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«ΑΞΙΟΛΟΓΗΣΗ ΤΗΣ ΠΡΟΓΝΩΣΤΙΚΗΣ ΣΗΜΑΣΙΑΣ ΤΩΝ ΚΥΚΛΟΦΟΡΟΥΝΤΩΝ ΚΑΡΚΙΝΙΚΩΝ ΚΥΤΤΑΡΩΝ ΚΑΤΑ ΤΗ ΔΙΑΡΚΕΙΑ ΤΗΣ ΠΑΡΑΚΟΛΟΥΘΗΣΗΣ ΑΣΘΕΝΩΝ ΜΕ ΠΡΩΪΜΟ ΚΑΡΚΙΝΟ ΜΑΣΤΟΥ»

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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ΗΡΑΚΛΕΙΟ ΜΑΪΟΣ 2013

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SHORT CURRICULUM VITAE OF THE CANDIDATE

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Posters and Oral Presentations

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Abbreviations used

95% CI: 95% Confidence Interval

AJCC: American Joint Committee on Cancer

ASCO: American Society of Clinical Oncology

BCT: Breast Conserving Therapy

BMI: Body Mass Index

CD: Cluster of Differentiation

cDNA: complementary DNA

CK: Cytokeratin

CMF: Cyclophosphamide Methotraxate Fluorouracil

CNS: Central Nervous System

CTC: Circulating Tumor Cells

DCIS: Ductal Carcinoma In Situ

DEPC: diethylpyrocarbonate

DFS: Disease Free Survival

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

DTC: Disseminated Tumor Cells

EBCTCG: Early Breast Cancer Trialists' Collaborative Group

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial Mesenchymal Transition

EORTC: European Organization for Research and Treatment of Cancer

EpCAM: Epithelial Cell Adhesion Molecule

ER: Estrogen Receptor(s)

FISH: Fluorescence In Situ Hybridization

HR: Hazard Ratio

IHC: immunohistochemistry

mRNA: messenger RNA

NCCN: National Comprehensive Cancer Network

NSABP: National Surgical Adjuvant Breast and Bowel Project

OS: Overall Survival

PAI: Plasminogen Activator Inhibitor

PBMCs: Peripheral Blood Mononuclear Cells

pCR: pathologic Complete Response

PFS: Progression Free Survival

PR: Progesteron Receptor

RNA: Ribonucleic acid

RR: Relative Risk

RT-qPCR: Reverse Transcriptase– quantitative Polymerase-Chain-Reaction

SEER: Surveillance Epidemiology and End Results

SWOG: Southwest Oncology Group

TNM: Tumor Nodes Metastases

uPA: urokinase Plasminogen Activator

uPAR: urokinase Plasminogen Activator Receptor

US: United States

VEGF: Vascular Endothelial Growth Factor

WHI: Women's Health Initiative

Abstract

Background — the detection of CK-19 mRNA-positive circulating tumor cells (CTC) before and/or after adjuvant chemotherapy in patients with operable breast cancer is associated with poor clinical outcome. Reliable prognostic markers for late disease relapse are not available. In this study we investigated the value of CTC detection during the first 5 years of follow-up in predicting late disease relapse.

Patients and Methods — blood was analyzed from 312 women with operable breast cancer who had not experienced disease relapse during the first two years of follow-up. A real-time RT-PCR for CK-19 mRNA was used for CTC detection three months after the completion of adjuvant chemotherapy and every six months thereafter for a 5-year follow-up period.

Results — eighty (25.6 percent) patients remained CTC-free throughout the 5-year period. A change in CTC status was observed in 133 (42.6 percent) patients; 64 (20.5 percent) patients with initially CK-19 mRNA-positive CTC during the first 24 months turned CTC-negative afterwards while 69 (22.1 percent) who were initially CTC-negative became CTC-positive. Ninety-nine (31.7 percent) patients remained persistently CK-19 mRNA-positive. After a median follow-up period of 107 months (range: 38-161 months), the persistently CTC-positive patients with either hormonal receptor positive or negative tumors, had a higher risk of late disease relapse compared to the persistently CTC-negative patients (36.4 percent versus 11.2 percent, $p<0.001$). Multivariate analysis revealed that persistently CTC-positive patients also had a shorter disease-free ($p=0.001$) and overall survival ($p=0.001$).

Conclusion — persistent detection of CK-19 mRNA-positive CTC during the first 5 years of follow-up is associated with an increased risk of late relapse and death for patients with operable breast cancer, indicating the presence of chemotherapy- and hormonotherapy-resistant residual disease. This prognostic evaluation may be useful when deciding on subsequent adjuvant systemic therapy.

Εκτενής περίληψη στα Ελληνικά

Εισαγωγή: Ο διηθητικός καρκίνος του μαστού είναι η πιο συχνή κακοήθεια στις γυναίκες, αντιπροσωπεύοντας το 28% των νέων περιπτώσεων καρκίνου και το 15% των θανάτων από καρκίνο. Λόγω της μείωσης των ποσοστών θνησιμότητας, τα οποία οφείλονται στην ευρεία εφαρμογή του προσυμπτωματικού ελέγχου με μαστογραφία και στην επικουρική θεραπεία, περισσότερες γυναίκες επιβιώνουν στις μέρες μας από τη νόσο. Δεδομένου ότι η μεταστατική νόσος θεωρείται ανίατη, η έγκαιρη αναγνώριση και θεραπεία της δυνητικά ακόμα ιάσιμης ελάχιστης υπολειπόμενης νόσου είναι ένας από τους σημαντικότερους στόχους της κλινικής έρευνας των ασθενών και προϋποθέτει την κατανόηση σε βάθος των προτύπων υποτροπής.

Η παρουσία καρκινικών κυττάρων στο μυελό των οστών και κυκλοφορούντων καρκινικών κυττάρων (ΚΚΚ) στο περιφερικό αίμα των ασθενών με πρώϊμο καρκίνο του μαστού έχουν αποδειχθεί ως ανεξάρτητοι δυσμενείς προγνωστικοί παράγοντες για υποτροπή και θάνατο από τη νόσο. Η ανοσοκυτταροχημεία με τη χρηση αντισωμάτων έναντι πρωτεϊνών που εκφράζονται σε επιθηλιακά αλλά όχι σε μεσεγχυματικά κύτταρα χρησιμοποιείται ευρέως για την ανίχνευση των ΚΚΚ. Ωστόσο, η ανίχνευση της γονιδιακής έκφρασης συγκεκριμένων επιθηλιακών δεικτών με τη χρήση qPCR φαίνεται να έχει υψηλότερη διαγνωστική ευαισθησία.

Η κυτταροκερατίνη-19 (Cytokeratin-19, CK-19) έχει χρησιμοποιηθεί ευρέως για την ανίχνευση των καρκινικών κυττάρων του μαστού σε μεσεγχυματικούς ιστούς και φαίνεται να είναι ευαίσθητος και αξιόπιστος βιοδείκτης σε ασθενείς με πρώϊμη και μεταστατική νόσο. Αρκετές μελέτες έχουν δείξει την προγνωστική σημασία της ανίχνευσης CK-19 mRNA-θετικών ΚΚΚ σε ασθενείς με πρώϊμο καρκίνο του μαστού. Ωστόσο, σε όλες αυτές τις μελέτες έχει μελετηθεί η προγνωστική αξία των ΚΚΚ κατά την αρχική διάγνωση και πριν από την έναρξη ή/και μετά την ολοκλήρωση της συμπληρωματικής χημειοθεραπείας. Μόνο λίγα δεδομένα υπάρχουν σχετικά με την κλινική σημασία της ανίχνευσης καρκινικών κυττάρων στο μυελό των οστών, αλλά καμία για τα ΚΚΚ κατά τη διάρκεια της περιόδου παρακολούθησης μετά την ολοκλήρωση της συμπληρωματικής χημειοθεραπείας. Οι μελέτες αυτές ανέδειξαν την δυσμενή κλινική έκβαση των ασθενών με ανιχνεύσιμα κύτταρα του όγκου στο μυελό των οστών.

Στην παρούσα μελέτη, επιδιώξαμε να αξιολογήσουμε την κλινική σημασία της ανίχνευσης των CK-19 mRNA-θετικών ΚΚΚ με την εφαρμογή RT-qPCR σε διαφορετικά χρονικά σημεία κατά την περίοδο παρακολούθησης μετά την ολοκλήρωση της συμπληρωματικής χημειοθεραπείας, σε ασθενείς με πρώϊμο καρκίνο του μαστού. Υποθέσαμε ότι οι ασθενείς που παρουσιάζουν ανιχνεύσιμο CK-19 mRNA κατά τη διάρκεια της παρακολούθησης, μετά τη χορήγηση επικουρικής θεραπείας έχουν αυξημένο κίνδυνο όψιμης υποτροπής της νόσου (υποτροπή τουλάχιστον δύο χρόνια μετά τη λήξη της συμπληρωματικής χημειοθεραπείας) και θανάτου από τη νόσο.

Ασθενείς – Μέθοδοι: Πραγματοποιήθηκε μια αναδρομική ανάλυση δεδομένων που συλλέχθηκαν προοπτικά στο πλαίσιο μιας συνεχιζόμενης μελέτης που έχει ξεκινήσει πριν πολλά χρόνια στο Εργαστήριο Βιολογίας του Καρκίνου. Γυναίκες με χειρουργήσιμο καρκίνο του μαστού (στάδιο I έως III), οι οποίες ήταν υπό παρακολούθηση και δεν είχαν παρουσιάσει υποτροπή της νόσου κατά τη διάρκεια των δύο πρώτων ετών μετά το πέρας της επικουρικής χημειοθεραπείας ήταν κατάλληλες για τη μελέτη. Μετά την ολοκλήρωση της συμπληρωματικής χημειοθεραπείας, οι ασθενείς έλαβαν συμπληρωματική ακτινοθεραπεία και ορμονική θεραπεία σύμφωνα με τα χαρακτηριστικά της νόσου. Η εξέταση δειγμάτων για CK-19 mRNA-θετικών ΚΚΚ γίνονταν σε συγκεκριμένα χρονικά σημεία μετά την ολοκλήρωση της συμπληρωματικής χημειοθεραπείας για μια 5-ετή περίοδο παρακολούθησης. Το πρώτο δείγμα αίματος λαμβάνονταν 3 μήνες μετά το τέλος της χημειοθεραπείας και στη συνέχεια κάθε 6 μήνες έως τη συμπλήρωση 5 ετών.

Οι ασθενείς ταξινομήθηκαν σε τέσσερις ομάδες ανάλογα με την ανίχνευση ή όχι ΚΚΚ κατά τα δύο πρώτα χρόνια και τα επόμενα τρία χρόνια παρακολούθησης (όπως επίμονα αρνητική, επίμονα θετική, αρνητικό μετατροπή σε θετικό και το αντίθετο). Χρησιμοποιώντας τον ορισμό αυτό, οι ασθενείς ταξινομήθηκαν στην "επίμονα ΚΚΚ-αρνητική" ομάδα, αν δεν είχαν κανένα θετικό δείγμα αίματος για CK-19 mRNA σε όλη την 5-ετή περίοδο παρακολούθησης. Από την άλλη πλευρά, η «επίμονα ΚΚΚ-θετική» ομάδα περιελάμβανε ασθενείς με τουλάχιστον ένα θετικό δείγμα αίματος για CK-19 mRNA στα πρώτα 2 έτη και τουλάχιστον άλλο ένα κατά τα επόμενα 3 χρόνια παρακολούθησης. Κατά συνέπεια, οι ασθενείς στην "CTC-αρνητική/θετική" ομάδα δεν είχε θετικά δείγματα στα δύο πρώτα χρόνια, αλλά τουλάχιστον ένα θετικό δείγμα στα επόμενα 3 χρόνια. Το αντίθετο ίσχυε για την "CTC-θετική/αρνητική ομάδα.

Είκοσι χιλιοστόλιτρα (ml) περιφερικού αίματος συλλέγονταν σε κάθε επίσκεψη σε σωληνάρια που περιείχαν EDTA. Για να αποφεύγεται η επιμόλυνση με τα επιθηλιακά κύτταρα από το δέρμα, όλα τα δείγματα αίματος λαμβάνονταν στα μέσα της παρακέντησης φλέβας και αφού τα πρώτα 5 mL αίματος απορρίπτονταν. Μονοπύρηνα κύτταρα του περιφερικού αίματος λαμβάνονταν μετά από διαβαθμιζόμενη φυγοκέντρηση χρησιμοποιώντας Ficoll-Hypaque. Η απομόνωση του RNA γίνονταν με τη χρήση των αντιδραστηρίων Trizol LS (Gibco-BRL, Grand Island, NY, Η.Π.Α.). Το απομονωμένο RNA διαλύονταν σε diethylpyrocarbonate επεξεργασμένο νερό και αποθηκεύονταν στους -80°C μέχρι να χρησιμοποιηθεί. Η αντίστροφη μεταγραφή του RNA γίνονταν με την μέθοδο Thermoscript RT-PCR (Life Technologies-Invitrogen, Ηνωμένο Βασίλειο). Το συμπληρωματικό DNA (cDNA) συνθέτοταν σύμφωνα με τις οδηγίες του κατασκευαστή. Η μέθοδος RT-qPCR μέθοδος για την ανίχνευση της έκφρασης της CK-19 έχει περιγραφεί προηγουμένως.

Η επιβίωση ελεύθερη νόσου (EEN), η οποία ορίζεται ως ο χρόνος από την έναρξη της μελέτης μέχρι την ημέρα υποτροπής της νόσου και η συνολική επιβίωση (ΟΕ) που μετριέται από την έναρξη της μελέτης μέχρι το θάνατο ανεξαρτήτως αιτιολογίας ήταν τα κύρια καταληκτικά σημεία της μελέτης. Κλινικοπαθολογοανατομικοί παράγοντες που είναι γνωστό ότι σχετίζονται με την πρόγνωση, όπως η εμμηνοπαυσιακή κατάσταση (προεμμηνοπαυσιακές vs μετεμμηνοπαυσιακές), το μέγεθος του όγκου (T1 vs T2-3), ο αριθμός των διηθημένων μασχαλιαίων λεμφαδένων (0-3 vs ≥ 4), ο βαθμός διαφοροποίησης (grade: 1-2 vs 3), οι οιστρογονικοί υποδοχείς (ER) (αρνητικοί έναντι θετικών), οι υποδοχείς προγεστερόνης (PR) (αρνητικοί έναντι θετικών) και η ενίσχυση του *HER-2/neu* (αρνητικό έναντι θετικού) εξετάστηκαν σε μονοπαραγοντική και πολυπαραγοντική ανάλυση.

Αποτελέσματα: Συνολικά 455 διαδοχικοί ασθενείς με διάγνωση καρκίνου του μαστού που αντιμετωπίστηκαν θεραπευτικά στην κλινική Παθολογικής-Ογκολογίας του Πανεπιστημιακού Νοσοκομείου Ηρακλείου μεταξύ Ιανουαρίου 1997 και Δεκεμβρίου 2004 εξετάστηκαν για την ένταξη τους στη μελέτη αυτή. Εκατόν σαράντα τρεις ασθενείς αποκλείστηκαν και 312 συμπεριλήφθηκαν στη μελέτη. Οι ασθενείς που βρέθηκαν θετικοί στην παρουσία ΚΚΚ δεν διέφεραν σε σχέση με όσες παρέμειναν αρνητικές σε ότι αφορά στα χαρακτηριστικά τους [ηλικία (p=0.197), εμμηνοπαυσιακή κατάσταση (p=0.372)] ή στα χαρακτηριστικά των όγκων

τους [μέγεθος (p=0.637), διήθηση λεμφαδένων (p=0.082), ιστοπαθολογική διαφοροποίηση (p=0.746) και κατάσταση των ορμονικών υποδοχέων (p =0.156)].

CK-19 mRNA-θετικά κύτταρα ανιχνεύθηκαν σε 232 ασθενείς (74.4%) σε οποιοδήποτε χρονικό σημείο κατά τη διάρκεια της 5ετούς παρακολούθησης, ενώ 80 ασθενείς (25.6%) παρέμειναν «αρνητικοί» καθ 'όλη την ίδια περίοδο (επίμονα αρνητική ομάδα). Πιο συγκεκριμένα, 99 ασθενείς (31.7%) είχαν επίμονα ανιχνεύσιμα CK-19 mRNA-θετικά κύτταρα τόσο κατά τη διάρκεια των δύο πρώτων όσο και τα επόμενα τρία χρόνια παρακολούθησης (επίμονα θετική ομάδα). Μια αλλαγή του CK-19 mRNA παρατηρήθηκε σε σχεδόν μισές από τις ασθενείς (133 ασθενείς ή 42.6%). Από αυτές, 64 ασθενείς (20.5%) με ανιχνεύσιμα αρχικά CK-19 mRNA-θετικά κύτταρα κατά τη διάρκεια των πρώτων 24 μηνών έγιναν «CTC αρνητικές» στη συνέχεια (θετική/αρνητική ομάδα), ενώ 69 ασθενείς (22.1%) που ήταν αρχικά «CTC αρνητικές» έγιναν «CTC θετικές» (αρνητική/θετική ομάδα).

Μετά από μια διάμεση περίοδο παρακολούθησης 107 μηνών (εύρος: 38 - 161 μήνες), 63 ασθενείς (20.2%) είχαν αναπτύξει απομακρυσμένη (n=56; 88.8%) ή τοπική υποτροπή (n=7; 11.2%) . Σε σύγκριση με την «επίμονα αρνητική» ομάδα ασθενών, μόνο η ομάδα των «επίμονα θετικών» είχε σημαντικά υψηλότερο κίνδυνο υποτροπής της νόσου (36.4% έναντι 11.2%, p<.001). Στην πραγματικότητα, ο κίνδυνος επανεμφάνισης της νόσου ήταν υψηλότερος σε ασθενείς με επίμονα θετικά ΚΚΚ (36.4% έναντι 7.8%, p<0.001 και 36.4% έναντι 18.8%, p=0.016 σε σύγκριση με θετική/αρνητική και αρνητική/θετική ομάδα, αντίστοιχα).

Τα ποσοστά 5-ετούς ΕΕΝ ήταν 82.5% έναντι 92.7% για τις επίμονα θετικές και επίμονα-αρνητικές ασθενείς, αντίστοιχα. Επιπλέον οι επίμονα θετικές ασθενείς είχαν σημαντικά μικρότερη ΕΕΝ συγκριτικά με τις επίμονα αρνητικές ασθενείς (p<0.001). Παρά το γεγονός ότι καμία ομάδα δεν έχει φθάσει στη διάμεση ΕΕΝ, υπήρξε μια σταδιακή μείωση της ΕΕΝ για τις τέσσερις ομάδες των ασθενών, σύμφωνα με την ανίχνευση των CK-19 mRNA-θετικών ΚΚΚ κατά τα 5 χρόνια παρακολούθησης.

Σαράντα-μία ασθενείς (13.1%) απεβίωσαν κατά τη διάρκεια της παρακολούθησης, ως συνέπεια της εξέλιξης της νόσου. Είκοσι τέσσερις (58.5%) και πέντε (12.2%) από αυτούς τους θανάτους συνέβησαν στην επίμονα-θετική και επίμονα-αρνητική ομάδα, αντίστοιχα (p=0.001). Η 10-ετής συνολική επιβίωση ήταν 81.4% για τις επίμονα θετικές έναντι 96.7% για τις επίμονα αρνητικές ασθενείς. Η εκτιμώμενη διάμεση συνολική επιβίωση ήταν

σημαντικά μικρότερη για τις επίμονα θετικές σε σύγκριση με τις επίμονα αρνητικές ασθενείς (*p*=0.013).

Καθώς οι ασθενείς υποβλήθηκαν σε σειρά μετρήσεων των CK-19 mRNA-θετικών ΚΚΚ, αναλύθηκαν τα δεδομένα για να απαντηθεί το ερώτημα αν το αθροιστικό αποτέλεσμα των θετικών μετρήσεων ανά ασθενή προσφέρει επιπλέον προγνωστική πληροφορία. Μεταξύ των ασθενών με θετικές μετρήσεις 38.7% (κατά τη διάρκεια των δύο πρώτων ετών της παρακολούθησης), 24.4% (κατά τα επόμενα 3 έτη) και 57.3% (κατά τη διάρκεια όλων των 5 ετών) είχαν δύο ή περισσότερα θετικά αποτελέσματα. Δεν βρέθηκε διαφορά στην ελεύθερη νόσου επιβίωση μεταξύ των ομάδων με διαφορετικό συνολικό αριθμό θετικών τεστ, πιθανώς λόγω του μικρού αριθμού των ασθενών και συμβάντων σε κάθε ομάδα.

Δεδομένης της προγνωστικής σημασίας της ανίχνευσης των ΚΚΚ πριν από τη χορήγηση συμπληρωματικής χημειοθεραπείας, εξετάσθηκε το ερώτημα αν θα μπορούσε να προσφέρει πρόσθετη προγνωστική πληροφορία με αυτό της διαδοχικής μέτρησης των ΚΚΚ κατά τη διάρκεια της παρακολούθησης. Για το σκοπό αυτό, μελετήθηκε η κατάσταση των ΚΚΚ προ-χημειοθεραπείας, όλων των ασθενών που περιλαμβάνονται στην παρούσα ανάλυση. Δεν προέκυψε διαφορά στο ποσοστό ανίχνευσης των CK-19 mRNA-θετικών ΚΚΚ μεταξύ των τεσσάρων ομάδων (Pearson Chi-square, *p*=0.320). Είναι ενδιαφέρον, ότι οι «επίμονα θετικές» ασθενείς με ανιχνεύσιμα CK-19 mRNA-θετικά ΚΚΚ πριν από τη χορήγηση συμπληρωματικής χημειοθεραπείας είχαν μικρότερη EEN, αλλά όχι συνολική επιβίωση (OE) σε σύγκριση με τους ασθενείς της ίδιας ομάδας που βρέθηκαν αρνητικές για ΚΚΚ σε μέτρηση πριν την έναρξη της χημειοθεραπείας.

Τέλος, έγινε ανάλυση υποομάδων ανάλογα με την ορμονική κατάσταση του πρωτοπαθούς όγκου. Είναι ενδιαφέρον, ότι η «επίμονα θετική» ομάδα ασθενών με όγκους είτε θετικούς είτε αρνητικούς για ορμονικούς υποδοχείς είχαν σημαντικά υψηλότερο ποσοστό υποτροπής, κίνδυνο θανάτου και μικρότερη EEN συγκριτικά με τις «επίμονα αρνητικές» ασθενείς (*p*=0.039 και *p*=0.004 για επίμονα θετικές vs επίμονα αρνητικές ασθενείς με ER/PR αρνητικούς και ER ή/και PR θετικούς όγκους, αντίστοιχα). Ωστόσο, η συνολική επιβίωση ήταν μικρότερη μόνο για τις «επίμονα θετικές» ασθενείς με ER/PR αρνητικούς όγκους (*p* =0.035).

