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# Meeting Abstracts
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## KEYNOTE LECTURES

Abstracts not available at time of publication.

### L1

### Chromosomal instability in the pathogenesis and treatment of breast cancer

**A Venkitaraman**
*Breast Cancer Res* 2008, **10(Suppl 2):**L1 (doi: 10.1186/bcr 1877)

### L2

### Molecular diversity of human breast cancer: clinical and therapeutic implications

**D Slamon**
*Breast Cancer Res* 2008, **10(Suppl 2):**L2 (doi: 10.1186/bcr 1876)

### L3

### Surviving breast cancer: can women expect to 'get back to normal'?

**P Hopwood**
*Breast Cancer Res* 2008, **10(Suppl 2):**L3 (doi: 10.1186/bcr 1878)

## SPEAKER PRESENTATIONS

### O1

### Use of BRCA1 protein:protein interactions to classify cancer risk

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*Breast Cancer Res* 2008, **10(Suppl 2):**O1 (doi: 10.1186/bcr 1879)

Germline loss-of-function mutations in *BRCA1* are associated with a high lifetime risk of breast and ovarian cancer. Most mutations in the gene are 'truncating': in the main these induce premature termination codons, resulting in nonsense-mediated decay, loss of the transcript and/or the entire protein. The improved screening methods now in use across the UK will identify many carriers of unclassified *BRCA1* variants. These are chiefly missense mutations, introducing an amino acid change in the context of an expressed protein. Indeed more than one-quarter of entries recorded in the Breast Cancer Information Core dataset of BRCA1 sequence variants collected from patients worldwide are unclassified missense alterations (http://research.nhgri.nih.gov/bic/). Currently, discovery of the majority of missense variants leaves both variant carriers and their families in an ambiguous position. These variants remain unclassified because in the majority of cases it is not possible to follow variants by cosegregation analysis, and the number of appropriate controls required to be certain that a variant is absent in unaffected individuals is prohibitive. Currently, *in silico* algorithms try to distinguish between missense substitutions that are likely to be pathogenic and those that are not. These algorithms compile a multicomponent likelihood ratio that integrates assessment methods ranging from conservation analysis, co-occurrence of a deleterious allele in trans, and immunohistochemical analysis [1-3]. What is missing from these analyses is the relationship between loss of protein function and detriment to patient health.

We have focused on the N-terminal region of BRCA1. This region has a high density of missense substitutions, including those of known pathogenic status, and many currently unclassified variants. We have shown that experimental missense variants, generated randomly and selected for loss of interaction with the BRCA1 ubiquitin ligase components, BARD1 and the E2 enzyme UbcH5, identify variants reported within the Breast Cancer Information Core database of individuals with a personal or family history of breast cancer [4]. The E2 component is particularly sensitive to missense alteration in BRCA1, with the majority of currently unclassified variants in the region inhibiting interaction, whereas the BARD1 component is disrupted by a smaller, but overlapping, subset restricted to substitution of the structurally detrimental zinc-ligation residues. Variants that inhibited the E2 also prevented the enzymatic activity. These data strongly suggest that the ligase activity of BRCA1, through interaction with E2 and BARD1, is related to breast cancer predisposition.

Using yeast two-hybrid analysis for BRCA1:BARD1 and BRCA1:E2 interaction, we have tested the most chemically different substitutions achievable by single nucleotide change in all of the most highly conserved amino acids of the region (invariant from human to sea urchin), and have also tested all currently identified patient missense variants. These data have been combined with Grantham variation and Grantham deviance scores (a measure of how conserved an amino acid is, together with how different the protein change is) to assess the relationship between protein:protein interaction and measures of disease risk. Risk measures were based on the results of full sequence tests of *BRCA1* and *BRCA2* from 68,000 BRACAnalysis subjects (Myriad Genetics, Salt Lake City, UT, USA), and used estimates of the odds of developing breast cancer for a carrier of a *BRCA1* missense substitution [2], together with enrichment ratios achieved by comparing the variants observed in the dataset with the variants expected on the basis of known substitution rates.

