (21%) and basal-like (13%). Basal-like and Her-2/neu subtypes had significant inferior survival rates than luminal subtypes. Basal-like and Her-2/neu subtypes, but not luminal subtypes had a significant benefit from HDC in terms of EFS and OS. In multivariate analysis, HD, tumor size <2 cm and positive PR, as conventional factors and luminal A/B subtypes as new marker were associated with favorable outcome. Interaction analysis showed that triple negativity (ER/PR/HER-2/neu) and G3 status of tumors, but not basal-like cluster group predicted benefit from HD.

Conclusion: For the first time molecular subtypes of BC could be reconfirmed in the HRBC population. Tandem HD improves both EFS and OS in HRBC. This therapy effect may be partly attributable to superior efficacy in the subgroup of triple negative tumors and /or G3. Patients with basal-like and Her-2/neu subtypes derived the most benefit from HD, compared to luminal subgroups, but the predictive value was inferior compared to “conventional” factors such as triple-negative subtype or G3 status.