Η επίμονη ανίχνευση των CK-19 mRNA-θετικών ΚΚΚ κατά τη διάρκεια της παρακολούθησης μετά την ολοκλήρωση της συμπληρωματικής χημειοθεραπείας, το

μέγεθος του όγκου μεγαλύτερο από 2.0 cm, η διήθηση περισσοτέρων από 3 μασχαλιαίων λεμφαδένων και η μετεμμηνοπαυσιακή κατάσταση σχετίζονταν με σημαντικά μικρότερη EEN και OE σε μονοπαραγοντική ανάλυση. Η πολυπαραγοντική ανάλυση έδειξε ότι η επίμονη ανίχνευση των CK-19 mRNA-θετικών ΚΚΚ, το μέγεθος του όγκου και περισσότεροι από 3 διηθημένους μασχαλιαίους λεμφαδένες ήταν ανεξάρτητοι προγνωστικοί παράγοντες για μικρότερη EEN και OE.

Συμπέρασμα: Τα αποτελέσματα αυτά αποτελούν την πρώτη ένδειξη για την προβλεπτική αξία της ανίχνευσης των CK-19 mRNA-θετικών ΚΚΚ κατά την παρακολούθηση ασθενών που ολοκλήρωσαν την τοπική θεραπεία και συστηματική χημειοθεραπεία. Τα ευρήματα αυτά υποστηρίζουν το ρόλο της παρακολούθησης των ΚΚΚ, ως συμπλήρωμα του ιστορικού, της φυσικής εξέτασης και των ακτινολογικών μεθόδων για την αξιολόγηση της κατάστασης της νόσου κατά τη διάρκεια της παρακολούθησης ασθενών με πρώιμο καρκίνο του μαστού.

Part A

Minimal Residual Disease in Breast Cancer

1. Epidemiology and risk factors for breast cancer

In Greece, breast cancer is the most common cancer and the second most common cause of cancer death in women. Approximately 4,400 new cases of invasive breast cancer were diagnosed in 2008, and almost 2000 women died from the disease [1]. About one-half of cases can be explained by known risk factors, such as age at menarche, first live birth, menopause, and proliferative breast disease. An additional 10 percent of cases are explained by a positive family history. Understanding the risk factors for breast cancer permits us to identify women at increased risk and to intervene to modify their risk. These risk factors are many and the major ones will be discussed briefly.

1.1 ***Age/Gender*** — Age and gender are among the strongest risk factors for breast cancer. Breast cancer occurs 100 times more frequently in women than in men [2]. However, in both sexes the incidence rates rise sharply with age until the age of 45 to 50 years after which the rise is less steep. The impact of hormonal change (menopause) that occurs around that time, probably explains this change of the slope, although alternative hypotheses have been proposed. At age 75 to 80 the curve flattens and slightly decreases thereafter only in women (figure 1) [3].

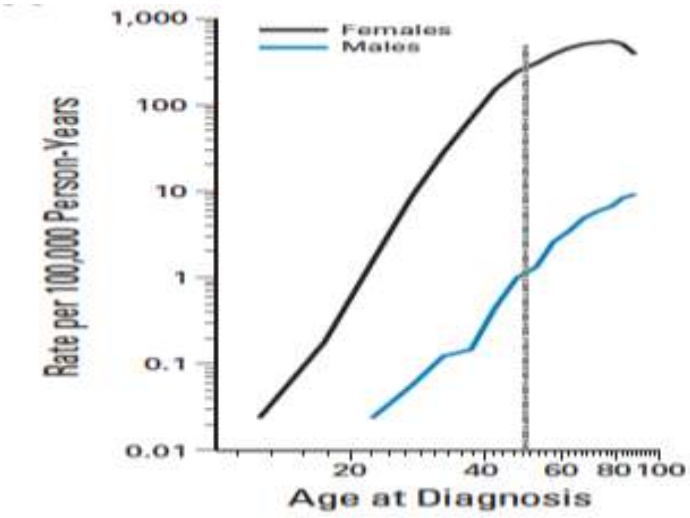

Figure 1: Age-specific incidence rates for breast cancer
(adapted with revision from Korde et al, Journal of Clinical Oncology 2010;28:2114).

1.2 ***Benign breast disease*** — Benign breast diseases include a wide spectrum of pathologic entities. Single non-proliferative lesions (fibrocystic change, simple fibroadenoma) are not associated with an increased risk for breast cancer. However, multiple non-proliferative lesions may be associated with an increased risk for breast cancer [relative risk (RR) 1.8 at 10 years] [4]. Proliferative lesions are risk factors for both noninvasive and invasive breast cancer, especially if cytologic atypia is present. The risk of invasive breast cancer is slightly increased (RR 1.3 to 2) for proliferative lesions without atypia (complex fibroadenoma, sclerosing adenosis, intraductal papillomas, moderate or florid hyperplasia), but it is significantly higher (RR 4 to 6) for if atypia is present (atypical lobular or ductal hyperplasia) and when the atypia is multifocal the risk is the highest (10-fold) [5].

1.3 ***Personal history of breast cancer*** — A personal history of invasive or in situ breast cancer is associated with a subsequent increased risk of an invasive breast cancer in the contralateral breast. In case of personal history of ductal carcinoma in situ (DCIS), the annual risk of developing a contralateral invasive breast cancer is 0.5 percent [6], meaning that the risk in 10 years is approximately 5 percent. Invasive breast cancer increases the risk of developing contralateral breast cancer to 1 and 0.5 percent per year for premenopausal and postmenopausal women, respectively.

1.4 ***Lifestyle and dietary factors***

1.4.1 Socioeconomic status — Higher socioeconomic status is associated with a greater risk for breast cancer, with a twofold increase in incidence from lowest to the highest strata. However, socioeconomic status does not appear to be an independent risk factor. Differences in reproductive patterns with respect to parity, age at first birth, smoking, and utilization of screening mammography seem to explain the increased incidence of breast cancer in women of high socioeconomic status (educational, occupational, and economic level) [7].

1.4.2 Body size — Weight and body mass index (BMI) have different effects on postmenopausal and premenopausal breast cancer risk. More specifically, postmenopausal women with higher weight/BMI or women who gained weight after menopause have been found to have an increased risk for breast cancer in multiple studies. In a pooled analysis of

seven prospective studies in the United States a 25 percent higher breast cancer risk has been shown for women who weighed at least 80 kg as compared to those weighing less than 60 kg, after adjusting for height [8]. In other words, women with a BMI >33 kg/m^2 had a 27 percent increased breast cancer risk compared to those with a BMI <21 kg/m^2 [8]. Increased peripheral conversion of estrogen precursors to estrogen by the adipose tissue resulting in higher circulating levels of estrogens might explain the higher risk. In addition to that, obesity is also associated with higher levels of insulin that is a known growth factor, and hyperinsulinemia may also explain the obesity-breast cancer relationship. In the same study premenopausal women with a BMI ≥31 kg/m^2 were 46 percent less likely to develop breast cancer than those with a BMI <21 kg/m^2. The higher BMI associated with irregular or longer menstrual cycles or with polycystic ovary syndrome could be an explanation for this association. The anovulation, associated with decreased levels of estradiol and progesterone and subsequent lower risk of breast cancer ("see hormonal factors" below) might be caused by these characteristics. Finally, increased height has been associated with a higher risk for breast cancer in both premenopausal and postmenopausal women. Women who were at least 175 cm tall were 20 percent more likely to develop breast cancer than those less than 160 cm tall according to the previously described pooled analysis of seven prospective cohort studies [8]. Prenatal as well as childhood exposures, such as birth weight and diet/energy balance most probably explain this association.

1.4.3 Fat intake — Despite the positive correlation between fat consumption and increased breast cancer risk shown in multiple animal studies, the results of case-control and prospective cohort studies have been conflicting. This might be due to interaction between reproductive variables, menopausal status, and fat intake. In particular a pooled analysis of more than 300,000 women (the majority postmenopausal) showed no association between fat (total, saturated, monounsaturated, or polyunsaturated) and breast cancer risk [9]. Similarly, the intervention to reduce total fat intake had no meaningful effect on breast cancer risk according to the Dietary Modification arm of the Women's Health Initiative [10]. The investigators randomly assigned 48,835 healthy postmenopausal women aged 50 to 79 to decrease fat intake via monthly group sessions in the first year followed by quarterly maintenance sessions thereafter versus a control group that received a packet of nutrition information without specific instructions. No difference in breast cancer risk between the two groups [hazard ratio (HR)=0.91; 95%CI 0.83-1.01] was found after a

median follow up of 8.1 years. In contrast, the data linking higher risk of breast cancer with postmenopausal increased BMI and weight gain have been consistent and demonstrate a stronger association.

1.4.4 Alcohol — More than 40 epidemiologic studies have tested the possible association between alcohol intake and breast cancer. Compared to no drinkers, women who consume moderate to high levels of alcohol (≥three drinks/day) have a higher breast cancer risk. There is a significant dose response relationship beginning with intakes as low as three to six drinks per week. In the Nurses' Health Study, which is the largest cohort study examining the association between alcohol and breast cancer 105,986 women were followed from 1980 until 2008 [11]. The study was powered enough to detect a small increased risk in breast cancer (RR=1.15; 95%CI 1.06-1.24) at levels of alcohol consumption equivalent to three to six drinks per week, compared to no drinkers. A 10 percent increase in risk with each 10 g per day of alcohol intake has been shown. Cumulative lifetime alcohol intake was linearly correlated with breast cancer risk and was most strongly associated with drinking patterns in both early and late adult life. It is noteworthy that the proportion of breast cancer cases attributed to alcohol intake varies widely. Only 2 percent of the population breast cancer risk in the United States is estimated to be due to alcohol consumption [12]. However, in Europe the risk for breast cancer has been reported to be higher. In the EPIC (European Prospective Investigation into Cancer and Nutrition) study that enrolled over 250,000 women from eight European countries a 5 percent of breast cancer risk was attributed to alcohol consumption [13].

1.5 *Reproductive/Hormonal risk factors* — Both prolonged exposure to and higher concentrations of endogenous estrogen increase the risk of breast cancer. The production of estrogen (estradiol, estriol, estrone) is modulated by ovarian function and influenced by the menarche, pregnancy, and menopause. But after menopause, the main source of estrogen is dehydroepiandrosterone (DHEA), which is produced by the adrenal gland and metabolized in peripheral fat tissue to estradiol and estrone. Age at menarche, age at first live birth, age at menopause, and possibly parity and breastfeeding are the key reproductive factors that influence breast cancer risk.

1.5.1 Age at menarche and menopause — Earlier menarche is associated with a higher risk of breast cancer. A 10 percent reduction in cancer risk for every two-year delay in the onset of menarche was reported [14]. In another interesting case control study of disease-concordant monozygotic twin pairs, the twin with earlier onset of menses was found to be five times more likely to be diagnosed with breast cancer supporting the hypothesis that age at menarche may influence the biology of breast cancer [15]. Higher cumulative lifetime estrogen exposure may explain the association between age of menarche and breast cancer. This hypothesis is supported by other observations for menstrual factors like late menopause that increases breast cancer risk. Each year older at menopause, increases the relative risk by 1.03 percent, while bilateral oophorectomy before the age of 40 reduces lifetime risk by 50 percent [16]. However, in case of hormonal replacement therapy this risk reduction is eliminated.

1.5.2 Pregnancy-related factors — Nulliparous women have an increased risk for breast cancer compared with parous women with a relative risk as high as 1.7 [17]. It takes 10 years following delivery for the protective effect of pregnancy to be seen [18]. However, breast cancer risk increases transiently after a full-term pregnancy. Whether multiparity confers protection against breast cancer has been a matter of controversy with the majority of studies suggesting a decreased risk with increasing number of pregnancies.

The younger a woman completes her first full-term pregnancy, the lower her breast cancer risk but advanced age at first full-term birth can be associated with an increased risk [17]. The cumulative incidence of breast cancer up to age 70 for parous versus nulliparous women was 20 percent lower if the first birth was before the age 20, 10 percent lower for first birth before age 25, and 5 percent higher if the first birth was after age 35 based on data from the Nurses' Health Study [19]. In other words, if a woman has her first full term birth at age 30 then the protective effect is eliminated and is similar to a nulliparous woman. The full cellular differentiation, which occurs in the gland during and after pregnancy, is protective for the breast and explains the effect of early first live birth. The protective effect of younger age at first birth pregnancy is hypothesized to be because of the additional proliferative stimulation placed on breast cells that have already become initiated and are at a later stage in development and perhaps more prone to cell damage.

The protective effect of breastfeeding has been also shown in multiple case-control and cohort studies. The magnitude of breastfeeding may be dependent on the duration of breastfeeding, and on the confounding factor of parity. A reduction by 4.3 percent for every 12 months of breastfeeding, in addition to a decrease of 7 percent for each birth, was reported in a large pooled analysis included individual data from 50,302 women with invasive breast cancer and 96,973 controls from 47 epidemiologic studies [20]. The protective mechanism has been hypothesized to be the delay in reestablishment of the ovulatory cycles. Increase in prolactin secretion and the concomitant decrease in estrogen production could contribute to this protective effect as well.

1.5.3 Endogenous estrogen levels — Obese postmenopausal women have higher estrogen levels than non-obese postmenopausal women due to conversion of adrenal androgens to estrogens in fatty tissue. The higher risk of breast cancer for the obese postmenopausal women has already been discussed. Furthermore the reduction of estrogen levels both in premenopausal and postmenopausal women, lowers breast cancer risk. These observations suggest that serum estrogen levels are linked to the risk of breast cancer and there are epidemiologic data in support of this association. In the MORE trial (Multiple Outcomes of Raloxifene Evaluation), women in the highest tertile of serum estradiol levels (>12 pmol/L) have had double risk of breast cancer compared to women with lower levels. [21]. Fortunately, the women in the highest estradiol tertile experienced greater reduction in the risk of breast cancer with raloxifene compared to women in the low estradiol subgroup (79 versus 64 percent).

The association is the strongest for hormone receptor-positive breast cancers [22]. When endogenous hormone levels were measured in 322 breast cancer patients and in 643 age-matched controls without breast cancer, there was a significant direct association between breast cancer risk and levels of both estrogens and androgens. Breast cancer risk was higher for the women in the highest quartiles of serum hormone concentration. The association was the strongest when the analysis was restricted to ER and PR-positive tumors.

In contrary, the findings in premenopausal women are less clear. This might be due to the fact that most epidemiologic studies have used a single blood sample that was not timed to the menstrual cycle. The variability of hormone concentrations during menstrual cycles

makes reproducible measurements difficult in the years before menopause. When samples were timed to the follicular phase of the menstrual cycle in a case control study from the Nurses' Health Study a significant association between serum estrogen levels and breast cancer risk was shown [23]. Similarly to the previous studies conducted in the postmenopausal setting, women in the highest (versus lowest) quartiles of total and free estradiol measured during the follicular phase had significantly higher rates of breast cancer (RR 2.1 and 2.4, respectively). Once again, the association was stronger for hormone receptor-positive tumors (RR 2.7 and 2.8 for total and free estradiol, respectively).

1.5.4 Breast density — The presence of dense breast tissue is independently associated with an increased risk of breast cancer. Women with mammographically dense breasts (usually defined as ≥75 percent density) have a four to five fold greater risk for breast cancer compared to women of similar age with less or no dense tissue [24]. The increased risk is not because of the difficulty of mammographic detection. A significant association between longitudinal increases or decreases in breast density on serial screening mammography and an increased or decreased risk of breast cancer has also been shown [25]. The association between increased breast density and higher rates of breast cancer appears to be independent of estrogen-mediated effects, but remains not completely understood. A stronger association of higher breast density with ER-negative than with ER-positive tumors, supporting the lack of estrogen-mediated effects, was reported in one nested case-control study of postmenopausal women from the Nurses' Health Study [26]. Bone mineral density in addition to breast density is considered a surrogate marker for long-term exposure to endogenous estrogen. However, in another case-control study, breast density was strongly associated with an increased risk of breast cancer even after controlling for reproductive and hormonal risk factors, while high bone mineral density (another marker for cumulative exposure to estrogen) was neither associated with increased risk of breast cancer nor with breast density [27]. Growth factors other than estrogen (eg, insulin-like growth factor-1) could be the mechanism for this association.

1.5.5 Exogenous hormone factors — Postmenopausal hormone replacement therapy has been shown to increase breast cancer risk in multiple observational studies. Both unopposed estrogen and combined estrogen-progestin therapy have been linked to breast cancer. For each year a woman uses postmenopausal hormones, her risk of breast cancer

increases by 2.3 percent according to an analysis including 52,705 women with and 108,411 women without breast cancer enrolled in 51 epidemiologic studies [28]. However the risk may be underestimated or overestimated in epidemiologic studies due to a number of biases. For example, women who have been taking hormone therapy for longer durations in epidemiologic studies tend to have had earlier menopause (and therefore a lower risk of breast cancer) than those taking hormones for shorter durations. In addition to that women who take hormonal replacement therapy may have higher rates of screening mammography and therefore more likely to receive a breast cancer over-diagnosis. The Women's Health Initiative (WHI) a randomized, placebo-controlled trial was specifically designed to overcome those limitations of epidemiologic studies. In this trial women randomized to receive combined hormone therapy had a significantly increased risk of invasive breast cancer (HR=1.24; 95%CI 1.01-1.54) after an average follow-up of 5.6 years [29]. In this study women taking combined estrogen-progestin therapy were more likely to be diagnosed with slightly larger primary cancers at the time of diagnosis and a higher percentage of positive lymph nodes when compared with women taking placebo. This observation was in contrary with the postulation that the cancers developed in women taking estrogen have a relatively favorable prognosis. However, unopposed estrogen replacement therapy found to be associated with a trend towards lower rate of breast cancer risk (HR=0.77 for unopposed estrogen versus placebo; 95%CI 0.59-1.01; *p*=0.06) [30]. This is in contrast to the results of the combined hormone therapy trial. From the woman's perspective, the critical issue is the absolute or attributable risk per 10.000 person-years associated with estrogen plus progestin. Practically speaking there were 8 more invasive breast cancers in addition to 7 more heart events, 8 more strokes and 8 more pulmonary embolisms. On the other hand the absolute risk reductions per 10 000 person-years were 6 fewer colorectal cancers and 5 fewer hip fractures. The absolute excess risk of events included in the global index was 19 per 10 000 person-years [31].

1.6 ***Family history and genetic factors*** — A positive family history in a first degree relative is reported by 15 to 20 percent of women with breast cancer and it is an important risk factor for breast cancer development. The risk associated with having an affected first or second degree maternal or paternal relative is modulated by the age of both the case patient and the family member at diagnosis, and the number of female first-degree relatives with and without cancer. The risk is highest for women with young affected relatives. Thus,

a woman whose relative was diagnosed before age 30 has an increased risk by 2.9 fold, but only increased by 1.5 fold if the affected relative was diagnosed after age 60 [20]. In addition to family history, specific genetic mutations predispose to breast cancer. However, the proportion of all breast cancers that are directly attributable to inheritance of a breast cancer susceptibility gene is only 5 to 6 percent. The two most important breast-cancer genes, *BRCA1* and *BRCA2,* confer a risk of breast cancer among carriers that is 10 to 30 times higher than the risk of women in the general population. Less frequent mutations associated with a relative risk of breast cancer of 2.0 or greater have been identified (figure 2) [32]. Other genes with a population frequency and risk profile similar to *BRCA1* or *BRCA2* are unlikely to exist [32].

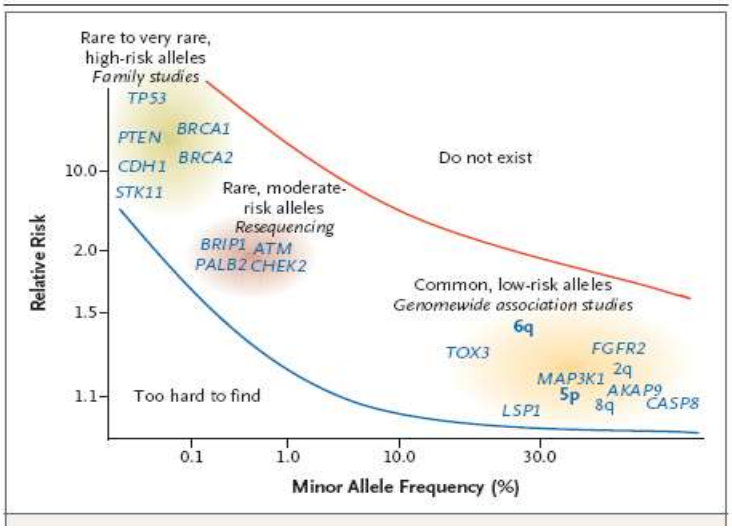

Figure 2: Breast-Cancer Susceptibility Loci and Genes
(adapted from Foulkes WD, New England Journal of Medicine 2008; 359:2143).

2. Prognostic factors for breast cancer recurrence

All patients with breast cancer require a multidisciplinary treatment approach that includes surgery, radiation therapy, and systemic therapy (chemotherapy and/or endocrine therapy). Despite the recent advances of treatment, women who were treated for invasive breast cancer remain at risk for both locoregional recurrence (at the chest wall and axillary or supraclavicular lymph nodes) and/or distant metastasis. The risk for locoregional recurrence is between 4 to 7 percent with mastectomy or breast conserving therapy (BCT),

respectively [33], with women treated with BCT to tend to recur later compared with the patients who were treated with mastectomy. In addition to locoregional recurrence breast cancer has the potential to metastasize to almost every organ in the body. The most common sites of metastases are the bone, liver, and lung. Approximately 50 to 75 percent of patients who relapse distantly do so in a single organ; the remainder will develop diffuse metastatic disease. Central nervous system (CNS) involvement as the first site of metastatic disease will be developed by fewer than 5 percent of patients.

The development of methods for determining prognosis in patients with breast cancer has been the focus of extensive research. There are several factors that are important for early breast cancer prognosis.

2.1 ***Tumor grade*** — This is an important prognostic variable. The higher the grade, the more unfavorable the prognosis. The Nottingham histological grade is recommended by the American Joint Committee on Cancer (AJCC) staging system. A tumor is graded by assessing three morphological features (tubular formation, nuclear pleomorphism and count of mitoses). A value of 1 (favorable) to 3 (unfavorable) is assigned to each feature. A combined score of 3 to 5 points is designated grade 1, 6 to 7 points is grade 2 and 8 to 9 is grade 3.