Classification methods in the past have attempted to place variants in either the pathogenic or the little-clinical significance categories. The results of this analysis suggest that some classes of variant may confer an intermediate risk. If so, these data have considerable implications for the counselling and clinical management of women found to be positive for missense variants in future.

**Acknowledgement** JRM is funded by Breast Cancer Campaign.

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## O2

### Dietary patterns across the life course, mammographic density and implications for breast cancer: results from a British prospective cohort

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**Background** Previous epidemiological studies have investigated the relationship between individual nutrients such as vitamin D and vitamin $B_{12}$ and mammographic density, a strong marker of breast cancer risk [1], with varied results. There has been limited research on overall dietary patterns and most studies have focused on adult dietary patterns [2]. We examine prospective data to determine whether dietary patterns from childhood to adult life affect mammographic density.

**Methods** The Medical Research Council National Survey of Health and Development is a national representative sample of 2,815 men and 2,547 women followed since their birth in March 1946 [3]. A wealth of medical and social data has been collected in over 25 follow-ups by home visits, medical examinations and postal questionnaires. Dietary intakes at age 4 years were determined by 24-hour recalls and in adulthood (ages 36, 43 years) by 5-day food records. Copies of the mammograms (two views for each breast) taken when the women were closest to age 50 years were obtained from the relevant NHS centres. A total of 1,319 women were followed up since birth in 1946 for whom a mammogram at age 50 years was retrieved, and the percentage mammographic density was measured using the computer-assisted threshold method for all 1,161 women. Breast cancer incidence for the whole cohort is being ascertained through the National Health Service Central Register.

*Statistical analysis* Reduced rank regression analysis, a relatively new approach to dietary pattern analysis, is being used to identify dietary patterns associated with mammographic density [4]. This approach identifies patterns in food intake that are predictive of an intermediate outcome of the disease process, such as mammographic density, and subsequently examines the relationship between the identified dietary patterns and breast cancer risk.

**Results** Preliminary analyses so far suggest that variations in dietary patterns in adulthood might explain more than 10% of the variation in percentage mammographic density at age 50 years (age 36 years: 13%; age 43 years: 14%), with variations in patterns in childhood explaining slightly less. Further work is being carried out on the characteristics of these dietary patterns and their effects on percentage mammographic density and its two components (that is, absolute areas of dense and nondense tissues) and on breast cancer risk, after adjusting for socioeconomic status, anthropometric variables and reproductive factors.

**Conclusion** The present study will provide for the first time information on the relationship between dietary patterns across the life course and mammographic density, and will help to clarify the pathways through which diet may affect breast cancer risk.

**Acknowledgements** Breast Cancer Campaign UK–World Cancer Research Fund International and the Medical Research Council fund this project. IdSS and VM are members of the Cancer Research UK Epidemiology and Genetics Group; VM is funded by a Cancer Research UK training fellowship.

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## O3

### Loss of oestrogen receptor alpha in long-term antioestrogen-resistant cells: reversal by a c-src inhibitor

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*Breast Cancer Res* 2008, **10(Suppl 2):**O3 (doi: 10.1186/bcr 1883)

**Background** Tamoxifen still remains the most frequently used antioestrogen for the treatment of breast cancer. However, its efficacy is often limited by the emergence of acquired resistance and it has been suggested that, in some instances, this may involve oestrogen receptor (ER) loss. This study addresses this issue by examining long-term tamoxifen treatment of breast cancer cells and identifies that progressive ER loss does occur, leading to greatly increased aggressive tumour cell behaviour. Encouragingly, even after 30 months treatment, ER loss is reversible by a c-src inhibitor. Our data therefore provide a new model to study the cellular mechanisms associated with antihormone promoted ER loss and its possible prevention/reversal by signal transduction inhibitors.