Tumor size	Number of involved lymph nodes	
	None	1-3 Nodes
T1a: <0.5	99%	95%
T1b: 0.5-0.9cm	98%	94%
T1c: 1.0-1.9cm	96%	87%

Table 1: Effect of tumor size according to the number of involved lymph nodes (SEER database).

2.2 ***Pathological stage*** — clearly impacts expected survival.

2.2.1 Tumor size — The risk of recurrence increases linearly with tumor size for patients with fewer than four lymph nodes involved with metastases; thereafter, the prognostic weight of lymph nodes metastases generally supersedes tumor size. The effect of tumor size on prognosis is reflected by the following SEER 5-year survival data (Table 1) [34].

2.2.2 Lymph node involvement

— is the greatest prognostic indicator for breast cancer recurrence. Since 2002 the TNM staging system does address this issue. The current (seventh edition) is shown below [34]:

Primary tumor classification

Tx — Primary tumor cannot be assessed

T0 — No evidence of primary tumor

Tis — Carcinoma in situ

Tis (DCIS) — Ductal carcinoma in situ

Tis (LCIS) — Lobular carcinoma in situ

Tis (Paget) — Paget disease of the nipple is **not** associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma. Carcinoma in the breast parenchyma associated with Paget disease is categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget disease should still be noted

T1 — Tumor ≤20 mm in greatest dimension

T1mi — Tumor ≤1 mm in greatest dimension

T1a — Tumor >1 mm but ≤5 mm in greatest dimension

T1b — Tumor >5 mm but ≤10 mm in greatest dimension

T1c — Tumor >10 mm but ≤20 mm in greatest dimension

T2 — Tumor >20 mm but ≤50 mm in greatest dimension

T3 — Tumor >50 mm in greatest dimension

T4 — Tumor of any size with direct extension to the chest wall and/or the skin (ulceration or skin nodules)*

T4a — Extension to chest wall, not including only pectoralis muscle adherence/invasion

T4b — Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma

T4c — Both (T4a and T4b)

T4d — Inflammatory carcinoma**

*Note: Invasion of the dermis alone does not qualify as T4.

**Note: Inflammatory carcinoma is restricted to cases with typical skin changes involving a third or more of the skin of the breast. While the histologic presence of invasive carcinoma

invading dermal lymphatics is supportive of the diagnosis, it is not required, nor is dermal lymphatic invasion without typical clinical findings sufficient for a diagnosis of inflammatory breast cancer.

Pathologic classification of regional lymph nodes

pNX — Regional lymph nodes cannot be assessed (eg, previously removed, or not removed for pathologic study)

pNO— No regional lymph node metastasis identified histologically

pNO(i-) — No regional lymph node metastases histologically, negative IHC

pN0(i+) — Malignant cells in regional lymph node(s) no greater than 0.2 mm (detected by H&E or IHC including ITC)

pNO(mol-) — No regional lymph node metastases histologically, negative molecular findings (RT-PCR)

pNO(mol+) — Positive molecular findings (RT-PCR), but no regional lymph node metastases detected by histology or IHC

pN1 — Micrometastases, or metastases in one to three axillary lymph nodes, and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected**

pN1mi — Micrometastases (greater than 0.2 mm and/or more than 200 cells, but none greater than 2.0 mm)

pN1a — Metastases in 1 to 3 axillary lymph nodes, at least one metastasis greater than 2.0 mm

pN1b — Metastases in internal mammary nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected**

pN1c —Metastases in one to three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected**

pN2 — Metastases in four to nine axillary lymph nodes, or in clinically detected**** internal mammary lymph nodes in the absence of axillary lymph node metastases

pN2a — Metastases in four to nine axillary lymph nodes (at least one tumor deposit greater than 2.0 mm)

N2b — Metastasis only in clinically detected*** ipsilateral internal mammary nodes and in the absence of clinically evident axillary node metastases

pN2b — Metastases in clinically detected*** internal mammary lymph nodes in the absence of axillary lymph node metastases.

pN3 — Metastases in 10 or **more** axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected******* ipsilateral internal mammary lymph nodes in the presence of 1 or more positive level I, II axillary lymph nodes; or in more than 3 axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected**; or in ipsilateral supraclavicular lymph nodes

N3a — Metastases in 10 or more axillary lymph nodes (at least one tumor deposit greater than 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes

pN3b — Metastases in clinically detected*** ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected**

pN3c — Metastases in ipsilateral supraclavicular lymph nodes

* Classification is based on axillary lymph node dissection with or without sentinel lymph node biopsy. Classification based solely on sentinel lymph node biopsy without subsequent axillary lymph node dissection is designated (sn) for "sentinel node," for example, pN0(sn).

** Note: Not clinically detected is defined as not detected by imaging studies (excluding lymphoscintigraphy) or not detected by clinical examination.

*** Note: Clinically detected is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy or a presumed pathologic macrometastasis based on fine needle aspiration biopsy with cytologic examination. Confirmation of clinically detected metastatic disease by fine needle aspiration without excision biopsy is designated with an (f) suffix, for example, cN3a(f). Excisional biopsy of a lymph node or biopsy of a sentinel node, in the absence of assignment of a pT, is classified as a clinical N, for example, cN1. Information regarding the confirmation of the nodal status will be designated in site-specific factors as clinical, fine needle aspiration, core biopsy, or sentinel lymph node biopsy. Pathologic classification (pN)

**** Note: Isolated tumor cell clusters (ITC) are defined as small clusters of cells not greater than 0.2 mm, or single tumor cells, or a cluster of fewer than 200 cells in a single histologic cross-section. ITCs may be detected by routine histology or by immunohistochemical (IHC) methods. Nodes containing only ITCs are excluded from the total positive node count for purposes of N classification, but should be included in the total number of nodes evaluated.

Distant metastasis (M)

M0 — No clinical or radiographic evidence of distant metastases (no pathologic M0; use clinical M to complete stage group)

cMO(i+) — No clinical or radiographic evidence of distant metastases, but molecularly or microscopically-detected tumor cells in circulating blood, bone marrow or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases

M1 — Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm

is used for excision or sentinel lymph node biopsy only in conjunction with a pathologic T assignment.

Stage	Tumor	Nodes	Metastases
0	Tis	N0	M0
I	T1	N0	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Table 2: Tumor node metastases (TNM) staging system for carcinoma of the breast.

2.3. ***Estrogen and progesterone receptors*** — Are both members of the nuclear hormone receptor superfamily that includes the androgen and retinoid receptors. The receptors are located in the cytosol of target cells and operate as ligand-dependent transcription factors. Attachment of a lipid-soluble hormone to the ligand-binding domain results in unmasking of the DNA-binding sites on the receptor, followed by migration into the nucleus, and binding to specific hormone-responsive elements near the genes that are responsible for the physiologic actions of the hormone. Subsequent steps include transcription of messenger RNA and ribosomal RNA, and the eventual synthesis of new proteins. The prognostic significance of hormone receptor expression has been a matter of extensive research. Women with stage I ER-positive breast cancer who receive no systemic therapy but surgery have a 5 to 10 percent lower likelihood of recurrence at five years than those who have ER-negative tumors. However, with longer follow up, the advantage of ER-positivity in terms of relapse and death grows smaller [36]. This might reflect sequential improvements in adjuvant chemotherapy (which benefits mostly the hormone receptor-negative patients) over time.

However, expression of hormone receptors is associated with a number of other well-established prognostic indicators, but not with nodal metastases [37]. ER-positive tumors

are more likely to be well differentiated (lower grade) and to have a lower fraction of dividing cells [38], but less likely to be associated with mutations, loss, or amplification of breast cancer-related genes such as *P53* [39], and *HER2* [36], all of which have been associated with a poorer prognosis. Hormone receptor status is also associated with specific site(s) of metastatic spread. For not completely understood reasons, ER-positive tumors tend to metastasize to the bone, soft tissue, or the reproductive/genital tracts, in contrast to ER-negative tumors that metastasize more commonly to brain and liver. The latter sites are associated with shorter survival [40].

2.4 *HER2 overexpression* — The *HER2-neu* oncogene (thereafter *HER2*) is amplified or overexpressed in approximately 18 to 20 percent of primary invasive breast cancers. Overexpression of *HER2* [3+ by immunohistochemistry (IHC) or gene copy amplification by fluorescence in situ hybridization (FISH)] represents an important predictive factor, identifying those patients who benefit from targeted therapies, such as trastuzumab, both in the adjuvant and metastatic setting. However the prognostic value of *HER2* remains controversial. In most studies, *HER2* overexpression (as determined by IHC) in primary tumor tissue is associated with a worse prognosis in patients who didn't receive trastuzumab [41]. The overexpression of *HER2* (as assessed by IHC and with FISH confirmation for those with 2+ IHC staining) was independently associated with significantly worse 10-year relapse free (66 versus 76 percent) and breast cancer-specific survival (76 versus 86 percent) in a cohort of 2026 breast cancer patients, of whom 70 percent did not receive adjuvant systemic therapy [42]. Almost 90 percent of the tumors in this series were >1.0 cm in size. However, all of the relevant studies were performed prior to the introduction of the monoclonal antibody trastuzumab in addition to anthracycline and taxane-based adjuvant chemotherapy in women with high-risk *HER2*-overexpressing tumors. That was the reason for the recommendation against the use of *HER2* overexpression and/or amplification as a prognostic marker in early breast cancer by an expert panel convened by the American Society of Clinical Oncology (ASCO) [43].

2.5. *Urokinase plasminogen activator system* — urokinase plasminogen activator (uPA) is a serine protease with an important role in cancer invasion and metastases. It acts by bounding to its receptor (uPAR) and converts plasminogen into plasmin that in turn

mediates degradation of the extracellular matrix during invasion. Specific inhibitors of uPA (plasminogen activator inhibitors [PAI] types 1 and 2) have been identified [44].

The prognostic role of the urokinase plasminogen activator system has been studied initially at the retrospective setting. High levels of uPA, uPAR, and PAI-1 have been associated with shorter survival in women with breast cancer while the opposite was found for high levels of PAI-2 [44-45]. The prognostic value of this system's molecules was supported further by a pooled analysis of individual patient data from 8377 women treated in clinical trials sponsored by the EORTC (European Organization for Research and Treatment of Cancer) [46]. The uPA and PAI-1 levels in the primary tumor tissue extracts were the strongest predictors of disease-free and overall survival, beside nodal status (relapse-free survival hazard ratio 2.3 for uPA and 1.9 for PAI-1) in the multivariate analysis. A prospective trial was undertaken using the cytosolic uPA and/or PAI-1 levels to dictate adjuvant treatment for 556 women with node-negative breast cancer based on this promising data [47]. The 241 women who had low levels of uPA (≤3 ng/mg of protein) and PAI-1 (≤14 ng/mg of protein) were followed without adjuvant treatment. In contrast, elevated intratumoral levels of uPA and/or PAI-1 were measured in 315 patients, who were randomized to six cycles of CMF chemotherapy (cyclophosphamide, methotrexate, and fluorouracil) or observation. Patients from the group without chemotherapy (n=374) with low expression of uPA and PAI-1 (6.7 versus 14.7 percent) have had a significantly lower three-year recurrence rate, after 32 months of median follow-up. The adjuvant chemotherapy was associated with a non-significantly lower risk of disease recurrence (RR= 0.27; 95%CI 0.09-0.78) for the women with high expression of either protein. Based on these promising results a larger validation trial is now ongoing.

2.6. *P53* analysis — Mutations in the *P53* tumor suppressor gene or accumulation of P53 protein (as a product of mutated *P53* which produces a protein more resistant to degradation than the wild type one leading to protein accumulation) have been reported in 20 to 50 percent of breast cancers. Either high P53 protein expression assessed by IHC or mutations in the *P53* represent an independent predictive factor of decreased disease-free and overall survival in both node-positive and node-negative patients according to a meta-analysis [48]. However, other studies have failed to confirm these findings. [49]. The use of adjuvant systemic therapy may explain this discrepancy. *P53* abnormalities might also be

associated with sensitivity to some therapeutic agents and resistance to others and worse prognosis only in patients who received no chemotherapy. Thus, *P53* mutations or deletions might confer a favorable or unfavorable prognosis depending on the specific type of treatment. At present, there is no role for *P53* gene analysis in women with breast cancer according to the ASCO panel on tumor markers [43].

2.7. *Gene-expression signatures* — Gene-expression profiling with the use of microarrays allows measurement of thousands of messenger RNA (mRNA) transcripts in a single experiment and has been used to develop genomic tests that may provide better predictions of clinical outcome than the traditional clinical and pathological standards. The 21-gene recurrence score (Oncotype Dx®) and the Amsterdam 70-gene prognostic profile (Mammaprint®) are both well studied and enhance our ability to predict breast cancer outcome and response to treatment.

2.7.1 The Oncotype Dx® measures the expression of *ER* and *HER2*, as well as that of ER-regulated transcripts and several proliferation-related genes, with the use of the reverse transcriptase–quantitative polymerase-chain-reaction (RT-qPCR) assay. Based on the results, a quantitative continuous "recurrence score" is calculated which can be used to stratify patients into low-risk, intermediate-risk, and high-risk group for 10-year disease distant recurrence. This recurrence score was examined retrospectively in 668 patients with ER-positive, node-negative tumors treated with adjuvant tamoxifen in the contest of National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 clinical trial. The 10-year distant recurrence rates were 7, 14, and 30 percent for the low-risk, intermediate-risk, and high-risk categories, respectively [50]. More interestingly, the Oncotype DX® assay appears to identify patients who are more likely to benefit from adjuvant chemotherapy in addition to endocrine therapy with tamoxifen. This hypothesis has been tested in 651 patients (ER-positive, node-negative) enrolled in the NSABP B-20 randomized clinical trial [51]. Higher recurrence scores were associated with greater benefit from adjuvant chemotherapy (with cyclophosphamide, methotrexate, and fluorouracil) and lack of any chemotherapy benefit was associated with lower recurrence scores. A subgroup analysis of postmenopausal women with node-positive breast cancer treated with or without anthracycline-based chemotherapy and tamoxifen led to similar results [52]. The effect of the Oncotype DX® assay on medical oncologist adjuvant treatment recommendations, patient treatment choice, and patient anxiety has been reported [53]. The molecular information may change

treatment recommendations for approximately 30 percent of patients, usually dictating against the use of chemotherapy and lowering patient anxiety. The Oncotype DX® assay has been recommended as a tumor marker only for patients with ER-positive, node-negative breast cancer by both ASCO and the breast cancer panel of the National Comprehensive Cancer Network (NCCN) [43].

2.7.2 The Amsterdam 70-gene prognostic profile or MammaPrint® is another signature developed by Dutch researchers from the Netherlands Cancer Institute. Data from a selected retrospective series of 78 patients with node-negative breast cancer who had received no systemic adjuvant therapy were analyzed [54]. The expression of 70 genes was measured to calculate a prognostic score that categorizes patients into “good” or “poor” risk groups. The MammaPrint® signature has been tested in a validation study of a retrospectively collected consecutive series of 307 breast tumors, from patients with both node-negative and node-positive cancers [55]. Patients with the poor-prognosis signature experienced time to distant metastases hazard ratios ranging from 2.13 to 2.15 after adjustment for various estimates of clinical risk, while their 10-year overall survival was 69 percent versus 88 percent for the patients with the good-prognosis signature. Similar to the Oncotype Dx® assay the use of MammaPrint® in combination with clinical guidelines led to altered adjuvant treatment recommendations in 26 percent of patients enrolled in a prospectively conducted multicenter study that included 427 patients [56]. Recently, the prognostic value of this study has been reported [57]. After 61.6 months of median follow-up, only fifteen percent (33/219) of the low-risk patients received adjuvant chemotherapy versus 81 percent (169/208) of high-risk signature patients. The 5-year disease-free survival rates for the 70-gene signature low-risk (n=219) and high-risk (n=208) patients were 97.0 and 91.7 percent respectively. According to authors, this prospective community-based observational study confirmed the additional prognostic value of the 70-gene signature and suggested that omission of adjuvant chemotherapy as judged appropriate by doctors and patients and instigated by a low-risk 70-gene signature, appeared not to compromise outcome.

2.7.3 The Rotterdam 76-gene prognostic signature was developed in an original set of 115 node-negative primary breast cancers from women who did not receive adjuvant therapy and had been followed for more than eight years [58]. Separate prognostic gene sets were developed for ER-negative (ER-, 16 genes) and ER-positive (ER+, 60 genes)

disease, in contrary to the Oncotype Dx® and MammaPrint® assays. The assay demonstrated prognostic significance for both distant metastasis-free and overall survival in the multivariate analysis of a study that put together an independent set of 171 tumors (25 percent ER negative) [59]. Interestingly, a strong time dependence of this signature, leading to an adjusted hazard ratio of 13.58 (95%CI 1.85-99.63) and 8.20 (95%CI 1.10-60.90) at 5 years and 5.11 (95%CI 1.57-16.67) and 2.55 (95%CI 1.07-6.10) at 10 years for time to distant metastasis and overall survival, respectively was observed. This finding highlights the time dependence of these genomic signatures, meaning that they predict early relapse far better than late relapse (after five years). This is due to the methodology of development that used early relapse as the endpoint. It is reasonable to assume that the biology of late relapse differs from early relapse and has been inadequately studied. Table 3 summarizes the characteristics of the discussed gene-signatures.

Assay	Prognostic in				Fixed tissue	Indication
	ER(+)	ER(-)	N(+)	N(-)		
Oncotype Dx®	√√	–	√	√√	√	**Prediction of relapse despite endocrine therapy**
MammaPrint ®	√√	–	√	√√	–	**Prognostication in N-**
Rotterdam 76	√√	?	?	√√	–	**Prognostication in N-**

Table 3: Characteristics of the three well-tested genomic profiles in early breast cancer [60].

3. Metastatic Pathways

3.1 Why do some patients with axillary node metastases develop metastatic disease, while others do not?

— At the end of nineteenth century William Halsted developed the basic concept that dictates breast cancer surgery to this day. According to this concept, breast cancer metastasis pathway follows a linear pattern; cancer cells spread from the breast to the lymphatic vessels and then to the systemic circulation whereby they can seed distant organs. Therefore, radical mastectomy that includes surgical removal of the whole breast that is affected by the tumor, as well as the regional axillary lymph nodes would prevent the development of metastatic disease. This concept was proven to be true in clinical practice, based on the evidence that radical mastectomies continue to be the cure of many breast cancer patients. The adverse prognosis of the patients with lymph nodes involvement further supports this concept [34]. The theory besides sentinel lymph node biopsy (if the first nodes draining lymphatic flow are free of cancer cells, then the rest of the axilla is almost always free of involvement - false negative rate of sentinel lymph node biopsy 4-5 percent) is based on this concept also [61]. Lastly, long-term experience continues to show that improved local control achieved with the addition of radiation therapy to breast conserving surgery decreases the risk of local and distant recurrence [62]. The better outcome of patients after treatment with the aforementioned clinical practices (mastectomy, sentinel-node mapping and adjuvant radiation therapy to the breast) and improved local control seem to support the concept of linear cancer progression.

However, we all know that metastasis is not that a simplistic process. Some women without axillary lymph nodes involvement still develop distant metastasis, while other women with extensive axillary lymph nodes metastases never recur in distant organs. To explain this clinical observation, the hypothesis that both hematogenous and lymphatic pathways are operable for metastatic disease development was made by Fischer et al [63]. The systemically administered therapies (chemotherapy and tamoxifen) required to improve breast cancer survival favors their hypothesis [64]. In addition several more recent studies seemingly support further the latter hypothesis. First, the finding of isolated tumor cells in axillary lymph nodes does not affect overall survival [65]. Second, patients treated with breast conserving surgery and radiotherapy for small, hormone receptor-positive breast cancer and two or fewer involved axillary lymph nodes, do not have increased rates of local

recurrence if they don't undergo a complete axillary dissection [66]. Finally, two recent studies highlight the imperfect relationship between tumor size and lymph node status to clinical outcome. In case of extensive lymph node metastases, very small tumors may confer a more aggressive subtype than larger tumors with the same lymph node involvement, as shown by Wo et al [67]. Similarly, triple negative breast cancers with lymph node involvement may have worse outcome irrespectively of the absolute number of positive lymph nodes [68].

It appears that both Halsted and Fischer hypotheses are valid. The first of an anatomic pathway for metastatic spread is correct in many cases but also Fischer's hypotheses that malignant spread does not require necessarily a linear pathway is also true in others.

3.2 Circulating tumor cells and self-seeding in cancer — Early dissemination of tumor cells is common even in early stages of breast cancer and usually remains undetected by conventional histopathological analysis and high-resolution imaging technologies. These cells that are present in the blood and present antigenic and/or genetic characteristics of a specific tumor type are known as Circulating Tumour Cells (CTC) [43].

The self-seeding model of malignant growth contains the idea that cancer cells leave the primary tumor and circulate in the blood seeding metastases in regional lymph nodes and/or distant sites. The concept of tumor self-seeding by CTC was introduced in 2009 after demonstration of the theory in diverse experimental models including colon and breast adenocarcinomas as well as melanomas [69]. To prove this hypothesis, the investigators first used the MDA-MB-231 (MDA231) human breast cancer cell line for which a metastatic subpopulation (MDA231-LM2) had already been characterized. Metastatic cells labeled with a green fluorescent protein were implanted into one mammary gland as the 'donor' tumor and either the metastatic or the more indolent parental cells into the contralateral gland as the 'recipient' tumor. Cells from the donor tumor appeared as small patches in 85 percent of recipient tumors, after 60 days. Results were generalizable to the metastatic donor breast tumors and the parental-line recipients; the metastatic donor colon tumor SW630-LM1 and its less aggressive recipient counterpart SW630; and the syngeneic donor breast tumor 4T1 (metastatic) and its recipient 67NR (poorly metastatic) in immunocompetent mice. Mammary tumors could also be seeded by metastases, as was demonstrated *in vivo*.

Injecting labeled MDA231-LM2 cells through the tail vein generated lung metastases and primary mammary tumors. Seeding of the mammary tumors with cells shed from the lung colonies was observed in ten out of 11 mice.

To establish whether self-seeding can affect tumor growth, MDA231 tumors were implanted, followed 10 days later by intracardiac injection of MDA231-LM2 cells. Tumor growth was accelerated in a manner well beyond what could be accounted for by either population alone. It was concluded that the MDA231-LM2 cells enhance the growth of MDA231 tumors through a stromal interaction [69].