**Methods** Using quantitative PCR based on SYBR Green fluorescence, the expression of total ERα mRNA and its constituent mRNA variants were quantified in MCF7 cells and in our *in vitro* developed tamoxifen-resistant breast cancer cells (TamR), which have been cultured in the presence of tamoxifen for 30 months. Specific PCR amplification of all ERα mRNA variants was possible using forward primers designed to bind specifically to the 5′ untranslated regions of ERα mRNA and used separately with a common reverse primer that anneals to the 5′ end of the protein

encoding region of exon 1 of ERα cDNA. Expression of ERα protein was assessed by western blot and immunohistochemistry.
**Results** In MCF7 cells, the ERα mRNA isoforms A, B and C were detected as the most predominant variants, with C ERα mRNA showing the highest expression level. In TamR cells, about a 40% fall in total ERα mRNA was observed in comparison with MCF7 cells and was most apparent for the C variant. Extension of the tamoxifen treatment period to 30 months produced a further dramatic decrease in ERα mRNA (all variants) and protein levels, resulting in ER negativity being recorded in >90% of the cells by immunohistochemistry. These cells show increased levels of phosphorylated Erk 1&2, AKT, PKCα and src, and are highly aggressive in their growth behaviour, with increased cell motility and invasiveness. Treatment of the cells with the demethylating agent 5-azacytidine did not restore ERα expression, suggesting that epigenetic alterations are unlikely to be responsible for the reduced ER levels. However, Affymetrix data in the TamR cells showed that some positive regulators of ER expression, such as p53 and Foxo3A, are downregulated during the development of the resistant phenotype and their continued absence may contribute to the progressive ER loss. Significantly, pathway inhibitor studies revealed c-src to be an important regulator of ER loss, since its inhibition rapidly restored ER levels.
**Conclusion** Our data indicate that considerable ER loss can occur during antihormonal treatment of breast cancer cells and that this can lead to a more aggressive phenotype. Encouragingly, however, even after 30 months exposure to tamoxifen, the process is reversible by inhibition of c-src. These data suggest that combinations of antihormones with signal transduction inhibitors could retain ER functions in treated cells and prevent a drift towards more aggressive cancer cell behaviour.

## O4

## Suppression of the NF-κB cofactor Bcl3 inhibits mammary epithelial cell apoptosis and, in breast tumours, correlates with poor prognosis

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*Breast Cancer Res* 2008, **10(Suppl 2):**O4 (doi: 10.1186/bcr 1884)