By this model Kim and colleagues [69] demonstrated the importance of CTC for metastatic disease development. They showed meticulously that CTC could go back from primary and distant tumor sites. In other words, a primary tumor may not be only the cause of distant seeding, but also the result of self-seeding. If this is the case in human cancers, a large tumor may grow from the “outside in” as opposed to the “inside out” which is the conventional theory. Finally, Kim et al challenged another dogma in metastasis, that cells could lie dormant for years in such sites without growing, making self-seeding necessary but not sufficient for development of metastatic foci [70].

Circulating tumor cells need to pass through many barriers before infiltrating and growing in distant organs such as the tight vascular capillary endothelial walls and the unfamiliar microenvironment. Therefore, not all CTC are successful in distant organ seeding, but only the most adaptable of them. However, when CTC re-enter the primary tumor, they face a leaky neo-vasculature and a fertile concentration of all the tissue-specific factors that initially permitted their entry to circulation stream [71]. Several inflammatory cytokines like IL-6 and IL-8 act as attractants for the CTC. The self-seeding CTC also express MMP1/collagenase-1, the actin cytoskeleton complement fascin-1, and CXCL-1, which promote tumor growth, angiogenesis, and the recruitment of myeloid cells into the stroma [69].

The genetic profile of cancer cells capable to generate distant metastases has been shown to be site-specific, with unique gene signatures for lung, liver and brain dissemination [71-74]. The genes that compose these signatures for bone, lung and brain do overlap to some extend but they are not identical. There are not only animal models that support the site-specific origin of metastases, but also the analysis of survival data in patients whose tumors have been classified by the molecular signatures [51-59]. Interestingly, there are an

increasing number of pathology reports of tumor-to-tumor metastases further supporting the evidence from the self-seeding experiments [75].

The problem with the site-specific nature of self-seeding is that the detection of isolated cancer cells in a distant organ does not always coincide with clinical metastatic behavior. On the other hand the detection of unresected axillary nodal disease does not necessarily give rise to distant metastases, but neither does its absence secure the lack of development of metastatic disease at the time of diagnosis or in the future. Furthermore, in the case of patients who undergo breast-conserving surgery and are found to have 1-2 involved lymph nodes, it remains to be determined why axillary lymph node dissection does not improve the locoregional recurrence rate [66]. To make it more complicated, in the same setting it remains to be determined why radiation to the axilla improves overall survival [76]. Someone can speculate that in the case of radiation to the axilla, CTC can seed but not colonize the radiated area. There is clinical evidence supporting this speculation, with adjuvant radiation therapy to the breast after systemic therapy reducing systemic recurrences more than the same radiation treatment given in advance of adjuvant chemotherapy [77].

We have already cited the recent research indicating that some small tumors may have an aggressive clinical behavior [67-68]. The self-seeding capacity of the tumor may explain this observation. Small tumors with the aggressive clinical behavior may be poor self-seeders, but highly efficient distant seeders. In some cases actually, a tumor could be such a poor self-seeder that remains occult despite the development of extensive metastatic disease. This scenario may be the explanation for the adenocarcinomas of unknown origin or the classic presentation of pancreatic adenocarcinomas [78].

The self-seeding hypothesis may be the explanation of the observation that only 10 percent of patients with breast cancer present with de novo metastatic disease. It is not uncommon some patients to have a primary tumor growing for years without having disease in distant sites. It has been speculated that patients do not develop metastatic disease because CTC are attracted by the primary tumor. In this setting the primary tumor acts as a sponge for the CTC, giving rise to a vicious cycle leading to a large tumor of the breast [78].

An interesting question is what happens when the CTC are detected in the circulation of patients with resected primary tumor. This is the clinical setting of patients who are under surveillance for disease recurrence after treatment for primary breast cancer. We

hypothesized that in this setting since the CTC are not recruited by the chemoattractants that initially engendered growth in the breast, they are free to seed other organs and develop metastatic disease. If this is true then CTC detection during follow-up of patients with operable breast cancer could be useful for the early detection of patients at risk for disease recurrence and death. This hypothesis will be tested in this dissertation.

4. Detection and characterization of Circulating Tumor Cells

4.1 Assays for CTC detection — Different markers have been used for the detection of CTC, based on their expression on epithelial cells (epithelial-specific markers) or their specific expression in breast tissue (breast tissue-specific markers). Cytokeratins (CKs) are intermediate filament keratins found in the cytoskeleton of epithelial cells that have been extensively used among others for this purpose [79].

Cytokeratin-19 (CK-19) is among the most well studied markers [80] while many others have also been evaluated separately or in combinations (CK18, mucin-1, carcinoembryonic antigen, mammaglobin) [81-83]. Cytokeratin-19 is stably and abundantly expressed on epithelial breast tumors but not on mesenchymal hemopoietic cells and has been successfully used for the detection of breast cancer cells in the bone marrow, lymph nodes and peripheral blood. In addition to the appropriate marker selection, the unambiguous identification and characterization of CTC requires extremely sensitive and specific analytical methods. Given that CTC are usually found at very low frequencies among the normal peripheral blood mononuclear cells (PBMCs), tumor cell enrichment techniques, including density gradient centrifugation (Ficoll–Hypaque separation), and immunomagnetic or size filtration procedures are often used to enrich tumor cells before their detection [84,85].

Monoclonal antibodies directed against histogenic proteins [80] and PCR-based molecular assays amplifying tissue-specific transcripts [81-84] are the two main approaches for the detection of CTC. The molecular assays have generally been considered more sensitive, while immunocytochemistry has the advantage of allowing the morphological assessment of the stained cells [86,87]. One problem with these methods is that the sensitivity might be suboptimal, especially for the detection of CTC in peripheral blood

because these cells are usually present at very low frequencies e.g. 1-5 cancer cells amongst a million peripheral blood mononuclear cells (PBMCs) [88].

Recently, automated immunomagnetic enrichment and staining system for CTC (CellSearch® system) has been introduced [89,90]. It performs automated immunomagnetic epithelial cell adhesion molecule-based (EpCAM) enrichment followed by cytokeratin staining of CTC in blood samples [90]. Epithelial cells are distinguished from leukocytes by using fluorescently labeled anti-leukocyte (CD45) and anti-epithelial (cytokeratin 8, 18, 19) specific monoclonal antibodies [92]. This standardized method has been associated with high intra-observer, inter-observer and inter-instrument concordance and has been approved by the US Food and Drug Administration for in vitro enumeration of CTC from blood samples of metastatic breast, colon and prostate cancer patients [89, 93].

The AdnaTest Cancer Select/Detect is a new CTC detection system that uses a first step where cancer cells are enriched *in vitro* from cancer patients' blood samples using magnetic bead-conjugated antibodies that are optimized for the specific cancer type. The isolated mRNA is transcribed into cDNA that can be amplified in a subsequent multiplex PCR. The multiplex PCR detection step analyses the tumor-associated gene expression of a variety of relevant tumor markers [94].

Another method for the detection of CTC from whole unseparated blood uses laser-scanning cytometry after staining with anti-EpCAM and anti-CD45 fluorescent antibodies (MAINTRAC®) [95].

More recently, a microfluidic platform ('CTC chip') mediating the interaction of target CTC with antibody EpCAM-coated microposts, under precisely controlled laminar flow conditions in whole blood, has been developed [96]. Surprisingly a high number of cytokeratin-positive CTC was detected using this device, in nearly all tested patients with lung, prostate, pancreatic, breast and colon cancer, [97].

Nevertheless, the expression of EpCAM on tumor cells, including CTC, varies widely and depends on the tumour type. This variation can restrict the detection ability of the described devices and alternative methods have been developed to circumvent this problem [88]. Ultra-speed automated digital microscopy, in a system called fiber-optic array scanning technology (FAST), applies laser-printing techniques to the rare-cell detection system. Three hundred thousand cells are excited by this method and decorated by fluorescent dye-conjugated antibodies [98, 99]. A much simpler approach is based on separation by cell size

(membrane microfilter devices) [84, 100]. It is also unclear whether this approach will have the potential to increase the sensitivity and reproducibility of CTC diagnostics considering that size and cell shape of CTC is rather heterogeneous.

Finally, the EPISPOT assay is a completely different antibody-based approach, used to detect proteins released by CTC [101, 102]. Only viable, protein-excreting cells are detected using this method.

False positive results can been obtained using either nucleic acid-based or antibody-based assays [103]. Indeed, contaminating genomic DNA during RNA extraction, illegitimate expression or stimulated expression CTC markers on normal leukocytes and the presence of CK-19 pseudogenes, have been considered responsible for the false positive results when using nucleic acid-based assays [103-107]. The specificity of the molecular methods may be increased by using quantitative RT-PCR, which can discriminate between the higher levels found in breast cancer and the low background expression of "normal" cells by designing primers that do not amplify genomic DNA or pseudogenes [108]. The major disadvantage of this approach is that it does not allow a direct enumeration of CTC in the blood sample, since the number of transcripts expressed from different cells is variable, and only the number of target transcripts present can be estimated.

Similar limitations have been described using antibody-based techniques. Many of the antibodies designed for epithelial breast cancer cells, occasionally stain hematopoietic cells, displaying illegitimate expression of cytokeratins or MUC1. Plasma cells can also be stained due to alkaline phosphatase reaction against the kappa and lambda light chains expressed on the cell surface [103]. Optimizing the antibodies and using the appropriate negative controls in staining experiments have been proposed to overcome these limitations.

4.2 Assays for the molecular characterization of CTC — In addition to enumeration, there is great interest in the molecular characterization of the CTC. Characterization of the specific subtypes of CTC based on the expression of different genes can provide information about the biology of metastasis and improve patient management. Molecular assays for the characterization of CTC are based on the extreme analytical sensitivity and specificity of PCR. Similarly to the use of PCR for the detection of CTC, the success of the molecular characterization requires the use of appropriate mRNA markers. Given the large background and diversity of circulating cells, it is probably necessary to detect 1 cancer cell

in the presence of more than 1000 leukocytes and to characterize this cell meticulously. By using a set of genes with no or minor expression on leukocytes, Sieuwerts et al succeeded to perform quantitative gene expression profiling for as little as one breast cancer CTC present in a CTC-enriched environment typically containing over 800 contaminating leukocytes [109]. Another major advantage of molecular methods in addition to sensitivity is their flexibility, especially for these multiplex assays, which reduces the required sample size, time, and analysis cost.

Using RT-qPCR, Obermayr et al. showed that a panel of six genes was superior to EpCAM and mammaglobin (*SCGB2A2* or secretoglobin, family 2A, member 2 also known as *hMAM*) for the detection of CTC in breast cancer [110]. Mammaglobin in conjunction with B305D-C has been shown by Reinholz et al. to offer potential advantage for the molecular characterization of circulating epithelial cells and early detection of invasive breast cancer [111]. Recently, the majority of CTC from patients with metastatic breast cancer were shown to express markers of epithelial-to-mesenchymal transition (EMT) and tumor stemness by the use of a commercially available kit (AdnaTest BreastCancer, AdnaGen AG) that detects EpCAM, mucin-1 (MUC1), and HER2 transcripts [112].

Interestingly, when ER and PR expression were assessed in CTC by RT-PCR, detection of CTC was mostly found in patients with triple-negative primary tumors. In general CTC phenotype was mostly triple negative regardless of the ER, PR, and HER2 status of the primary tumor [113].

Different markers with adequate sensitivity individually, have been combined together in an effort to improve CTC detection and to define clinically relevant subpopulations of CTC with aggressive biological behaviour. Three of these markers, detected by real-time (*CK19*) and nested (*SCGB2A2* and *HER2*) RT-PCR were evaluated in a study of 175 women with stage I to III breast cancer before the initiation of adjuvant chemotherapy [114]. The worst OS was reported for patients with CK19 mRNA+ and SCGB2A2 mRNA+ cells (p=0.044 and p=0.034, respectively) whereas patients with HER2 mRNA+ cells experienced shorter DFS (p<0.001). Detection of CK-19 mRNA+ and SCGB2A2 mRNA+ cells and the estrogen receptor–negative status of the tumor were independently associated with worse DFS in the multivariate analysis. Other investigators have reported on the negative prognostic value of the detection of HER2-positive CTC also [115].

Recently, researchers from our group developed a multiplexed PCR-coupled liquid bead array to detect the expression of multiple genes in CTC [116]. Using this approach, six established CTC gene targets [*HER2*, *SCGB2A2*, *CK19*, *MAGEA1* (melanoma antigen family A, 1) *TWIST-1* (twist homolog 1) and *HMBS* (hydroxymethylbilane synthase, also known as *PBGD*)] are simultaneously amplified and detected in the same reaction. A very limited amount of biological sample was used, thereby saving precious material and reducing the costs and time of analysis. This assay forms an efficient basis for a multiplex approach to study the expression of up to 100 genes in CTC. Table 4 summarizes the presented analytical approaches currently used for CTC analysis.

Assay	Assay System	Enrichment	Detection	Advantages	Disadvantages
Molecular	RT-PCR	Ficol gradient centrifugation	CK-19, HER2, h-MAM, CEA, maspin, GABA A, B726P	High sensitivity Detects only viable cells	No morphological analysis
	RT-qPCR	Ficol gradient centrifugation	CK-19, BST1, PTPRC	Quantification High sensitivity	No morphological analysis
	RT-qPCR	OncoQuick enrichment and RNA preamplification	CCNE2, DKFZp762E1312, EMP2, MAL2, PPIC and SLC6A8, hMAM,	Quantification High sensitivity	No morphological analysis
	Multiplex RT-PCR AdnaTest BreastCancer	EpCAM antibodies and MUC1 antibodies coupled to ferrofluidics	Multiplex PCR for: mucin-1, HER2, EpCAM, actin	High sensitivity Detects only viable cells Saves sample and time. Reduces cost	No morphological analysis EpCAM and MUC1 positivity dependent assay No quantification
	Liquid bead array	Ficol gradient centrifugation and positive selection with EpCAM antibody–coupled ferrofluidics	Multiplex PCR for CK-19, HER2, MAGE-A3, hMAM, PBGD, TWIST1	Saves sample and time, reduces cost Detects only viable cells Saves sample and time,	No morphological analysis EpCAM positivity–dependent assay End-point PCR
Cytological	CellSearch CTC test	Positive selection: EpCAM antibodies coupled on ferrofluids	Specific markers: CTC: CK-19 Leukocytes: CD-45 Cell viability: DAPI	FDA cleared Visual confirmation of CTC Visual confirmation of CTC	EpCAM-positivity dependent Limited number of markers
	CTC chip	Positive selection: EpCAM antibodies coupled to microspots	Positive markers: CKs Negative marker: CD45 Nucleus: DAPI	High detection rate Visual confirmation of CTC	Further investigation on assay specificity EpCAM-positivity dependent
	EpiSpot assay	Negative selection: $CD45^+$ cells	Immunological detection of secreted proteins: CK-19, mucin-1, cathepsin-D	Detects only viable cells High sensitivity	Clinical relevance not demonstrated

Table 4: Selected methodologies for the detection and molecular characterization of CTC. (adapted with revision from Lianidou ES, Clinical Chemistry 2011; 57:124.

5. Minimal residual disease in early breast cancer prognosis

5.1 Disseminated tumor cells — One of the first compartments where the minimal residual disease was studied is the bone marrow. The occult cancer cells when found in the bone marrow are defined as disseminated tumor cells (DTC). A large pooled analysis conducted by Brown et al, that put together data from over 4500 patients and found 31 percent of them to be DTC-positive in the bone marrow at the time of diagnosis [117]. In this analysis the detection of DTC was a prognostic factor for adverse overall survival in the multivariate analysis.

There are several trials incorporating different technologies for the detection of bone marrow DTC, all of them showed a worse prognosis for DTC-positive patients both at the time of diagnosis and during the course of the disease [118]. Despite the administration of systemic therapy (hormonal treatment and/or chemotherapy) these cells seem to survive. One explanation for this observation is the theory of DTC dormancy. According to this theory the DTC escape systemic therapy because they do not proliferate and therefore remain unaffected by the mechanism of action of most chemotherapeutic agents [119,120]. However, when DTC are examined regarding with their potential to proliferate in cell culture with growth factors-containing media, they demonstrate a capability of escaping their non-proliferating dormancy [121]. The bone marrow microenvironment seems to play an important role in the ability of DTC to switch from dormancy to overt metastases development [122]. Unfortunately, the exact mechanisms that control this phenomenon are still not well understood and remain a matter of extensive research. It might be a kind of selection dictated by the bone marrow microenvironment surrounding the DTC that eventually leads them to proliferate [123].

The detection rate of DTC has been reported to be as high as 40 percent in early breast cancer patients [124]. Unexpectedly, DTC have also been reported in the bone marrow of patients treated for ductal carcinoma in situ (DCIS). In a study limited to 30 consecutive women with DCIS, bone marrow aspirates were taken at the time of primary surgery, and DTC were detected by immunocytochemistry. DTC were detected in 4 of 19 cases of pure DCIS (21.1 percent) and in four of seven cases of DCIS with microinvasion (57.1 percent)! After a median follow-up time of 22 months, two initially DTC-positive patients

suffered from contralateral carcinoma and contralateral DCIS at months 12 and 30 respectively, whereas the remaining patients were relapse-free. The authors concluded that hematogenous tumor cell dissemination to the bone marrow is an early event in breast cancer development, supporting Fischer hypothesis for metastatic spread.

The effectiveness of adjuvant chemotherapy to eliminate DTC from the bone marrow of patients with high-risk breast cancer (inflammatory or more than four nodes involved) was reported by Braun et al. [119]. The presence of DTC in bone marrow after chemotherapy was an independent predictor for shorter overall survival (RR=5.0). Similar to the previous study, the bone marrow aspirates were examined immunocytochemically with the monoclonal anticytokeratin antibody. The same question was evaluated in a recent study focusing on triple-negative breast cancer a subgroup that is considered to be more sensitive to the most commonly used chemotherapy regimens [125]. In this study there was non-significant trend towards a higher rate of pathological complete responses (pCR) after neoadjuvant chemotherapy in patients with triple-negative breast cancers compared to luminal A or luminal B subtypes. Interestingly, all triple-negative breast cancer patients who achieved pCR had complete eradication of DTC, whereas 36 percent of luminal (A and B) subtypes had persistently detected DTC after the neoadjuvant treatment. A limitation of the study was that the receptor status of DTC was not evaluated but nonetheless the study suggests the role of DTC in the recurrence of patients with luminal subtypes, where chemotherapy does not seem to be very efficient in DTC eradication.

In contrary to overt metastases, locoregional relapse doesn't seem to be correlated with DTC detection. Only 20 percent of patients with locoregional relapse have disseminated tumor cells in the bone marrow, compared to 80 percent of patients with distant metastases [126,127]. Patients with skeletal metastases are more likely to have DTC in the bone marrow. The detection of 2.5 or more cancer cells/1 million bone marrow cells seem to be an independent risk factor for adverse overall survival in the setting of metastatic disease, irrespectively whether the patients had visceral or skeletal disease.

We have already reported the prognostic factors that are commonly used at the time of diagnosis to guide adjuvant treatment administration. However, reliable markers to estimate the risk of late disease relapse are lacking. The detection of DTC might be used as a new

approach for individualized treatment. On the basis of the strong independent prognostic significance of DTC at the time of primary diagnosis, it was hypothesized that DTC reflect the presence of minimal residual disease and may be the precursor of subsequent metastatic disease [117]. To elucidate the role of persistent DTC in a larger cohort, clinical follow-up data of 676 patients from 3 European academic breast cancer centers were pooled [118]. Tumor cells were detected by the standardized immunoassays and the patients were followed for a median of 89 months. In follow-up bone marrow aspirates 15.5 percent of patients had DTC. The presence of DTC was an independent indicator of poor prognosis for disease-free and overall survival during the first 5 years following cancer diagnosis, but not beyond that in the multivariate analysis. Whether CTC detection during the follow up of patients with operable breast cancer is predictive of late disease relapse will be the question to study in this dissertation.

Despite the prognostic role of DTC, there are only a few data to support changing therapeutic strategies based on serial bone marrow aspirates. It has been hypothesized that bisphosphonates or the new class of monoclonal antibodies against RANK ligand might alter the DTC and bone marrow microenvironment interaction. The effect of zoledronic acid on the clearance of DTC from the bone marrow was evaluated in 120 women who underwent neoadjuvant chemotherapy [128]. Patients were randomized to receive or not zoledronic acid every 3 weeks. Bisphosphonates administered with chemotherapy resulted in a decreased proportion of patients with DTC detected in the bone marrow at the time of surgery. Another randomized phase II study concluded that zoledronic acid added to standard adjuvant chemotherapy improves elimination of DTC [129].

In addition to bisphosphonates there is a need for further evaluation of the effect of endocrine or targeted therapies like the monoclonal antibody against the HER2 receptor (Herceptin®), on DTC. Given that *HER2* overexpression on disseminated tumor cells is associated with poor outcome [130], there is great interest for the potential benefit of anti-HER2 therapies in case of HER2-negative primary tumors, but HER2-positive disseminated tumor cells.

Disseminated tumor cells in the bone marrow represent an appealing source of prognostic information as well as a tool to monitor anticancer treatment. However, according

to the ASCO recommendation DTC should continue to be evaluated in more directed studies to establish their clinical significance [43].

5.2 Circulating tumor cells in metastatic breast cancer — the majority of research on CTC has been conducted in the metastatic disease setting. Ten years ago, Christofanilli et al. reported the prognostic utility of CTC, using the CellSearch® system in a prospective study of 177 patients with measurable metastatic disease [90]. Circulating tumor cells were enumerated at baseline and at the first visit after the administration of the first course of chemotherapy (3-4 weeks later). Patients with levels of CTC equal to or higher than 5 per 7.5 mL of whole blood, as compared with the group with fewer than 5 CTC per 7.5 mL, had a shorter median progression-free survival (2.7 months vs 7.0 months, $p<0.001$) and shorter overall survival (10.1 months vs >18 months, $p<0.001$). At the first follow-up visit after the initiation of therapy, this difference between the two groups persisted (progression-free survival, 2.1 months vs 7.0 months; $p<0.001$; overall survival, 8.2 months vs >18 months; $p<0.001$), and the reduced proportion of patients (from 49 percent to 30 percent) in the group with an unfavorable prognosis suggested that there was a benefit from therapy. However this trial could not answer the question whether a switch in therapies before disease progression according to imaging, would improve survival. A follow up study found that the association with PFS and OS based on CTC detection was significant at multiple time points after the initiation of treatment (range 3-20 weeks) [131]. The authors concluded that a switch in CTC levels from more than 5/7.5mL to less than 5/7.5mL was predictive of response to treatment, while the opposite was associated with lack of response to systemic therapy.