**Background** Several transcription factors have been shown to play important roles in the regulation of apoptosis at the onset of murine mammary involution. These include LIF-activated STAT3, c/ebpdelta, Ap-1 and IKK/NF-κB-mediated regulation of death receptor ligands. A study of STAT3 and STAT5 transcriptional targets in mammary epithelial cells *in vitro* showed that both c/ebpdelta and c-fos (a component of Ap-1) were upregulated by STAT3, suggesting a degree of interdependence between these transcription factor pathways in mediating their apoptotic effects. Interestingly, while no NF-κB or IKK genes were significantly regulated by STATs, the NF-κB cofactor gene, Bcl3, was found to be a principal transcriptional target of STAT3. This factor plays a role in altering the transcriptional capacity of specific NF-κB subunits and has previously been described as an oncogene in B-cell lymphomas. In this study we set out to establish whether Bcl3 had a role in regulating the cell fate of mammary epithelial cells either in the normal mammary gland or in mammary/breast cancer.
**Methods** Archived material representing a range of tumour grades and types was collected from breast cancer patients immediately after surgery (tumour tissues = 122, normal tissues = 32). The median follow-up of the patients was 120 months (range 12 to 156 months). QRT-PCR for Bcl3 was performed and this information was used to determine statistically significant correlations with the clinical data on breast pathology. MCF7, T47D and MDA-MB231 human breast cancer cell lines were subjected to Bcl3-specific siRNA knockdown and subsequently assessed for cell motility characteristics using ECIS technology. Bcl3-knockout mice were assessed histologically for alterations in apoptosis rate during the adult pregnancy cycle. Western blots, quantitative PCR and DNA binding assays were used to determine the activity of molecular markers of apoptosis in these animals. Bcl3-deficient animals were crossed with mmtv-*neu* (*c-erbB2*) mice to establish the role of Bcl3 in primary (*neu*-dependent) mammary tumour growth, and magnetic resonance imaging was performed on tumour-bearing animals, to establish metastasis rates in the presence/absence of Bcl3.
**Results** An analysis of 122 human breast cancer tissues showed that Bcl3 gene expression was suppressed in a significant proportion of invasive tumours, which correlated with poor prognosis. This also correlated with a significant decrease in Bcl3 gene expression in human breast cancer cell lines exhibiting increased motility characteristics. The effects of siRNA-mediated knockdown of Bcl3 are ongoing. In the mouse mammary gland, Bcl3 expression was restricted to epithelial cells during the first 24 hours of involution. Bcl3 deficiency resulted in a transient delay in the appearance of apoptotic bodies in the early involuting mammary gland in $Bcl3^{-/-}$ mice, while pSTAT3 levels were unchanged compared with equivalent timepoints in control animals. The activities of initiator/executor caspases of both intrinsic and extrinsic pathways were significantly decreased in $Bcl3^{-/-}$ tissues at this time, which correlated with decreases in the expression of key regulators of intrinsic/extrinsic apoptosis. Results from the ongoing magnetic resonance imaging study of tumour incidence/progression in mmtv-*neu*/$Bcl3^{-/-}$ mice will be presented.
**Conclusion** These observations suggest that Bcl3 promotes apoptosis in the mammary gland and provides preliminary evidence of cross-talk between STAT3 and NF-κB pathways, both of which have been implicated in breast cancer. Our current data on Bcl3 in primary breast tumours and breast cancer cell lines contrasts with other studies, to suggest that Bcl3 suppresses the metastatic progression of primary breast cancer and has a neutral role in breast cancer incidence or primary tumour growth.
**Acknowledgement** Funded by the Breast Cancer Research Trust.

## O5

## Activation of TGF-beta signalling in breast cancer metastatic cells

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*Breast Cancer Res* 2008, **10(Suppl 2):**O5 (doi: 10.1186/bcr 1880)

**Background** The onset of metastasis in organs such as the lung, bone and brain is a major cause of mortality in breast cancer patients. Many signalling pathways have been implicated in mediating progression to metastatic disease, including the transforming growth factor beta (TGFβ) signalling pathway. In many tissues TGFβ results in a growth inhibitory signal. This is mediated by transducers of the Smad family, which translocate to the nucleus and activate transcription. In tumour cells, however, TGFβ-dependent antiproliferative control is lost and cells acquire the ability to replicate in TGFβ-rich environments. Despite molecular and clinical evidence pointing to a role for TGFβ signalling in cancer progression and metastasis, it is unclear at which points of the metastatic process TGFβ signalling occurs and whether it is necessary and/or sufficient to elicit cancer cell motility.

**Methods** To address these questions, MTln3E rat breast cancer cells were used as a relevant model system. When injected into the mammary fat pad of nude mice these cells form a primary tumour from which motile cells will depart to form metastasis in the lymph nodes and the lungs. To gain insight into TGFβ signalling *in vivo*, MTln3E cells were engineered to express either GFPSmad2 or a Smad3 responsive promoter driving the expression of ECFP (CAGA::ECFP). This allowed the monitoring of Smad-dependent TGFβ signalling *in vivo* using multiphoton confocal microscopy.
**Results** Our results indicate that TGFβ signalling, measured by cytoplasmic to nuclear translocation of GFPSmad2 and by activation of CAGA ECFP, does not occur ubiquitously within the primary tumour. In particular, cells that have acquired a motile phenotype display active TGFβ signalling. As nuclear localisation of GFPSmad2 and activation of CAGA ECFP are also observed in nonmotile cells, however, TGFβ signalling may be necessary but not sufficient to elicit cell motility in primary tumour cells. Furthermore, activation of TGFβ signalling in motile cells is transient, as lymph node metastasis display little activation of the pathway. In addition, we have uncovered a second role for TGFβ signalling in the metastatic process. After intravenous injection in mouse tail vein, TGFβ pretreated cells colonise the lungs more efficiently than untreated controls and this results from the ability of these cells to survive clearance from the lungs.
**Conclusion** Together these data suggest that TGFβ signalling may positively influence two distinct steps of the metastatic cascade, first by enabling cells to become motile and second to enhance their survival during the lung colonisation.
**Acknowledgement** Supported by Breast Cancer Campaign.