Subsequent series of studies have been conducted to validate these findings. Bidard et al. studied 67 patients with metastatic breast cancer treated with chemotherapy in combination with bevacizumab [(a humanized monoclonal antibody that inhibits vascular endothelial growth factor A (VEGF-A)] and found that the same cut-off (5 CTC/7.5mL) was not predictive of tumor response or time to disease progression at baseline [132]. However, a level of 3 CTC/7.5mL was predictive of the same endpoints. By 6 weeks following treatment initiation, CTC count was not predictive of tumor response or time to disease progression irrespectively of the cut-off, suggesting that many time- and cut-off points need to be tested before establishing significance. The authors speculated that bevacizumab impaired tumor cells access to vessels through vessels endothelium. Giordano et al. similarly found no

predictive value for CTC enumeration in patients with HER2-positive disease treated with trastuzumab [133]. In the same study however, it was found that CTC strongly predicted survival in HER2-negative metastatic breast cancer and the prognostic value was independent of subtype and metastatic sites. In contrast to Christofanilli pivotal study, this finding was true for hormone receptor-positive disease and skeletal involvement. These two studies underline the variability of CTC detection by tumor subtypes and the improvement needed in methodology before this can be used in prime time. More importantly, since none of these studies was randomized, a switch in therapy based on persistence of CTC is not currently recommended. The ongoing Southwest Oncology Group (SWOG) 0500 trial is designed to address this important question.

The detection of *HER2* overexpression on CTC is less well studied, partly due to lack of a standardized method for determining the HER2 status of CTC. Using a semiquantitative RT-PCR for CK-19 in a group of HER2-positive metastatic breast cancer patients Nunes et al. reported the prognostic and predictive role of CTC for treatment response and disease progression [133]. Compared to circulating levels of HER2 extracellular domain – a more established tumor marker – CTC measurement showed a closer correlation. A question that arises when looking at the HER2 status of CTC is how to interpret the discordance between the HER2 status of primary tumor and that of CTC. Reuben et al. reported on the hormonal receptors and HER2 status both in primary and metastatic tumors as well as CTC [134]. Eighty percent of tumors were ER-positive, 55 percent were PR-positive and 15 percent HER2-positive. Interestingly, CTC from 11 patients (55 percent) overexpressed HER2! On the other side, two patients with HER2-negative primary tumors had overexpressed HER2 in their CTC and had a response to trastuzumab-containing regimens. In a larger study, using both the CellSearch® system and the AdnaTest® discordance was found. One-third and half of patients with HER2-negative primary tumors had HER2-positive CTC using CellSearch® and AdnaTest®, respectively [135]. The potential role of HER2-targeted agents in the setting of HER2-negative primary tumor with HER2-positive CTC is the question of an ongoing clinical trial with lapatinib (a dual tyrosine kinase inhibitor which interrupts the HER2 and epidermal growth factor receptor (EGFR) pathways) in advanced breast cancer patients with HER2-non-amplified primary tumors and HER2- or EGFR-positive CTC. Such a prospective trial is

necessary to understand if there is any value for CTC evaluation in the treatment of metastatic disease with HER2-directed targeted agents.

Since 50 percent of metastatic breast cancer patients have no detectable CTC, it has been suggested that this is in part due to lack of methods' sensitivity for the detection of CTC. Using the CellSearch® system, Mego et al. reported that an undetectable CTC status is associated with brain metastasis and inversely correlated with bone disease [136]. Overall, the lack of CTC detection is associated with superior outcome, suggesting a role of CTC for tumor burden estimation. The authors hypothesized that current detection methods may miss cells that are undergoing EMT, given that both EpCAM and cytokeratins are expressed on epithelial cells. A study by Aktas et al. reported that a major proportion of CTC from metastatic breast cancer patients show EMT characteristics [112]. Three EMT markers [Twist1, Akt2, PI3Kalpha] were used and the samples were considered positive if at least one of the markers was detected. The majority of CTC-positive patients (62 percent) expressed EMT marker(s), while the opposite was true for only 7 percent of the patients. In a more recent study it was reported that the presence of mesenchymal markers on CTC more accurately predicted worse prognosis than the expression of cytokeratins alone [137]. The authors suggested that the use of epithelial markers only for CTC detection might miss the most invasive cell population. Despite the fact that the detection of variable antigens/genes expression on CTC is appealing, it requires further research before incorporating into clinical trials or clinical practice for metastatic breast cancer patients.

5.3 Circulating tumor cells in early breast cancer — the tumour staging system TNM cannot accurately identify a significant proportion of women who despite having favorable clinical and tumor characteristics are at increased risk of relapse and death of breast cancer. Moreover, at the present time the efficacy of an adjuvant treatment is solely judged by the disease relapse rate and the time interval from initial diagnosis to development of metastases. Unfortunately, this strategy in many cases results in late diagnosis of disseminated metastatic disease, which is incurable with current therapies. Therefore, there is an urgent need of novel markers with independent prognostic value and biomarkers for real-time monitoring of the efficacy of systemic adjuvant therapy in individual patients before the development of overt metastases.

The measurement of CTC during adjuvant therapy and the follow up period could be used to diagnose early on the presence of resistant disease and to modify the treatment strategy in patients with breast cancer. The prevalence of circulating tumor cells in the peripheral blood of metastatic breast cancer patients has been extensively studied and shown to correlate with poor progression–free and overall survival and treatment response [90,131]. However, their prognostic role in primary breast cancer is still under investigation and intense discussion. Several techniques have been used to detect CTC, with the CellSearch® system and the molecular approaches to be the main ones.

5.3.1 Prevalence and prognostic significance in the setting of neoadjuvant treatment — several groups have been collecting blood samples before and after neoadjuvant chemotherapy in patients with large operable and locally advanced breast cancer and used the CellSearch® system for the enumeration of CTC. Riethdorf et al. evaluated blood samples from 287 patients from the German multicenter GeparQuattro trial [138]. The patients were treated with epirubic/cyclophosphamide and then randomized to docetaxel or docetaxel in combination with capecitabine or docetaxel followed by capecitabine. Patients, whose tumors overexpressed HER2, received additional trastuzumab treatment. The prevalence of ≥ 1 CTC was 22 percent before neoadjuvant chemotherapy, whereas only 10 percent of patients had detectable CTC after chemotherapy. A total of 15 percent of initially positive patients turned to be negative after chemotherapy, whereas the opposite was true for 8 percent of the patients. CTC detection did not correlate with primary tumor characteristics and there was no association with response to neoadjuvant chemotherapy. Survival data are still lacking. In a subgroup of patients additional CTC phenotyping was performed by immunocytochemistry and showed HER2 overexpression in the CTC from 14 out of 58 patients (24 percent), including eight patients with HER2-negative primaries.

5.3.2 Prognostic significance before adjuvant chemotherapy — using the CellSearch® system the prognostic value of CTC was evaluated in a large cohort of 2026 early breast cancer patients with node-positive or high risk node-negative disease within the German multicenter SUCCESS trial [139]. In contrary to the aforementioned neoadjuvant and metastatic studies, 23 mL of blood was drawn and processed for the detection of CTC. Twenty-two percent of patients were CTC-positive before the administration of chemotherapy (median number of CTC 1.3; range: 1-827). A total of 12 percent of patients had only one

circulating tumor cell per 23 mL of blood analysed, 4 percent had two, 2 percent had three or four CTC, while five or more CTC were detected in the blood from 3 percent of the patients. CTC-positive patients were significantly more frequently node-positive (p<0.001), but there was no correlation with other known clinicopathological parameters for adverse outcome like tumor size, histological grade or hormonal receptor status.

The CTC-positive patients had a shorter disease-free (p<0.001), distant disease-free (p<0.001) and overall survival (p=0.002) after a median follow up of 35 months (range: 0-54). In the multivariate analysis, CTC detection before treatment was confirmed to be an independent predictor for disease-free survival (HR=1.91) in addition to tumor size, histological grade, lymph node involvement and hormonal receptor status. In subgroup analysis according to the absolute number of CTC (>0, >1, >5) both disease-free and overall survival was significantly shorter compared to the group of patients with no CTC detected in their blood sample. Therefore, the cut-off point of 1 circulating tumor cell detected in 23 mL of blood before the initiation of adjuvant chemotherapy seems to be justifiable. Finally, the greater the number of CTC in the blood before chemotherapy administration, the worst the outcome was. Specifically, the outcome was worst in patients with 5 or more CTC who experienced a fourfold increased risk for disease recurrence and a three-fold risk for death (p<0.05, for both outcomes).

More recently, Lucci and colleagues used also the CellSearch® system in a US patient population and prospectively showed that the presence of CTC is an independent predictor of relapse and death in chemotherapy-naive patients with non-metastatic breast cancer [140]. In 302 individuals a single CTC sample was collected at the time of their definitive surgery, and CTC levels were correlated with survival and standard histological characteristics. Detection of one or more CTC in 73 patients (24 percent), predicted both decreased progression-free [HR=4.62; 95%CI 1.79-11.9; p=0,005] and overall (HR=4.04; 95%CI 1.28-12.8; p=0,01) survival. Furthermore, the presence of CTC could not be predicted by any primary tumour characteristics, suggesting that the worse outcome was not simply because of an association with previously validated poor prognostic factors such as tumour size or grade.

5.3.3 Evaluation immediately after chemotherapy and during surveillance — CTC were also evaluated both immediately after and following two years from the completion of adjuvant chemotherapy in the setting of the SUCCESS trial [141]. Cut-off positivity for this

analysis was more than 1 circulating tumor cell per 23 mL of blood. In 143 (10 percent) out of 1500 patients analysed before the administration of adjuvant chemotherapy, more than 1 CTC was detected (range: 2-827). After the completion of chemotherapy, the number of patients tested positive for more than one CTC was 130 (9 percent). Of the 143 patients who were initially CTC-positive, 15 remained persistently positive (10 percent), whereas 115 become CTC positive (10 percent) after chemotherapy despite the fact that they have been tested negative before the start of treatment. Preliminary results support the prognostic significance of persisting CTC after cytotoxic chemotherapy with shorter disease-free survival (p=0.0623). Patients with persistently positive CTC before and after chemotherapy had the highest risk of disease relapse (p<0.001).

In the subgroup of 579 patients with a follow up sample 2 years after the completion of adjuvant chemotherapy, 10 percent had at least one CTC detected, while 1.2 percent of the patients had more than 5 CTC [142]. This observation shows the long-term persistence of CTC in the peripheral blood. If this persistence is clinically meaningful, remains to be seen.

5.4 CK-19 mRNA-positive circulating tumor cells in breast cancer

— Cytokeratin-19 (CK-19), a cytoskeletal component present in normal and cancerous epithelial cells, has been extensively used for the detection of breast cancer cells in mesenchymal tissues and seems to be a sensitive and reliable tumor marker in both patients with operable and metastatic breast cancer [81-83]. Almost 22 years ago Slade et al. developed an RT-PCR methodology for the detection of micrometastases in patients with breast cancer based on the estimation of the number of cytokeratin 19 transcripts in blood and bone marrow samples [143]. Our group developed an RT-qPCR assay for CK-19 mRNA [108,144] and evaluated its analytical and diagnostic sensitivity as well as its specificity and clinical potential for the molecular detection of occult carcinoma cells in peripheral blood of breast cancer patients.

5.4.1 Prognostic value in early breast cancer before adjuvant chemotherapy

— the prognostic significance of CTC detection in patients with early stage breast cancer before the administration of adjuvant therapy was first reported by Stathopoulou et al. [145]. One hundred forty-eight patients with operable breast cancer (stages I and II) were included and a nested RT-PCR was used for the molecular CK-19 mRNA detection. Roughly, one-third of patients (44 patients or 29.7 percent) had CK-19 mRNA-positive cells. No significant

association between the detection of CTC and the patients' menstrual status, disease stage, tumor grade, or hormonal receptor status was noted. After 28 months of median follow-up, 19 patients (12.8 percent) experienced disease relapse and eight (5.4 percent) died of breast cancer. The CTC-positive patients had poorer progression-free survival (HR=4.64; 95%CI 1.74-12.38; p<0.001) and overall survival (HR=6.13; 95%CI 1.24-30.47; p=0.01) compared to CTC-negative patients. In a multivariate analysis, the detection of CK-19 mRNA-positive cells (in addition to tumor size and estrogen receptor status) was an independent prognostic factor for disease relapse and death.

Later on, researchers from our group developed the real-time RT-PCR for the quantification of CK19 mRNA transcripts mentioned before [108,144]. The assay was used to detect CTC in an expanded cohort of 444 women with stage I-III breast cancer before the administration of adjuvant chemotherapy [146]. The results verified the shorter DFS (p<0.001) and OS (p<0.001) of the CTC-positive patients (Figure 3).

Nevertheless, only 30 percent of CK19 mRNA-positive patients relapsed, while 15 percent of patients relapsed and died of breast cancer after a 5-year median follow-up, without having detectable CTC [146]. Relevant questions arose as to whether these cells were viable or apoptotic and proliferating or quiescent. The proliferative potential of DTCs from patients with breast cancer or other epithelial tumours has been documented since they could be cultured in vitro [147]. Furthermore, Kallergi et al. demonstrated that CTC express activated signalling kinases, which may regulate migration mechanisms, supporting the presumption of their malignant and metastatic potential [148].

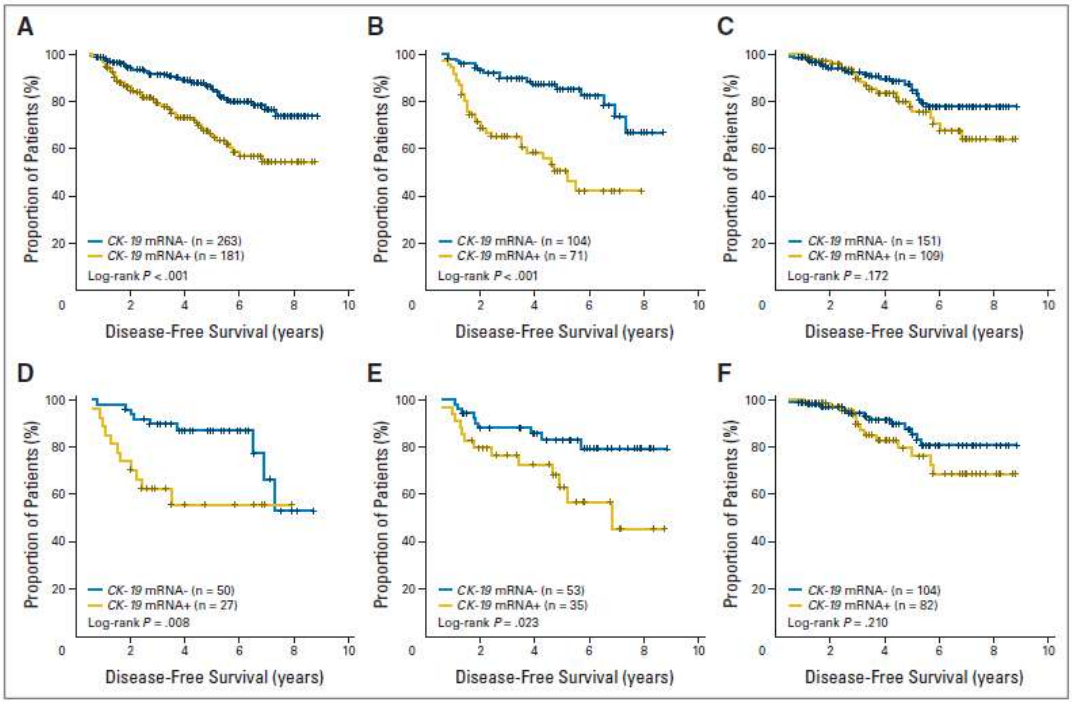

Figure 3: Overall survival in patients with and without cytokeratin-19 (CK-19) mRNA–positive circulating tumor cells (CTC) before the administration of adjuvant chemotherapy: (A) entire patient population, (B) ER-negative, (C) ER-positive, (D) triple-negative (E) HER2-positive, and (F) ER-positive/HER2-negative subgroups (adapted from Ignatiadis, M. et al. J Clin Oncol 2007;25:5194-5202).

5.4.2 Prognostic value in early breast cancer after adjuvant chemotherapy — Xenidis et al. evaluated the prognostic significance of CTC detection after the completion of adjuvant chemotherapy [149]. A nested RT-PCR was used for CK19 mRNA detection and patients with less than four involved axillary lymph nodes and detectable CTC were found to have an increased risk for disease relapse and shorter DFS.

Recently, the aforementioned results were confirmed using a real-time RT-PCR assay in an expanded cohort of patients and at the same time the sensitivity of CTC to adjuvant chemotherapy was evaluated [150]. Blood was drawn from 437 patients with early breast cancer before the administration and after the completion of adjuvant chemotherapy. One hundred seventy nine patients (41 percent) were CTC-positive before chemotherapy. Approximately one in two of these patients (51%) became CTC-negative after completion of

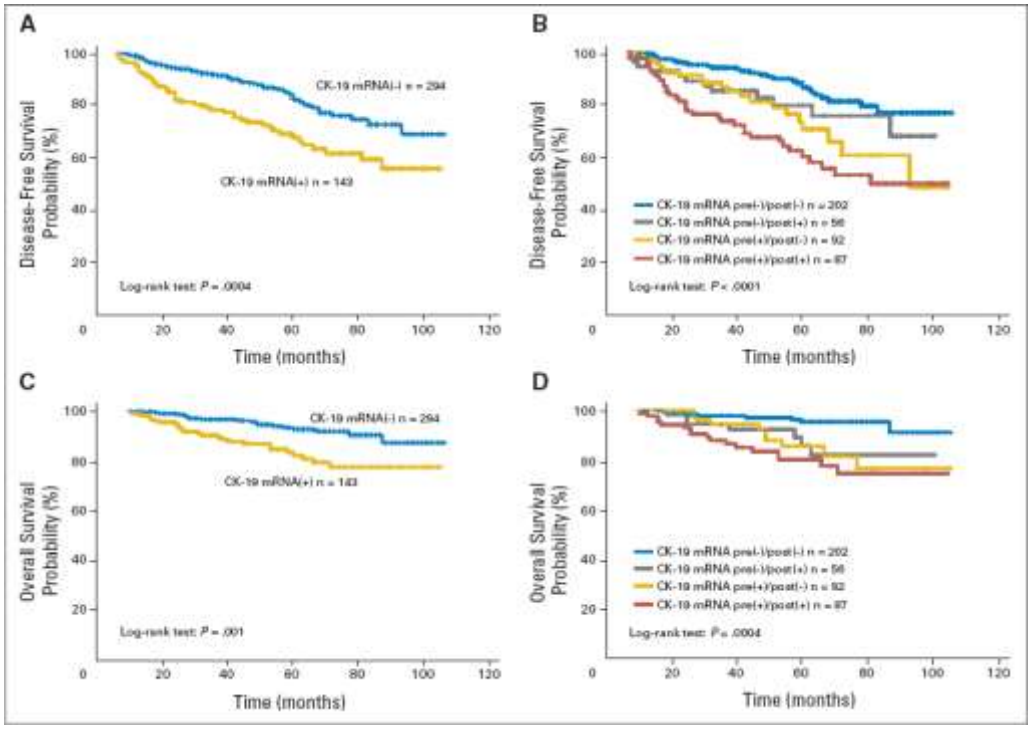

Figure 4: (A) Disease-free survival (DFS) of patients with or without detectable cytokeratin-19 (CK-19) mRNA-positive circulating tumor cells (CTC) after the completion of adjuvant chemotherapy. (B) DFS according to the detection of CK-19 mRNA-positive CTC both before the initiation and after the completion of adjuvant chemotherapy. (C) Overall survival of patients with or without detectable CK-19 mRNA-positive CTC after the completion of adjuvant chemotherapy. (D) Overall survival according to the detection of CK-19 mRNA-positive CTC both before the initiation and after the completion of adjuvant chemotherapy. (adapted from Xenidis N. et al. J Clin Oncol 2009; 27:2177-84).

More recently, taxane-based chemotherapy regimens have been showed to be more efficacious in CTC elimination [151]. The presence of CK-19 mRNA-positive CTC in the peripheral blood was evaluated before and after chemotherapy, using a real-time RT-qPCR assay, in a historical comparison of two cohorts of women with stage I-III breast cancer treated with adjuvant taxane-free and taxane-based chemotherapy. Taxane-based chemotherapy resulted in a higher incidence of CTC elimination than taxane-free regimens since 49.7 percent (74 of 149) and 33 percent (29 of 88) of patients with detectable CTC before chemotherapy, respectively, turned negative post-chemotherapy (p=0.015). More interestingly, only the patients with detectable CTC before chemotherapy derived benefit from

the addition of taxane. Patients treated with taxane-free regimens had a significantly lower DFS (p=0.035) than patients treated with taxane-based regimens; this difference was observed in patients with but not without detectable CTC before chemotherapy (p=0.018 and p=0.481, respectively). Similarly, the incidence of deaths was significantly higher in the taxane-free cohort of patients with but not without detectable CTC before chemotherapy compared with that of the taxane-based cohort (p=0.002). The taxane-based chemotherapy regimen was shown to be significantly associated with prolonged DFS in the multivariate analysis (HR: 2.00; 95% CI 1.20-3.34).

5.4.3 Prognostic value in node-negative early breast cancer — roughly 30 percent of patients with node-negative (N0) breast cancer will relapse in distant sites and eventually die as a result of disseminated disease [152]. Xenidis et al. reported the prognostic value of CTC detection in a group of 167 patients with node-negative early breast cancer [153]. The detection rate of CK-19 positive cells using the same real-time RT-qPCR assay was 21.6 percent. There was no correlation between CTC detection and known pathologic and clinical prognostic factors; only HER2 overexpression (IHC score 2 or 3+) on the primary tumor was associated with CTC positivity (p=0.033). Multivariate analysis revealed that CTC detection was associated with early distant disease relapse ($p<0$.001) and disease-related death (p=0.008). These results indicate that dissemination of tumor cells via hematogenous spread could be an early event in the disease course, occurring before the lymphatic metastasis.

5.4.4 Prognostic value during adjuvant hormonal therapy administration — the aim of adjuvant endocrine therapy is to prevent breast cancer cells stimulation from endogenous estrogens. In the most recent Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis (published in 2011) five years of tamoxifen was associated with a remarkable 15-year reduction in breast cancer mortality by about a third in all women with ER-positive disease [152]. No predictive markers for tamoxifen are available except from ER/PR expression.