## O6

## Development of breast cancer immunotherapy using MUC1-retargeted T lymphocytes

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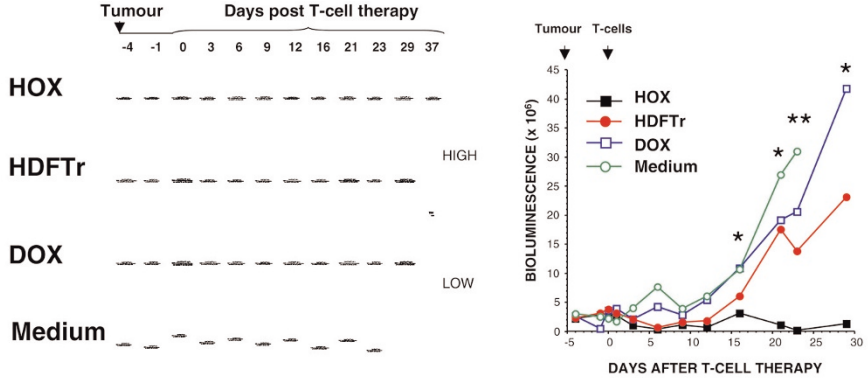
*Breast Cancer Res* 2008, **10(Suppl 2):**O6 (doi: 10.1186/bcr 1881)

**Background** MUC1 is a highly attractive target for immunotherapy of breast cancer owing to its overexpression, altered glycosylation and loss of polarity in over 90% of tumours. To exploit this, we are developing genetic approaches to retarget T-cell specificity to MUC1 using chimeric antigen receptor (CAR) technology.
**Methods** A panel of CARs have been generated using scFv derived from the SM3 and HMFG2 hybridomas. Using the SFG oncoretroviral expression vector, gene transfer was achieved in up to 75% of human T cells.
**Results** Two parameters proved crucial in engineering an optimized CAR ectodomain. First, we found that MUC1-mediated activation of engineered human T cells is subject to steric hindrance. This was observed using anchored but not soluble MUC1 and was independent of MUC1 glycosylation status. To circumvent this, we increased the flexibility and reach of CAR binding arms using the elongated hinge found in IgD. Second, CAR function was highly dependent upon strong binding capacity across a broad range of tumour-associated MUC1 glycoforms, including MUC1 Tn, T and sialylated derivatives. This was realized using an scFv cloned from the HMFG2 hybridoma. To optimize CAR signalling, tripartite endodomains were constructed that contain modules derived from TNF receptor family members in addition to CD28 and CD3ζ. Ultimately, this iterative design process yielded a potent MUC1-specific CAR termed HOX that contains a fused CD28/OX40/CD3ζ endodomain. HOX-expressing T cells proliferate vigorously *in vitro* upon repeated encounter with soluble or membrane-associated MUC1, mediate production of proinflammatory cytokines (IFNγ and IL-17) and elicit brisk antigen-dependent killing of MUC1$^+$ tumour cells. To test function *in vivo*, a human breast cancer xenograft model has been established using MDA MB 435 tumour cells engineered to coexpress MUC1 and firefly luciferase. When introduced into SCID/Beige mice by intraperitoneal injection, rapid tumour growth occurs that can be monitored longitudinally and noninvasively by bioluminescent imaging. Mice bearing established tumour have been treated intraperitoneally with a single dose of human T cells grafted with HOX, two control CARs (DOX: lacking the HMFG2 scFv; HDFTr: lacking a functional endodomain) or medium alone. We observed that treatment with HOX-expressing T cells resulted in a significant delay in tumour growth, as measured by bioluminescent imaging, compared with control mice (Figure 1). In addition, HOX-grafted T cells confer a significant survival advantage upon mice bearing MCF7 breast cancer xenografts.
**Conclusion** Despite its role in tumorigenesis and immune evasion, we show that the near-ubiquitous breast cancer antigen MUC1 can be targeted using CAR grafted T cells.