To address the clinical utility of CTC detection during hormonal therapy blood from 119 patients with hormone receptor-positive tumors treated with adjuvant tamoxifen, was analysed using the same real-time RT-qPCR assay for CK19 mRNA. Twenty-two patients

(18.5 percent) were CTC-positive after the completion of adjuvant chemotherapy and before the initiation of tamoxifen [154]. The majority of them (68.2 percent) were also resistant to adjuvant tamoxifen (persistently CTC-positive: 12.6 percent of the 119 patients). Sixty-eight patients (57.1 percent) remained CTC-negative throughout the follow-up period (persistently CTC-negative: 57.1 percent of the 119 patients). Failure of tamoxifen to eradicate CTC was an independent prognostic factor for short DFS and OS.

In the modern era, aromatase inhibitors are the preferable alternative to tamoxifen for upfront, sequential or extended administration in post-menopausal women with early breast cancer [155]. In this regard, CTC-positive patients after tamoxifen administration could also be switched to an aromatase inhibitor in an effort to eliminate minimal residual disease. Additional studies are necessary to determine the utility of CTC monitoring in this setting.

5.4.5 Prognostic value according to the molecular subtypes — breast cancer is not a single disease with variable morphologic features but rather a group of molecularly distinct neoplastic disorders. Gene-expression profiling has been used to classify breast cancer into four main molecular classes [156]. The "intrinsic" classification includes the basal-like type that corresponds mostly to ER-negative, PR–negative, and HER2-negative tumors (hence, "triple-negative"), luminal-A (ER-positive and histologically low-grade), luminal-B (ER-positive but may express low levels of hormone receptors and are histologically high-grade) and HER2-positive tumors, which show amplification and high expression of the *HER2* gene.

Apart from differences in gene expression profiles, the ER-negative and ER-positive tumors differ in their response to treatment and natural history of the disease. We have already mentioned that during the first 5 years after primary treatment, women with ER-negative early-stage breast cancer experience more often disease relapse than those with ER-positive tumors. On the other hand, it is not uncommon for patients with ER-positive tumors to relapse after 5-15 years [36].

A recent study by Ignatiadis et al. demonstrated that CTC detection predicts the prognosis in clinically relevant subgroups of early-stage breast cancer patients [146]. A total of 444 patients were enrolled in this study before the initiation of adjuvant chemotherapy and 181 (40.8 percent) were found to be CTC-positive. Among them, 109 (41.9 percent) of 260 ER-positive patients were CTC-positive, 71 (40.6 percent) of 175 ER-negative, 27 (35 percent) of 77 triple-negative, 35 (39.8 percent) of 88 HER2-positive, and 82 (44.1 percent) of

186 patients with ER-positive/HER2-negative tumors. After a median follow-up of 53.5 months, patients with detectable CTC experienced significantly reduced DFS and OS (Figure 3). Nevertheless, this was mainly observed in patients with ER-negative, HER2-positive or triple-negative tumors. On the other hand, despite the presence of CTC, patients with ER-positive or ER-positive/HER2-negative tumors did not have any significant difference in clinical outcome (Figure 3). In multivariate analysis, the interaction between CTC and ER status was the strongest independent prognostic factor for reduced DFS (HR=3.808; 95%CI 2.415-6.003; p<0.001) and OS (HR=4.172; 95%CI, 2.477-9.161; p<0.001). This study showed for the first time that CTC detected by a sensitive quantitative RT-PCR assay had different prognostic value among the molecular subtypes of early breast cancer.

5.4.6 Meta-analysis — the prognostic role of CTC detected by RT-PCR in early breast cancer was confirmed by a recent meta-analysis of the published literature [157]. Twenty-four studies were included which comprised 4.013 patients for final analysis. The presence of CTC was significantly associated with increased relapse (HR=2.6; 95%CI 2.09-3.42 p <0.001) and shorter OS (HR=3.00; 95%CI 2.29-3.94; p<0.001). When CK-19 was used as a marker the prognostic value of CTC detection was higher (relapse: HR=2.94 95%CI 2.17-3.98, p<0.001 and OS: HR=3.59; 95%CI 2.52–5.11; p<0.001). CK-19 mRNA-positive CTC were not associated with primary tumors' characteristics (histological grade, tumor size, axillary lymph nodes metastases, ER/PR negativity, *HER2* overexpression).

The evaluation of CTC status following standard therapy might be of great interest in the modern era of targeted therapy. This was clearly shown in a recent study by Georgoulias et al. [158]. Seventy-five women with early breast cancer without HER2 overexpression and detectable CK19 mRNA-positive CTC both before and after adjuvant chemotherapy were randomized to trastuzumab (n=36) or observation (n=39). The primary endpoint was 3-year disease-free survival rate. The vast majority of the patients (89 percent) had CTC that were positive for HER2. After trastuzumab administration, 27 out of 36 women (75 percent) turned CK19 mRNA-negative compared to only 17.9 percent (7 out of 39) in the observation arm (p=0.001). After 67.2 months of median follow-up, four (11 percent) and 15 (38 percent) relapses were observed in the trastuzumab and observation arm, respectively (p=0.008). The median DFS was also significantly prolonged for the patients treated with trastuzumab (p=0.008). The authors concluded that trastuzumab administration could eliminate

chemotherapy-resistant CK19 mRNA-positive CTC, reduce the risk of disease recurrence and prolong the DFS of patients with early breast cancer.

5.4.7 Prognostic value in metastatic disease — more recently the clinical relevance of CK-19mRNA-positive CTC detected before the initiation of front-line treatment in patients with metastatic breast cancer has been reported [159]. The presence of CTC was detected in 298 patients with metastatic breast cancer using a real-time RT-PCR assay. In 44 patients, both the CellSearch® system and the RT-PCR assay were used for the detection of CTC. There was a strong correlation between the detection of CTC by both assays. CK-19mRNA-positive CTC were detected in 201 (67 percent) patients and their detection was independent of various clinico-pathological characteristics. The median progression-free survival (9.2 vs 11.9 months, p=0.003) and the overall survival (29.7 vs 38.9 months, p=0.016) were significantly shorter in patients with detectable CK-19mRNA-positive CTC compared with patients without detectable CTC. Similar to the studies that used the CellSearch® system, the detection of CK-19mRNA-positive CTC in patients with metastatic breast cancer before front-line therapy could define a subgroup of patients with dismal clinical outcome.

6. Conclusions and perspectives — Despite increasing evidence supporting the detection of CTC as a biomarker, it still remains unclear how this information could be integrated into the daily clinical practice. Although biomarkers could simply be used in addition to the present methods to more accurately stratify patients in terms of proposed benefit of adjuvant treatments, their presence at diagnosis or after completion of adjuvant treatment might guide the use of additional therapies including those that specifically target CTC. Although there is the suggestion that CTC might represent a chemo-resistant stem cell-like phenotype [112], there are reports in the (neo)adjuvant and metastatic settings showing a reduction in CTC counts after standard chemotherapy [131, 141]. Therefore if complete CTC eradication is needed to achieve better clinical outcomes, whether this can be achieved with prolonged treatment with currently available drugs or the design of novel therapies, remains undetermined. Furthermore, the timing of CTC measurements might prove to be important prognostically, and it is unclear whether they should be taken before, during, or after surgery, or before or after completion of adjuvant treatment. Agreement is also needed as to which CTC cutoff should be considered significant.

Present clinical practice does not use CTC to stage the disease or guide adjuvant treatment decisions and this is exemplified by the fact that the recent ASCO guidelines do not recommend CTC measurements for staging non-metastatic breast cancer [43]. Although studies from our group and others support the notion that CTC detection should be included in the staging algorithms for patients with non-metastatic breast cancer, larger multicenter clinical studies are needed to confirm and further clarify their role. Therefore, until the completion of such studies, we cannot recommend that patients should be treated differently on the basis of these data.

Part B

CK-19 mRNA-positive circulating tumor cells during follow-up of patients with operable breast cancer. Prognostic significance for late relapse

1. **Hypothesis to be tested** — Invasive breast cancer is the most common malignancy in women. Due to declining mortality rates that are attributable mostly to the use of screening mammography and effective adjuvant therapy, more women nowadays survive their breast cancer [64]. Since metastatic disease is considered incurable, the early recognition and treatment of potentially still curable minimal residual disease is one of the major goals of care of breast cancer survivors and requires the in depth understanding of relapse patterns.

Depending on the specific breast cancer type, the majority of recurrences occur during years 2 to 5, although they can occur earlier or much later [36]. Especially, for women with hormone receptor-positive disease, more than one-half of all recurrences and deaths occur beyond five years from diagnosis. To date no tool is available for monitoring the effect of adjuvant treatment and in most cases the recurrence risk is calculated based on previous statistical analyses. Therefore, with existing methods prediction of the risk of relapse for the individual patient is limited.

We have already reviewed that circulating tumor cells in the peripheral blood of patients with operable breast cancer have been shown to be independent adverse prognostic factors for disease recurrence and disease-related death. Immunocytochemistry using antibodies against proteins that are expressed on epithelial but not on mesenchymal cells is widely used for the detection of DTC and CTC. However, the detection of mRNA transcripts for specific epithelial markers by using RT-PCR and, more recently, the RT-qPCR seems to have higher diagnostic sensitivity [86]. The major advantage of RNA-based approaches is related to the rapid degradation of RNA released from cells in the blood by RNAses; therefore, the origin of detectable RNA transcripts in blood is considered to be viable cells. Cytokeratin-19 a cytoskeletal component present in normal and cancerous epithelial cells, has been extensively used for the detection of breast cancer cells in mesenchymal tissues and seems to be the most sensitive and reliable tumor marker in both patients with operable and metastatic breast cancer [160,161].

Several studies have shown the prognostic significance of CK-19 mRNA-positive CTC in patients with operable breast cancer [145,149,150,151,153,162]. However, all these studies have investigated the prognostic value of CTC at initial diagnosis and before the initiation and/or following the completion of adjuvant chemotherapy. Only a few reports exist concerning the clinical relevance of DTC, but none for CTC, during the surveillance period

after the completion of adjuvant chemotherapy [118,163]. The unfavorable clinical outcome of patients with detectable isolated tumor cells in bone marrow has been shown in the latter studies. Given that DTC and CTC are theoretically the primary targets of adjuvant treatments, their fate after systemic therapy could be a potential useful marker permitting a direct and individualized assessment of treatment efficacy and a more accurate estimation of the risk of relapse.

In the present study, we sought to evaluate the clinical relevance of CK-19 mRNA-positive CTC detected by a quantitative real-time RT-PCR assay at different time points during the follow up period after the completion of adjuvant chemotherapy in patients with operable breast cancer. We hypothesized that patients having detectable CK-19 mRNA-positive cells during follow up despite the administration of adjuvant therapy could be at an increased risk of late disease relapse (defined as relapse at least two years after the end of adjuvant chemotherapy) and death.

2. **Patients** — A retrospective analysis of prospectively collected data in the context of an ongoing longitudinal study that has been previously reported was performed [150]. Women with operable breast cancer (TNM stage I to III) who were under surveillance and had not experienced disease relapse during the first two years of follow up, were eligible for this study. All patients had received adjuvant chemotherapy mostly in the context of research protocols of the Hellenic Oncology Research Group. The chemotherapy regimens that were administered are listed below:

- **CMF regimen:** cyclophosphamide 600mg/m^2, methotrexate 40mg/m^2 and 5-fluorouracil 600mg/m^2 given on days 1 and 8 of 28 days' cycle for total of 6 cycles.
- **FEC regimen:** 5-fluorouracil 500mg/m^2, cyclophosphamide 500mg/m^2 and epirubicin 75mg/m^2, given on day 1 of 21 days' cycle for total of 6 cycles.
- **T/EC regimen**: docetaxel 100mg/m^2. Given on day 1 of 21 days' cycle for total of 4 cycles, followed by epirubicin 75mg/m^2 and cyclophosphamide 700mg/m^2 given on day 1 of 21 days' cycle for total of 4 cycles.

After the completion of adjuvant chemotherapy, patients received adjuvant radiotherapy and hormonal therapy when indicated according to their individual disease characteristics. There were no subgroups of patients who received only adjuvant hormone therapy or no adjuvant systemic therapy at all.

Patients' follow-up consisted of medical history and physical examination, with laboratory and imaging studies restricted as indicated, every 3 months for the first 2 years, every 6 months for the next 3 years and yearly thereafter. Treating physicians were completely unaware of the CK-19mRNA results for their individual patients and all follow up laboratory and imaging studies to detect disease relapse were performed independently of the CK-19mRNA results. All patients signed an informed consent to participate in the study, which was approved by the ethics and scientific committees of our institution.

Cytokeratin-19 mRNA-positive CTC were monitored at specific time points after the completion of adjuvant chemotherapy for a 5-year follow up period. The first blood sample was obtained 3 months after the end of chemotherapy and subsequent samples were obtained every 6 months thereafter during the 5-year follow up.

Patients were classified into four groups based on their CTC status during the first two years and the subsequent three years of follow up (as persistently negative, persistently-positive, negative turn to positive and the opposite). At least one CK-19 mRNA-positive blood sample in the corresponding period of time was required for classifying the patient in the CTC-positive group. On the other hand, if all the collected blood samples were negative for CK-19 mRNA, the patient was characterized as CTC-negative. Using this definition, the patients were classified in the “persistently CTC-negative” group, if there were no positive blood samples for CK-19 mRNA throughout the 5-year follow up period. On the other hand, the “persistently CTC-positive” patients had at least one positive blood sample for CK-19 mRNA in the first 2 years and at least another positive one in the subsequent 3 years of follow up. Accordingly, patients in the “CTC-negative turn to positive” group had no positive samples in the first two years, but at least one positive sample in the next 3 years. The opposite was true for the “CTC-positive turn to negative” group.

3. **Clinical Samples** — Twenty milliliters (mL) of peripheral blood in EDTA was collected at each visit (3 months after the end of chemotherapy and subsequent samples were obtained every 6 months thereafter during the 5-year follow up). To avoid contamination with epithelial cells from the skin, all blood samples were obtained at the middle of vein puncture after the first 5 mL of blood was discarded.

4. Method

4.1 Peripheral Blood Mononuclear Cells isolation

— Peripheral blood was diluted (vol/vol for peripheral blood) in PBS (8,0 gr/l NaCl, 0,2gr/l KCl, 1,15 gr/l Na2HPO4, 0,2 gr/l KH2PO4, pH 7,3) and cells were dissociated by passing them through 25-gauge 5/8 needles. The protocol used for the isolation of the PBMCs consisted of the following steps:

- Step 1: the diluted cells suspension was carefully layered over Ficoll-Hypaque 1077 (Sigma) in a 50 mL conical tube. The volume of ficoll was half the volume of the suspended cells.
- Step 2: the two-phase mix was centrifuged at 1,200 *g* for 30 min at 4°C in a swinging-bucket rotor without brake.
- Step 3: the upper layer was aspirated, leaving the mononuclear cell layer (lymphocytes, monocytes, and thrombocytes) undisturbed at the interphase.
- Step 4: the mononuclear cell layer was carefully transferred to a new 50 mL conical tube which was filled with PBS, and centrifuged at 1,600 *g* for 10 min at 18°C. The supernatant was removed carefully. The tube was filled again with PBS and centrifuged at the same conditions.
- In between the two washes with PBS the viable cells were counted using a haemocytometer.
- Step 5: cells were resuspended in 1 mL of PBS and pelleted at 1,200 *g* for 2 minutes at 18°C. Cell pellets were kept at −80°C until RNA extraction.

4.2 RNA extraction

— Total RNA extraction was performed using Trizol LS reagent (Gibco) according to the manufacturer's instructions [164]. All RNA preparation and handling steps took place in a laminar flow hood, under RNAse-free conditions.

- Cells were lysed with TRIZOL reagent by repetitive pipetting or by passing through syringe and needle. We used 1 mL of the reagent per 5-10 $X10^6$ of cells.
- The homogenized sample was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.
- 0.2 mL of chloroform per 1 mL of TRIZOL was added and the sample was mixed vigorously by vortexing for 15 seconds and incubated them at room temperature for 2 to 3 min. The sample was centrifuged at 12,000 *g* for 15 minutes at 4°C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The upper aqueous phase was transferred carefully without disturbing the interphase into fresh tube. (The volume of the aqueous phase is about 60 percent of the volume of TRIZOL Reagent used for homogenization).
- The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. We used 0.5 mL of isopropyl alcohol per 1 mL of TRIZOL reagent used for the initial homogenization. The sample was incubated at room temperature for 10 minutes and centrifuged at 12,000 *g* for 10 minutes at 4°C. The RNA precipitated, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
- The supernatant was removed completely. The RNA pellet was washed once with 75 percent ethanol, and 1 mL of 75 percent ethanol per 1 mL of TRIZOL reagent used for the initial homogenization was added. The sample was mixed by vortexing and centrifuged at 7,500 *g* for 10 minutes at 4°C. The above washing procedure was repeated and the leftover ethanol removed.
- The RNA pellet was air-dried for 5-10 minutes and dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip and stored at −80°C until used. The RNA concentration was determined by absorbance readings at 260 nm with the Hitachi UV-VIS (U-2000) spectrophotometer (Tokyo, Japan). RNA integrity was tested by PCR amplification of the beta-actin housekeeping gene. As positive and negative controls, RNA samples were also prepared from the human tumor cell lines MCF-7 and ARH-77, respectively.

4.3 Complementary DNA (cDNA) synthesis — cDNA Reverse Transcription (RT reaction or first strand cDNA synthesis) is a process in which single-stranded RNA is reverse transcribed into by using total cellular RNA or poly(A) RNA, a reverse transcriptase enzyme, a primer, dNTPs and an RNase inhibitor. Three types of primers can be used for RT reaction: oligo (dT) primers, random (hexamer) primers and gene specific primers with each having its pros and cons. We used the oligo (dT) primers. The reaction was carried out with the Thermoscript RT-PCR system (Gibco). The reaction mix contained:

- 1-5 micrograms of extracted RNA.
- 1 microliter of primer [oligo (dT) 20 (50 μM)]
- DEPC-treated water to a total volume of 10 microliters.

The mix was first incubated at 70 °C to denature RNA secondary structure and then quickly chill on ice to let the primer anneal to the RNA. Then the other components of RT are added to the reaction including:

- 4 microliters of RT reaction buffer (5x)
- 1 microliter of Dithiothreitol (DTT, 0.1 M)
- 1 microliter of RNase inhibitor (RNaseOUT, 40 U/μL)
- 1 microliter DEPC-treated water
- 2 microliters of dNTP mix (10 mM)
- 1 microliter of reverse transcriptase (ThermOEcript RT, 5 U/μL)

The RT reaction was extended at 60 °C for 60 minutes and heated at the reaction at 70 °C for 5 minutes to inactivate the enzyme. Before using the reaction in real time RT- PCR, removal of the template RNA was necessary by treating the RT reaction with RNase H. The cDNA was stored at −20°C until used.

4.4 Real-Time RT-PCR for CK-19 mRNA — The principle of the proposed real-time RT-PCR assay for CK-19 is shown in figure 5. Quantification is based on real-time monitoring during PCR of fluorescently labeled specific hybridization probes for CK-19. The point where the fluorescence rises above background noise (Cq) is best quantified through the LightCycler software as the second derivative maximum of the curve. Real-time RT-PCR for CK-19 mRNA was performed using the LightCycler system (Roche Diagnostics). The primers

and the hybridization probes used for CK-19 were designed and synthesized by TIB MOLBIOL (Berlin, Germany; Table 5)

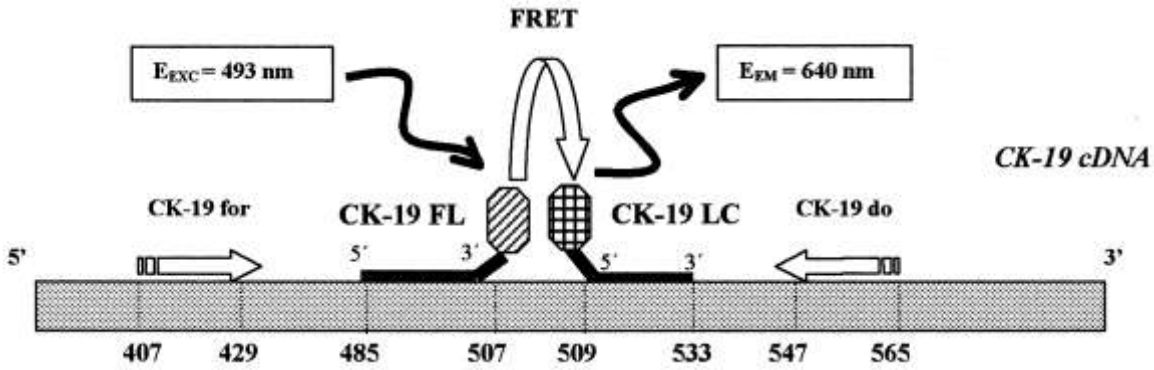

Figure 5: Principles of the real-time RT-PCR assay for CK-19 mRNA (adapted from Stathopoulou A. et al. Clin Cancer Res; 2003;9:5145).

Gene	Primer/Probe	Name	Oligonucleotide sequence (5′-3′)
CK-19	Forward primer	CK19-for	gCACTACAgCCACTACTACACgA
	Reverse primer	CK19-do	CTCATgCgCAgAgCCTgTT
	Hybridization probe	CK19-FL[1]	TgTCCTgCAgATCgACAACgCCC-FL
	Hybridization probe	CK19-LC2	CRed640-CTggCTgCAgATgACTTCCgAACC
GAPDH	Forward primer	GAPDH F	TTggTATCgTggAAggACTCA
	Reverse primer	GAPDH R	TgTCATCATATTTggCAggTTT
	Hybridization probe	GAPDH 3FL[1]	TgTCCCCACTgCCAACgTgTCAg-FL
	Hybridization probe	GAPDH 705[3]	LCRed705-ggTggACCTgACCTgCCgTCTAgA

Table 5: Sequences of primers and hybridization probes used in this study. [1]labeled with with fluorescein, [2] labeled with LC Red640 (TIB MOLBIOL) and [3] labeled with LC Red705 (TIB MOLBIOL).