**Figure 1 (abstract O6)**




*$P < 0.05$, HOX versus DOX, HOX versus medium if present; **$P < 0.05$, HOX versus DOX, HOX versus medium, HOX versus HDFTr.

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## POSTER PRESENTATIONS

### P1

### Role of the Hsp90 cochaperone, FKBPL, in oestrogen receptor signalling and breast cancer growth and survival

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*Breast Cancer Res* 2008, **10(Suppl 2):**P1 (doi: 10.1186/bcr 1885)

Hsp90 chaperone complexes are involved in maintaining the stability and signalling of Hsp90 client proteins such as the oestrogen receptor (ER). The ER is the primary mediator of breast cancer proliferation in response to oestrogen. Since increased ER levels and transcriptional activation are associated with over 50% of breast cancers, the ER is an attractive target for cancer treatment strategies. Hsp90 inhibitors such as 17AAG are known to destabilize these complexes by promoting proteasome-mediated degradation of the steroid hormone receptor leading to tumour growth inhibition [1] and sensitization to chemotherapy [2] and radiotherapy [3]. Using protein interaction assays, we have identified FKBPL, a novel gene that codes for an immunophilin-like protein, as an Hsp90 cochaperone associated with the ER and dynein motor protein complex. Overexpression studies have demonstrated that FKBPL modulates ER signalling and affects breast cancer growth and survival. Since most tumours become refractory to current hormonal therapies within a year of starting treatment, FKBPL represents a novel drug target that would enable the disruption of signalling pathways integral in maintaining ER-mediated tumour growth and survival.

**Acknowledgements** Funded by Breast Cancer Campaign, Action Cancer and DEL.

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### P2

### Two functionally distinct epithelial progenitors exist within the luminal cell compartment of the mouse mammary gland

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*Breast Cancer Res* 2008, **10(Suppl 2):**P2 (doi: 10.1186/bcr 1886)

**Background** The organization of the mammary epithelial cell hierarchy is poorly understood.

**Methods** To determine the cells that make up this hierarchy and the relationship between them, we used fluorescence-activated cell sorting in combination with *in vitro* colony-forming cell assays to examine the growth and differentiative properties of phenotypically distinct subsets of mouse mammary epithelial cells.

**Results** Our results indicate that >95% of all colony-forming cells present within the mammary epithelium are localized within the luminal cell compartment and that >90% of these have a $CD45^-Ter119^-CD31^-(Lin^-)CD24^{high}CD14^+$ phenotype. This progenitor cell population can be further resolved into two functionally distinct subpopulations based on the expression of Sca1. The $Lin^-CD24^{high}CD14^+Sca1^-$ progenitors, which express low levels of estrogen receptor alpha and intermediate levels of keratin 14 (K14), are perceived to be progenitors that produce $Lin^-CD24^{high}CD14^-Sca1^-$ alveolar cells during pregnancy. The $Lin^-CD24^{high}CD14^+Sca1^+$ progenitors, which express intermediate levels of estrogen receptor alpha and are $K14^-$, are perceived to be precursors of the steroid receptor expressing cells, of which the vast majority are terminally differentiated and have a $Lin^-CD24^{high}CD14^-Sca1^+$ phenotype.

**Conclusion** These results demonstrate the existence of two functionally distinct progenitor cells within the luminal compartment of the mammary gland and provide a framework for interpreting breast tumour gene expression profiles and the possible origins of breast tumours.