For the PCR, 2 μl of cDNA were placed into a 18-μl reaction volume containing 1 μl of the sense primer CK19-for (3 μm), 1 μl of the antisense primer CK19-do (3 μm), 2.4 μl of the LightCycler Fast Start DNA Master Hybridization Probes reagent (10× concentration), 1 μl of the probe CK19-FL (3 μm), and 1 μl of the probe CK19-LC (3 μm), and DEPC-H_2O was added to the final volume (Table 6). PCR reaction was initiated with a 10 minutes denaturation at 95°C and terminated with a 30 seconds cooling step at 40°C. The cycling protocol consisted of denaturation step at 95°C for 10 seconds annealing at 60°C for 10

seconds, and extension at 72°C for 20 seconds, and repeated for 50 times. Fluorescence detection was performed at the end of each annealing step (Table 7).

Reagent	Volume (microliters)
Forward primer (3 μM)	1
Reserve primer (3 μM)	1
Hybridization probe CK19-FL	1
Hybridization probe CK19-LC	1
BSA (10μg/μl)	0.3
dNTPs, (10 μM)	0.4
$MgCl_2$, 50 mM	1
PCR reaction mix (10x)	2
cDNA	1
Taq polymerase (5U/μL)	0.2
DEPC-treated H_2O	11.1
	Total 20

Table 6: Reagents' concentration for CK-19 mRNA real time RT-PCR reaction.

PCR stage	Temperature/duration
Denaturation	95°C/10 min
Denaturation Annealing Extension	95°C/30 sec 60°C/10 sec 72°C/20 sec 50 cycles
Termination	40°C/30 sec

Table 7: Conditions for CK-19 mRNA real time RT-PCR reaction.

For quantification, an external calibration curve was obtained by using external standard cDNA. Total RNA was prepared from 1×10^6 MCF-7 cells (as verified by a hemocytometer). Serial dilutions of this RNA preparation in DEPC-treated water, corresponding to 1–10,000 MCF-7 cells, were used for cDNA synthesis. These cDNA were kept in aliquots at −20°C and used throughout the study as external standards. This calibration curve was created by plotting the number of MCF-7 cells corresponding to each

external standard cDNA versus the value of its Cp. The number of circulating CK-19 mRNA-positive cells for all of the tested samples was expressed as MCF-7 cell equivalents per 5 μg of total-RNA, as determined by LightCycler software 3.1, according to the external standard calibration curve.

Real-time PCR for the housekeeping gene *GAPDH* was performed in all clinical samples to evaluate the quality of the cDNAs used in the study. The primers and the hybridization probes used for *GAPDH* were designed and synthesized by TIB MOLBIOL and are shown in Table 4.

The specificity, sensitivity, and reproducibility of the quantitative real-time RT-PCR methodology for CK-19 mRNA was optimized by Stathopoulou et al [86]. The presence of ≥0.6 MCF-7 cell equivalents/5 μg RNA in the patient blood sample was considered as a positive result. According to this cut-off, the specificity of the proposed method was evaluated by analyzing peripheral blood samples from 89 female healthy blood donors: only 2 samples (2.2 percent) were found positive.

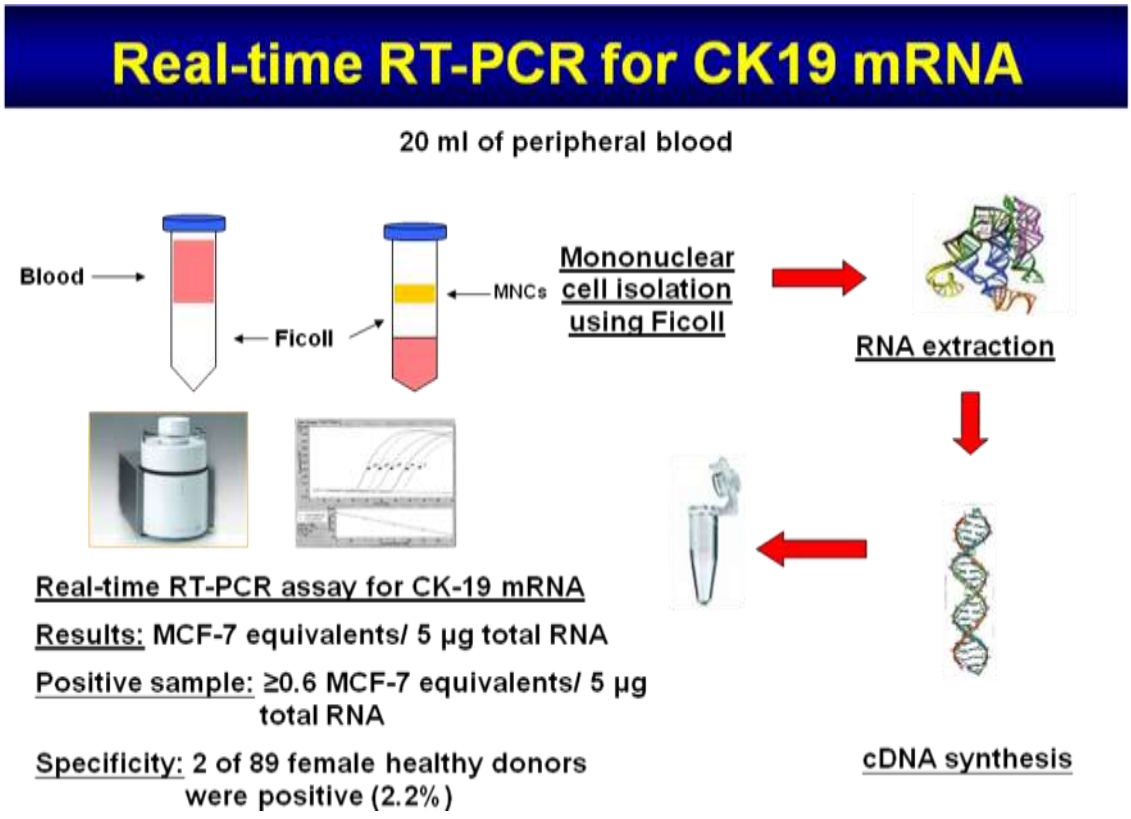

Figure 6: Graphic representation of the real-time RT-PCR assay used for the detection of CK19 mRNA-positive cells.

5. Statistical analysis — Disease-free survival, defined as the time from study entry until the day of the first evidence of disease recurrence and OS defined as the time from study entry to death were the main dependent variables of the study. The data cut-off date was the July 20th 2010. Kaplan-Meier curves for DFS and OS were compared using the log-rank test to provide a univariate assessment of the prognostic value of selected clinical risk factors. Clinicopathologic factors known to be associated with prognosis, such as menopausal status (premenopausal *vs* postmenopausal), tumor size (T1 *vs* T2-3), number of axillary lymph nodes involved (0-3 *vs* ≥4), histological grade (1-2 *vs* 3), ER status (negative *vs* positive), PR status (negative *vs* positive) and HER2 status (negative *vs* positive) were tested in univariate analysis. Variables that were found to be significant at the univariate screen were then entered in a stepwise multivariate Cox proportional hazards regression model to identify those with independent prognostic information. Entry into and removal from the model were set at 5 percent and 10 percent, respectively. All statistical tests were performed at the 5 percent level of significance. SPSS version 13 (SPSS Inc, Chicago, IL) statistical software was used for the analysis. This report is written according to the reporting recommendations for tumor marker prognostic studies (REMARK criteria) [165].

6. Results

6.1 Patients' characteristics — A total of 455 consecutive patients with diagnosis of operable breast cancer treated and followed at the department of medical oncology of the university hospital of Heraklion between January 1997 and December 2004 were screened for eligibility for this study. Four hundred twelve (91 percent) patients belong to the same cohort of patients that was used to evaluate the prognostic significance of CK-19 mRNA-positive CTC detection before initiation and/or after completion of adjuvant chemotherapy [150].

One hundred forty-three patients were excluded for reasons listed in Figure 7, and 312 were included in the study. Patients' characteristics at the time of primary diagnosis in relation to CTC status during follow-up are summarized in Table 8. The persistent detection of CTC during follow-up did not correlate with patients' and/or tumor's characteristics, such as age (p=0.197), menopausal status (p=0.372), tumor size (p=0.637), lymph node status (p=0.082), histopathological grade (p=0.746) and hormone receptor status (p=0.156). There

was a difference in the type of adjuvant chemotherapy administered, with more patients in the persistently positive group having received anthracycline-based regimens (p=0.011).

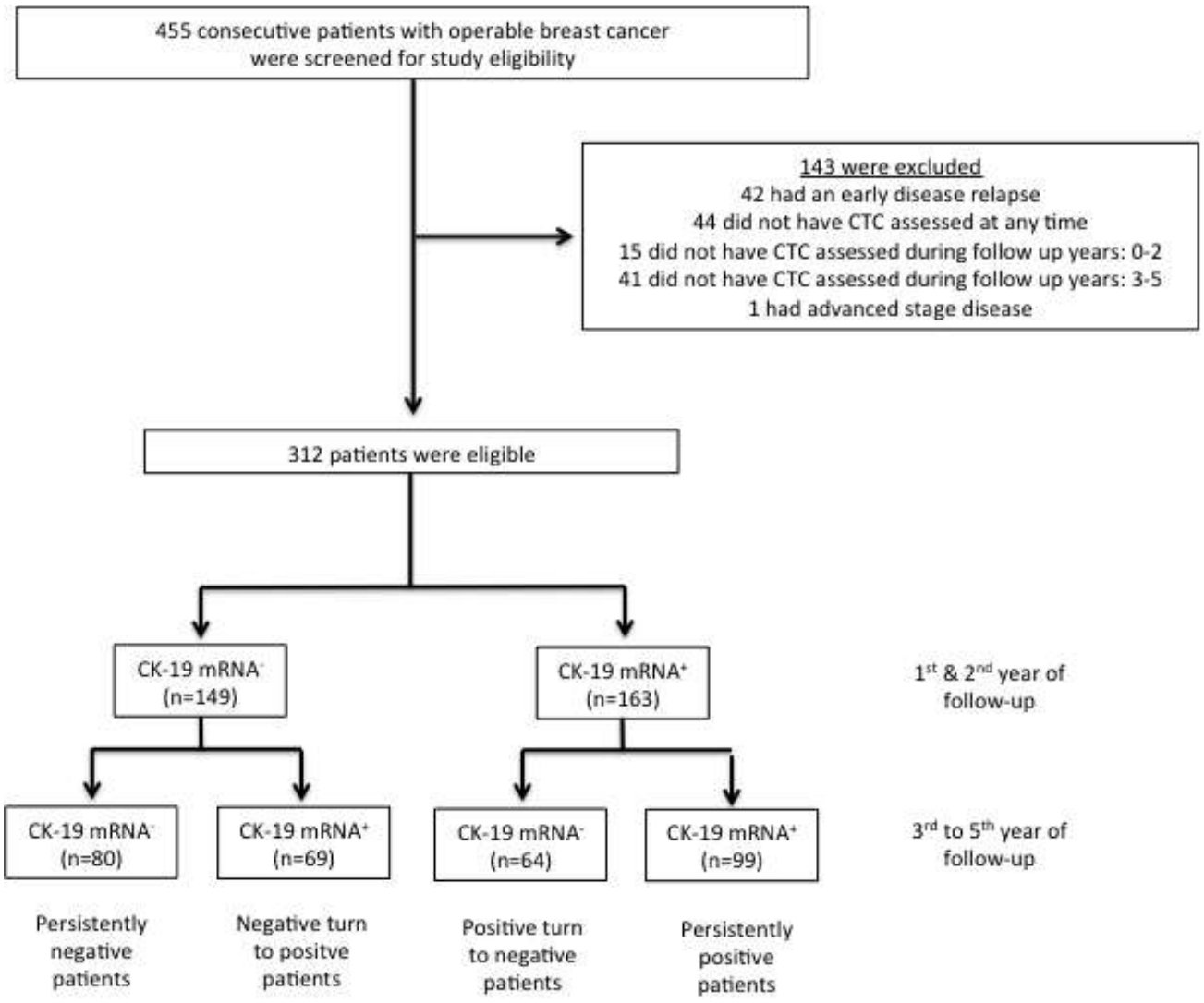

Figure 7: Study enrollment, reasons for patients' exclusion and patients' classification according to CK-19 mRNA CTC status.

Characteristics	All Patients		CK-19 mRNA-Persistently Negative		CK-19 mRNA-Persistently Positive		
	N	%	N	%	N	%	
Patients enrolled	312		80	25.6	99	31.7	
Age median (range)	54 (26-77)		51.5 (26-75)		54 (28-75)		Mann-Whitney p=0.197
Menopausal Status Pre-menopausal Post-menopausal	 145 167	 46.5 53.5	 41 39	 51.3 48.8	 44 55	 44.4 55.6	p=0.372
Tumor Size T1 T2 T3	 116 174 22	 37.2 55.8 7.1	 30 43 7	 37.5 53.8 8.8	 33 59 7	 33.3 59.6 7.1	T2/T3 vs T1 p=0.637
Lymph Nodes N0 N1-3 N>3	 107 119 86	 34.3 38.1 27.6	 33 29 18	 41.3 36.3 22.5	 28 35 36	 28.3 35.4 36.4	p=0.082
Histology Grade 1 / 2 3 lobular	 159 119 34	 51.0 38.1 10.9	 39 28 13	 48.8 35.0 16.3	 56 36 7	 56.6 36.4 7.1	p=0.746
HR ER(-)/PR(-) Other Unknown	 70 190 52	 22.4 60.9 16.7	 24 39 17	 30.0 48.8 21.3	 22 60 17	 22.2 60.6 17.2	p=0.156
HR and Her-2 ER(-)/PR(-)/HER-2(-) Other Unknown	 52 255 57	 16.7 65.1 18.3	 19 41 20	 23.8 51.3 25.0	 16 66 17	 16.2 66.7 17.2	p=0.116
Radiation therapy No Yes	 24 289	 7.7 92.3	 7 73	 8.8 91.2	 9 90	 9.1 90.9	p=0.937
Hormonotherapy No Yes	 23 289	 7.4 92.6	 10 70	 12.5 87.5	 5 94	 5.1 94.9	p=0.103
Type of Hormonotherapy No Hormonotherapy AIs T AIs & T LHRH LHRH + T or AIs	 23 33 50 57 32 117	 7.4 10.6 16.0 18.3 10.3 37.5	 10 10 10 9 11 30	 12.5 12.5 12.5 11.3 13.8 37.5	 5 11 19 22 8 34	 5.1 11.1 19.2 22.2 8.1 34.3	p=0.119
Chemotherapy CMF FEC T/EC	 30 149 133	 9.6 47.8 42.6	 11 31 38	 13.8 38.8 47.5	 4 57 38	 4.0 57.6 38.4	p=0.011

Table 8: Patients' characteristics at diagnosis categorized based on CK-19 mRNA-positive cells during follow up.

6.2 Detection of CK-19 mRNA-Positive Cells and Disease Recurrence — After a median follow-up period of 107 months (range: 38-161 months), 63 patients (20.2 percent) had developed a distant (n= 56; 88.8 percent) or locoregional recurrence (n=7; 11.2 percent) (Table 8). Compared to the persistently negative patients, only the group of CK-19 mRNA-persistently positive patients had a significant higher risk of disease relapse (36.4 versus 11.2 percent; Fisher's exact test $p<0.001$). In fact, risk of disease recurrence was the highest in patients with persistently-positive CTC (36.4 versus 7.8 percent; $p<0.001$ and 36.4 versus 18.8 percent; $p=0.016$ compared to positive turn to negative and negative turn to positive groups, respectively) (Table 9).

CK-19 mRNA	No of patients	Relapses			Deaths		
		Yes N (%)	No N (%)	Fisher's Exact test, p	Dead N (%)	Alive N (%)	Fisher's Exact test
Persistently Positive	99	36 (36.4)	63 (63.6)	$p<0.001$	24 (24.2)	75 (75.8)	$p=0.001$
Persistently Negative	80	9 (11.2)	71 (88.8)		5(6.3)	75 (93.8)	
Positive Turn to negative	64	5 (7.8)	59 (92.2)	$p<0.001$ versus persistently positive	3 (4.7)	61 (95.3)	$p=0.001$ versus persistently positive
Negative Turn to positive	69	13 (18.8)	56 (81.2)	$p=0.016$ versus persistently positive	9 (13.0)	60 (87.0)	$p=0.079$ versus persistently positive
While also, $p = 0.248$ for relapse of turn to positive versus persistently negative $p = 0.172$ for deaths of turn to positive versus persistently negative							

Table 9: Incidence of disease recurrence and deaths according to the detection of CK-19 mRNA-positive circulating tumor cells.

The 5-year DFS rates were 82.5 versus 92.7 percent for persistently-positive versus persistently-negative patients, respectively. As illustrated in Figure7A, persistently positive patients had a significantly shorter DFS than the persistently negative patients ($p<0.001$). Although no group has as yet reached the median DFS, there was a progressive decrease in

the DFS of the four groups of patients according to the detection of CK-19 mRNA-positive CTC during the 5 years of follow up (Figure 8A).

6.3 Detection of CK-19 mRNA-Positive Cells and Survival — Forty-one patients (13.1 percent) died during follow up as a result of disease progression. Twenty-four (58.5 percent) and five (12.2 percent) of these deaths occurred in the persistently-positive and persistently-negative groups, respectively (Fisher's exact test p=0.001; Table 9). The 10-year overall survival rates were 81.4 percent for persistently-positive versus 96.7 percent for persistently-negative patients. Estimated median overall survival was significantly shorter for persistently positive compared to persistently negative patients (p=0.013). Similar to DFS, there was a progressive decrease in the OS of the four groups of patients according to the detection of CK-19 mRNA-positive CTC during the 5 years of follow up (Figure 8B).

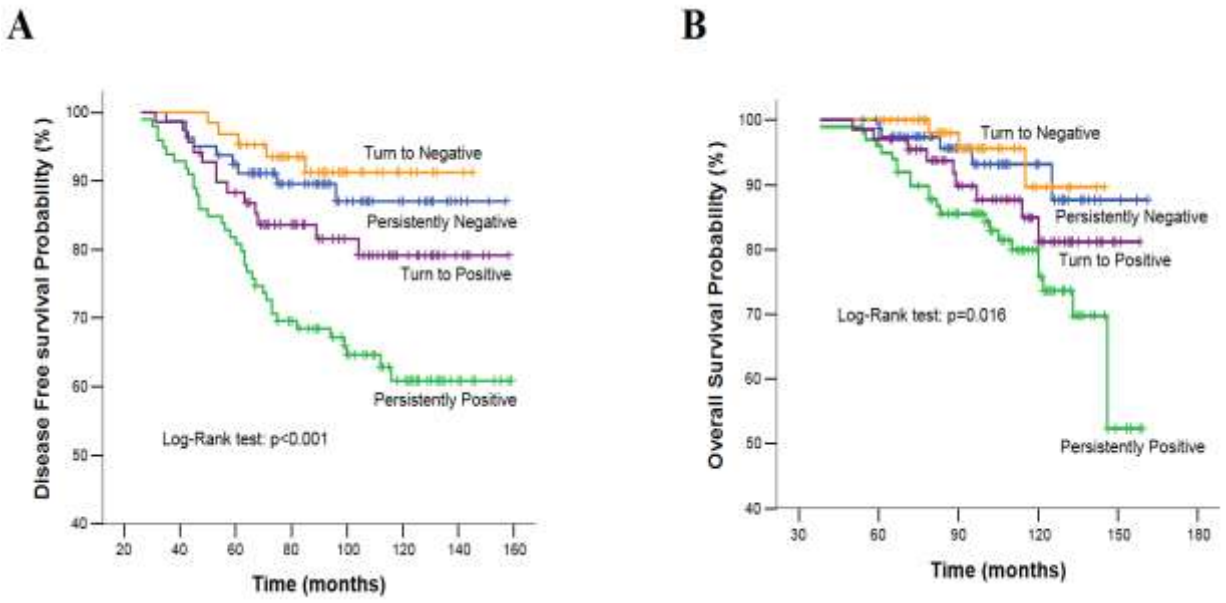

Figure 8 (A): Disease-free survival **(B):** Overall survival according to the detection of CK-19 mRNA-positive circulating tumor cells during the first two years and the subsequent three years of follow up.

6.4 Subgroup analysis based on cumulative number of positive tests, pre-chemotherapy CTC status and hormone receptor status

— Since patients underwent serial blood draws for the assessment of CK-19 mRNA-positive CTC, we analyzed our data to address the question whether the cumulative number of positive tests matters. Among patients with positive tests 38.7 percent (during the first two years of follow up), 24.4 percent (during the subsequent 3 years) and 57.3 percent (during all the 5 years) had two or more positive test results (Table 10). No difference was found in disease-free survival between the groups with different cumulative number of positive tests, probably due to the small number of patients and events in each group (Figure 9).

Number of positive tests	First 2 years of follow up N (%)	Subsequent 3 years of follow up N (%)	Entire 5 years of follow up N (%)
1	100 (61.4)	121 (72)	99 (42.6)
2	32 (19.6)	29 (17.3)	62 (26.8.)
3	25 (15.3)	13 (7.7)	28 (12.1)
≥4	6 (3.7)	5 (3)	43 (18.5)
Total	163	168	232

Table 10: Distribution of number of positive samples during the first 2 years, the subsequent 3 years and the entire 5 years of follow up.

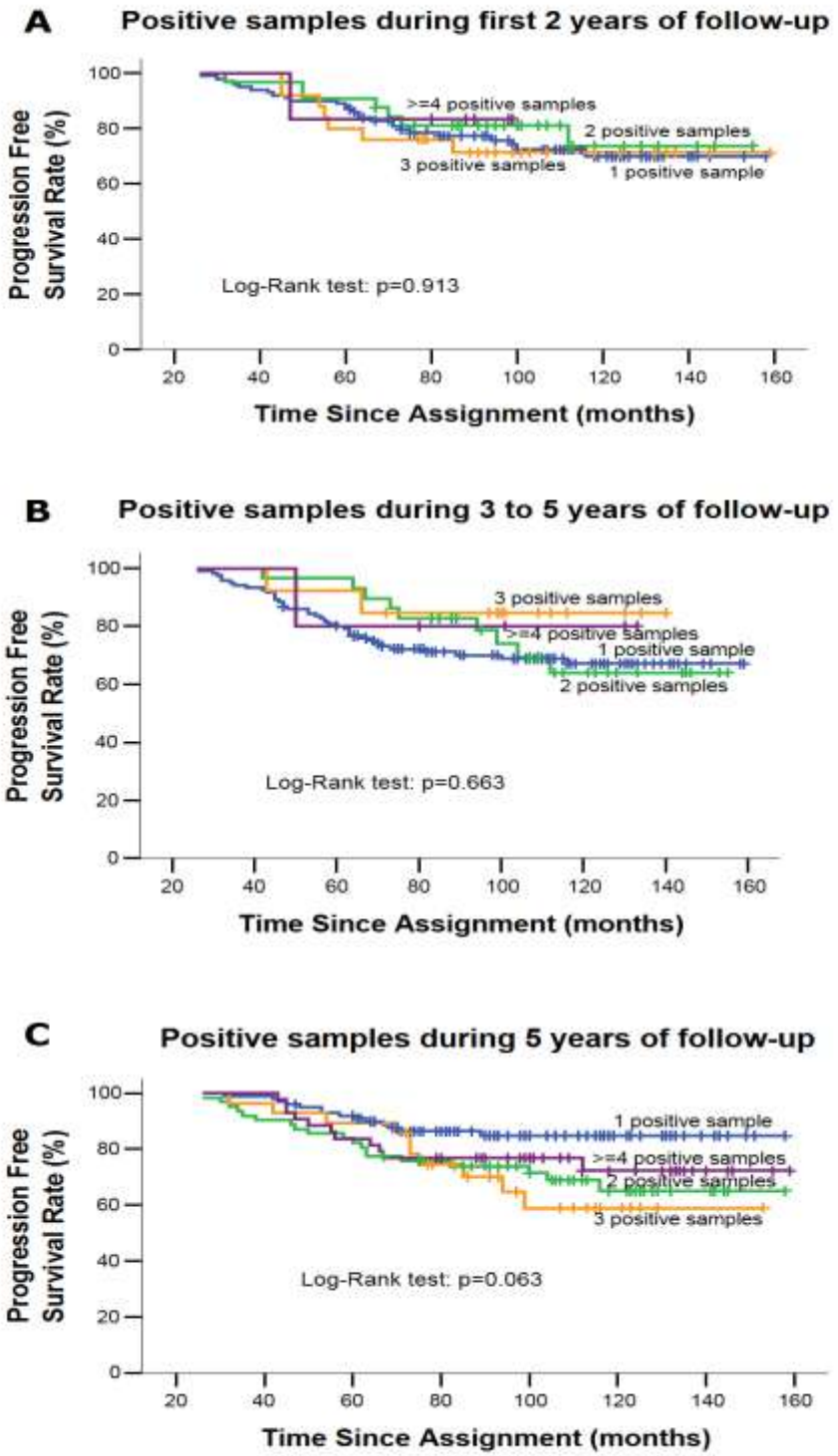

Figure 9: Disease-free survival according to the cumulative number of positive analyses during: (A) the first two years, (B) the subsequent three years and (C) the total five years of follow up.

Given that the prognostic role of the CTC detection before the administration of adjuvant chemotherapy has already been reported [151], we investigated whether it could

offer additional prognostic information to consider also the serial measurements of CTC during follow up. For this purpose, we reviewed the pre-chemotherapy CTC status of the patients included in this analysis (Table 11). No difference was found in the detection rate of the CK-19 mRNA-positive CTC between the four groups (Pearson chi-square *p*=0.320). Interestingly, the persistently positive patients with detectable CK-19 mRNA-positive CTC before the administration of adjuvant chemotherapy had shorter DFS but not OS compared to the patients of the same group who tested negative for pre-chemotherapy CK-19 mRNA-positive CTC (Figure 10).

CK-19 mRNA		Disease free survival			Overall survival	
CK-19 mRNA 5 years of follow up	**CK-19 mRNA Pre-chemotherapy**	**Patients**	**Relapses**	**Rate (%)**	**Deaths**	**Rate (%)**
Persistently Negative	Negative	47	8	17	4	8.5
	Positive	32	1	3.1	1	3.1
Turn to negative	Negative	32	1	3.1	1	3.1
	Positive	31	4	12.9	2	6.4
Turn to positive	Negative	32	5	13.5	3	7.9
	Positive	31	8	25.8	6	19.3
Persistently Positive	Negative	31	11	31.4	9	25.7
	Positive	53	24	45.3	15	28.3

Table 11: Incidence of disease recurrence and deaths according to the pre-chemotherapy detection of CK-19 mRNA-positive circulating tumor cells.

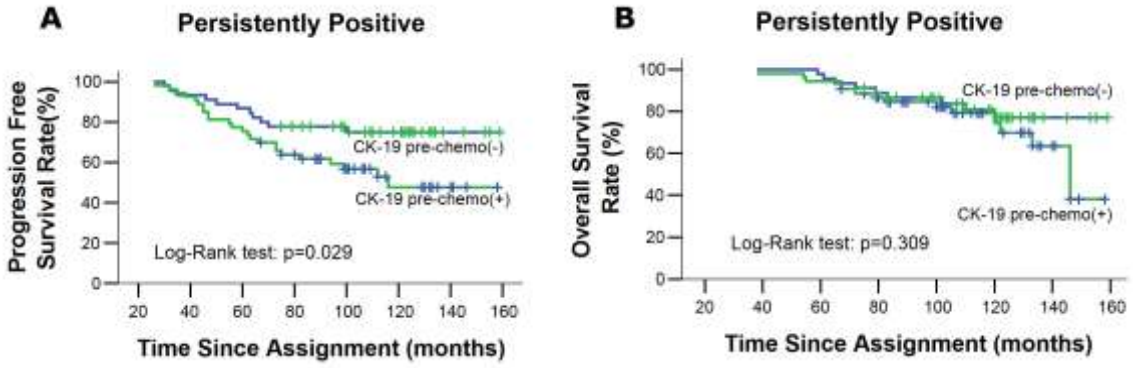

Figure 10: Disease-free (A) and overall (B) survival of the persistently positive patients according to the pre-chemotherapy CK-19 mRNA positive CTC status.

Finally, a subgroup analysis was performed according to hormone receptor status. Interestingly, the persistently positive patients with either hormone receptor positive or negative tumors had a significantly higher relapse rate (Table 12), risk of death and shorter DFS than the persistently negative patients (p=0.039 and p=0.004 for persistently positive vs persistently negative patients with ER/PR negative and ER and/or PR positive tumors respectively) (Figures 10a and 10b). However, the overall survival was shorter only for the persistently positive patients with ER/PR negative tumors (p=0.035; Figures 11c and 10d).

HR Status	CK-19 mRNA	No of patients	Relapses			Deaths		
			Yes N (%)	No N (%)	Fisher's Exact test,	Dead N (%)	Alive N (%)	Fisher's Exact test
ER(-)/PR(-)	Persistently Positive	22	9 (40.1)	13(59.9)	p=0.044	8 (34.4)	16 (63.6)	p=0.009
	Persistently Negative	24	3 (12.5)	21 (87.5)		1(4.1)	24 (95.9)	
ER(+) and/or PR(+)	Persistently Positive	60	26 (43.3)	34 (46.7)	p<0.001	15 (25)	45 (75)	p=0.007
	Persistently Negative	39	4 (10.2)	35 (89.2)		2 (5.1)	37 (94.9)	

Table 12: Incidence of disease recurrence and deaths according to the detection of CK-19 mRNA-positive circulating tumor cells and the hormonal receptor status of the primary tumor.

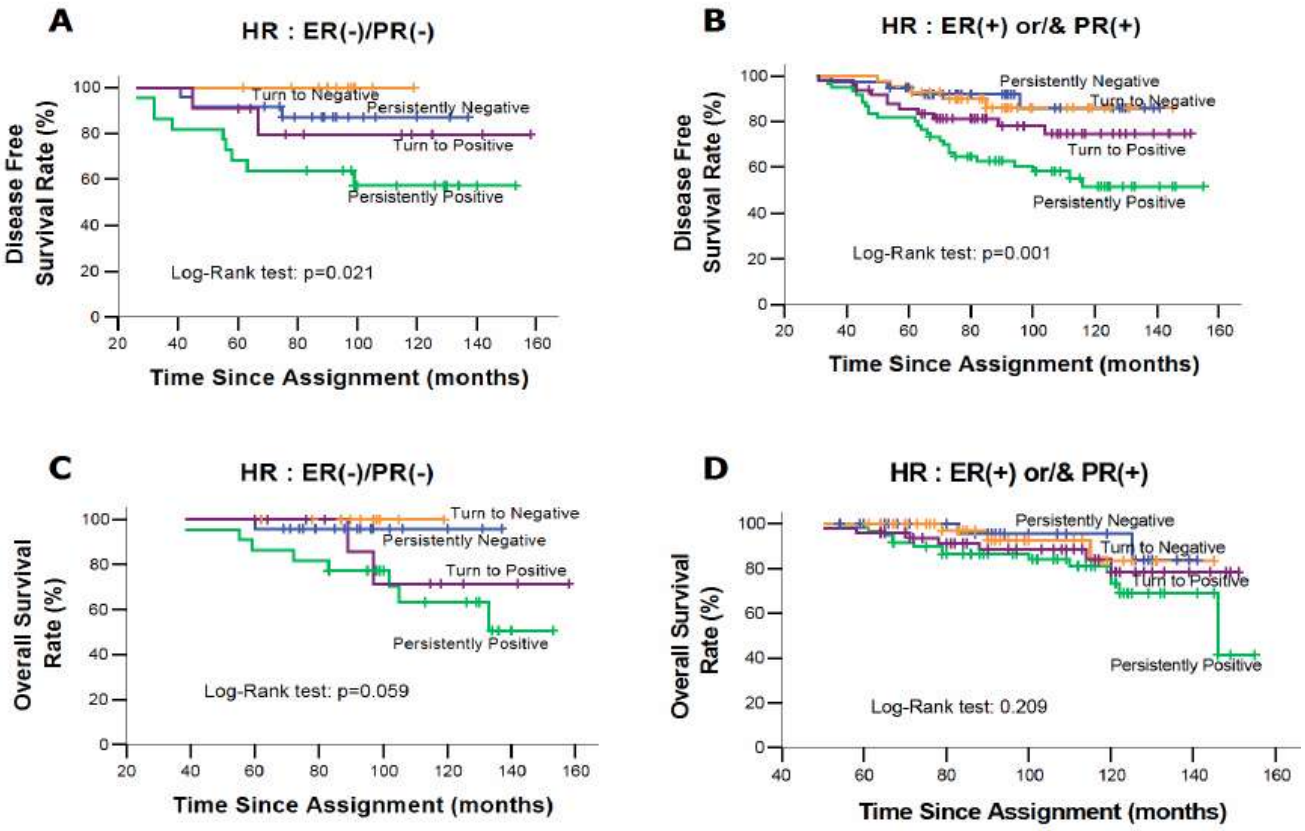

Figure 11: Disease-free survival and overall survival according to CK-19 mRNA positive circulating tumor cells' status in patients with hormone receptor negative (A,C) and hormone receptor positive tumors (B,D) during the first two years and the subsequent three years of follow up.

6.5 Univariate and Multivariate Analysis — Persistent detection of CK-19 mRNA-positive CTC during follow up after the completion of adjuvant chemotherapy, tumor size greater than 2.0 cm, more than three involved axillary lymph nodes and postmenopausal status were significantly associated with reduced DFS and OS in univariate analysis (Table 13). Multivariate analysis revealed that persistent detection of CK-19 mRNA-positive CTC, tumor size and more than three involved axillary lymph nodes were independent prognostic factors for shorter DFS and OS (Table 14).

	DFS			Overall Survival		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Menopausal Status Post vs Pre	1.815	1.075-3.064	0.026	1.985	1.027-3.836	0.041
Tumor Status T2/T3 vs T1	2.401	1.304-4.419	0.005	4.359	1.710-11.114	0.002
Nodes N>3 vs N0-3	2.331	1.418-3.832	0.001	2.819	1.527-5.207	0.001
Histology Grade 3 vs 1 / 2	1.665	0.989-2.805	0.055	1.240	0.644-2.390	0.520
HR ER(-)/PR(-) vs Other	0.826	0.454-1.504	0.533	1.182	0.585-2.387	0.641
Triple Negative ER(-)/PR(-)/HER-2 vs Other	0.874	0.454-1.681	0.686	1.075	0.492-2.348	0.856
Hormonotherapy No vs Yes	1.618	0.696-3.759	0.264	1.439	0.442-4.687	0.546
Chemotherapy CMF (ref) FEC T/EC	 1.498 1.034	 0.589-3.812 0.389-2.745	 0.339 0.396 0.947	 1.024 1.176	 0.352-2.976 0.388-3.564	 0.911 0.966 0.775
CK-19 at 5 years FU Persistently Negative Turn to Negative Turn to Positive Persistently Positive (ref)	 0.300 0.201 0.498	 0.144-0.623 0.079-0.513 0.264-0.939	 0.001 0.001 0.031 <0.001	 0.318 0.260 0.582	 0.121-0.836 0.078-0.868 0.271-1.253	 0.020 0.028 0.167 0.025

Table 13: Univariate Analysis (unadjusted relative risks) for Disease-Free and Overall survival.

	DFS			Overall Survival		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Menopausal Status **Post vs Pre**	1.462	0.858-2.492	**0.163**	1.365	0.794-2.347	**0.260**
Tumor Status **T2/T3 vs T1**	2.187	1.167-4.098	**0.015**	2.135	1.141-3.994	**0.018**
Nodes **N>3 vs N0-3**	1.801	1.080-3.003	**0.024**	2.150	1.109-4.168	**0.023**
CK-19 at 5 years FU **Persistently Negative** **Turn to Negative** **Turn to Positive** **Persistently Positive (ref)**	0.328 0.206 0.622	0.157-0.683 0.081-0.526 0.327-1.186	0.003 0.001 0.149 **0.001**	0.330 0.201 0.587	0.159-0.688 0.079-0.514 0.309-1.115	0.003 0.001 0.104 **0.001**

Table 12: Prognostic factors by multivariate analysis for Disease-free and Overall survival.

7. Discussion

— We provide, to our knowledge, the first clear evidence of a strong correlation between detection of CK-19 mRNA-positive CTC during follow up and increased risk of late disease relapse and death in patients with either hormonal receptor positive or negative operable breast cancer. These findings support the role of CTC monitoring as an adjunct to standard clinical and radiographic methods in the evaluation of disease status during follow up.

Although the prognostic role of DTC for disease relapse and death in early breast cancer is clearly documented [117], the assessment of tumor cells in peripheral blood is an easier, more broadly applicable than bone marrow aspirates and certainly more appropriate for repeated testing. Our group has previously reported that the detection of CK-19 mRNA-positive CTC in the blood of patients with node-negative operable breast cancer before the initiation of any systemic treatment was an independent prognostic factor associated with an increased risk of disease recurrence [152]. More recently, we demonstrated that patients' risk of relapse could be distinguished based on the response of their CTC to adjuvant chemotherapy [150,151] and we reviewed our experience regarding the prognostic role of

CK-19 positive CTC in operable breast cancer [166]. Other investigators have shown that longitudinal monitoring of CTC was superior to a single test analysis, and a more than 10-fold increase in the CTC numbers towards the end of therapy is highly predictive for early relapse [167].

The present study was designed to investigate the prognostic value of CTC detection during follow up in predicting the risk of late disease relapse. Accordingly, patients who experienced disease relapse during the first two years from diagnosis were excluded from this analysis and the median follow up period for the studied patients was extended to 107 months. The changes in CK-19 mRNA-positive CTC status were thus analyzed in 312 patients and four groups were distinguished. The first group included patients without detectable CTC throughout the follow up period only 11.2 percent of who experienced disease relapse. The second group included patients with CK-19 mRNA-positive CTC early during the first two years. These patients had similar relapse risk to the persistently negative patients and might indeed have derived a benefit from adjuvant therapy. In the third group patients with detectable CTC after, but not during, the first two years were included. The relapse risk for these patients was almost 50 percent higher compared to the risk of persistently negative patients, perhaps due to the growth of therapy-resistant residual disease, which could not be detected early on by our method. Finally, in the fourth group patients with CK-19 mRNA-positive CTC throughout the follow up period were included. One third of them experienced disease relapse, while one out of four patients died. This persistently positive group was by far the group with the highest relapse risk.

Almost 40 percent of the “positive at any time” patients had detectable CK-19 mRNA-positive CTC only in one single sample. This observation could theoretically be attributed at least in part to false positive results. However, given the very low false positive rate of our assay (approximately 2 percent) this occurrence should be rather limited.

Our results could be explained by the hypothesis that drug-resistant cancer cell clones generated during tumor evolution constitute the re-emerging dominant tumor cell population and may start proliferating under the selective pressure of drug exposure. The high probability of subsequent disease relapse indicates that these resistant cells have a proliferative and survival advantage. This hypothesis seems to be supported by the observation (Figure 12) that the vast majority of CTC detected during the follow up period from CK-19 mRNA

persistently positive patients who had experienced disease relapse, were not apoptotic since they did not express the M30 antigen which is a neo-epitope expressed only after caspase cleavage of cytokeratin 18 during early apoptosis [168-169].

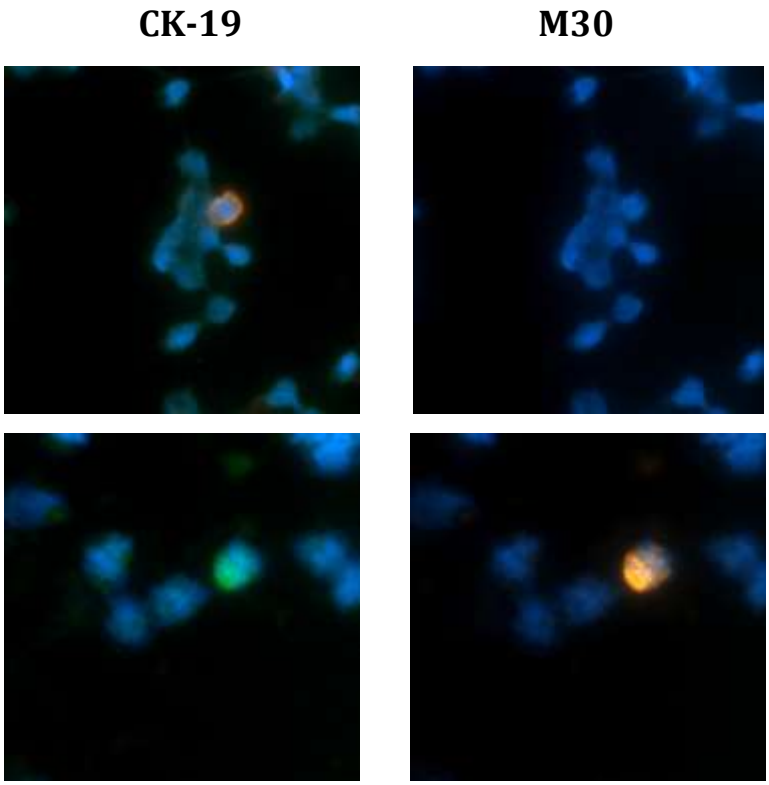

Figure 12: CK-19^{+}/M30^{-} CTC (first row) and: CK-19^{+}/M30^{+} CTC (second row).

An algorithm for the optimal timing of CTC detection during follow up is currently lacking. Our findings suggest that serial assessments every six months for up to five years represent an acceptable timetable. Furthermore, for hormone receptor positive patients, in whom half of the recurrences occur beyond five years, extension of the CTC serial assessments for even longer might be reasonable. These patients may represent the group that could derive benefit from extended adjuvant treatment approach or switch to another agent.

The RT-qPCR assay used in our study is not the only available assay for CTC detection. A semi-automated approach, the CellSearch® system, which has been approved

by the Food and Drug Administration for monitoring CTC in metastatic breast cancer setting, has gained considerable attention [89,90]. The prognostic relevance of CTC detection in peripheral blood of operable breast cancer patients, using the CellSearch® system, has been evaluated in the SUCCESS trial. According to the most updated results, detection of at least one CTC in 23 mL of peripheral blood after surgical resection of the primary tumor and before the start of adjuvant systemic treatment was an independent predictor for worse DFS and OS in multivariate analysis [139]. The prognostic significance of CTC detection before and/or after the completion of adjuvant chemotherapy [150,151], as well as the high specificity/sensitivity represent some advantages of the RT-qPCR assay compared to the CellSearch®. On the other hand, the ability for direct enumeration, morphological analysis and isolation of CTC for further analysis are important advantages of the latter [170].

Our study has some potential limitations that should be taken into account when considering the results. This is a single institution study and the analysis was performed in one laboratory. Therefore, before the establishment of our assay as a clinically relevant test, sample analysis must be performed in several laboratories and stability during shipment must be demonstrated. Also, despite the fact that our assay has been validated in multiple patients' cohorts and data analyses [145,149,150-151,153,162], neither survival advantage, nor improvement in quality of life have been demonstrated in a prospective interventional randomized trial in which the serial assessment of CTC is used to modify the treatment strategy and thus improve clinical outcome. In this regard, the SWOG and the Breast Cancer Intergroup of North America have initiated a prospective trial in metastatic setting to test whether patients with elevated CTC count (using the CellSearch® system) after one cycle of first-line chemotherapy will be benefit from switching to a different chemotherapeutic regimen (SWOG protocol S0500). However, for patients with operable breast cancer the lower CTC detection rate post chemotherapy makes this strategy far more challenging [138, 139].

Additionally, the patients of our study received various types of adjuvant therapy based on available clinical and disease data at the time of enrolment. This heterogeneity may be a confounding variable, but the similarity between our findings on relapses (20.2 percent) and those published by the EBTCG is encouraging [152]. Finally, the cellular heterogeneity of CTC was not analysed using the RT-qPCR detection method. This is very important since various studies have already confirmed that CTC present significant genetic and phenotypic

heterogeneity [171] that could explain why not all patients who have detectable CTC actually experience disease relapse while some patients relapse although they do not present detectable CTC.

In conclusion our data support a prognostic role and potential clinical utility of monitoring CTC in conjunction with standard surveillance strategies in the follow up of patients with operable breast cancer. Given their independent unfavorable prognostic value for reduced DFS and OS, the detection of CTC after therapy could be considered as indirect evidence for the presence of chemotherapy and hormonal therapy resistant disease. Analyzing a 20-mL blood sample at various time points during follow up might enable clinicians to assess the efficacy of administered adjuvant therapy, limit patient exposure to ineffective agents with unnecessary toxicity, assist in the identification of patients who are most likely to benefit from clinical trials of novel therapeutics and perhaps making eradication of cancer cells more feasible, when the tumor burden is still low and before the appearance of clinically overt metastases. Since only one third of patients with persistent CK-19 mRNA-positive CTC experience disease relapse, additional prognostic markers are needed to better define those patients who indeed might benefit from novel extended adjuvant therapies. These hypotheses can be addressed only in the context of well-designed, adequately powered, prospective, randomized clinical studies. In this way, definitive proof will be provided as to whether monitoring of CTC can be used to improve clinical outcome in patients with operable breast cancer.

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