### P3

### Interactions between BRCA2 protein and the meiosis-specific recombinase DMC1

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*Breast Cancer Res* 2008, **10(Suppl 2):**P3 (doi: 10.1186/bcr 1887)

Homologous recombination has a dual role in eukaryotic organisms. Firstly, it is responsible for the creation of genetic variability during meiosis by directing the formation of reciprocal crossovers that result in random combinations of alleles and traits. Secondly, in mitotic cells, it maintains the integrity of the genome by promoting the faithful repair of DNA double-strand breaks. In vertebrates it therefore plays a key role in tumour avoidance. Mutations in the tumour suppressor protein BRCA2 are associated with predisposition to breast and ovarian cancers, and loss of BRCA2 function leads to genetic instability, as BRCA2 is required for regulation of double-strand break repair by homologous recombination. BRCA2 protein regulates recombinational repair by interacting directly with RAD51 recombinase via a series of degenerate BRC repeat motifs encoded by exon 11 (BRCA2996-2113), and an unrelated C-terminal domain (BRCA23265-3330). Recent observations show that BRCA2 is also required for homologous recombination at meiosis. We show that human BRCA2 binds directly to the meiosis-specific recombinase DMC1 and define the primary DMC1 interaction domain to a 26 amino acid region located at BRCA22386-2411. This region is highly conserved in BRCA2 proteins from a variety of mammalian species, but is absent in BRCA2 from *Arabidopsis thaliana*, *Caenorhabditis elegans*, and other lower eukaryotes. Within this region, we demonstrate the critical importance of Phe2406, Pro2408, and Pro2409 at the conserved motif 2404KVFVPPFK2411, and define this novel DMC1 interaction domain the PhePP motif. The PhePP motif promotes specific interactions between BRCA2 and DMC1, and no interactions take place between this region of BRCA2 and RAD51. Thus, the RAD51 and DMC1 interaction domains on BRCA2 are distinct from each other, allowing coordinated interactions of the two recombinases with BRCA2 at

meiosis. These results lead us to suggest that BRCA2 is a universal regulator of RAD51/DMC1 recombinase actions.

## P4

### Lineage commitment in mammary epithelium is regulated by type 2 cytokines and Stat6

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*Breast Cancer Res* 2008, **10(Suppl 2):**P4 (doi: 10.1186/bcr 1888)

Naïve T-helper cells differentiate into Th1 and Th2 subsets that have unique cytokine signatures, activators, and transcriptional targets. The Th1/Th2 cytokine milieu is a key paradigm in T-cell lineage commitment and IL-4/IL-13 and Stat6 are known to be important mediators of Th2 development. We have now demonstrated that this paradigm applies also to mammary epithelial cells, which undergo a switch from Th1 to Th2 cytokine production upon the induction of differentiation. Thus, the Th1 cytokines IL-12, IFNγ, and TNFα are downregulated concomitantly with the upregulation of the Th2 cytokines IL-4, IL-13 and IL-5 as epithelial cells commit to the luminal alveolar lineage. Moreover, we show that Th2 cytokines play a crucial role in mammary gland development *in vivo*, because differentiation and alveolar morphogenesis are reduced in both Stat6 and IL-4/IL-13 doubly deficient mice during pregnancy. This unexpected discovery demonstrates a role for immune cell cytokines in epithelial cell fate and function, and adds an unexpected tier of complexity to the previously held paradigm that steroid and peptide hormones are the primary regulators of mammary gland development.

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## P5

### Topoisomerase II expression as a determinant of chromosomal radiosensitivity and possible susceptibility in breast cancer

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*Breast Cancer Res* 2008, **10(Suppl 2):**P5 (doi: 10.1186/bcr 1889)

**Background** Elevated chromosomal radiosensitivity in lymphocytes of breast cancer patients is thought to be an indicator for the presence of one or more as yet unidentified genes of low penetrance that promote susceptibility to the disease in up to 60% of cases [1,2]. One such gene may be *TOPO2A*, encoding for the DNA processing enzyme topoisomerase IIa. The involvement of topoisomerase IIa is predicted from the author's model for formation of chromatid breaks [3]. In the model the DNA double-strand break is not directly involved in the chromatid break, but acts as an initiator in a sequence of events leading to a chromatid break. It is thought that a chromatid break may be formed by a misjoining of chromatin ends during topoisomerase IIa decatenation of chromatids as the cell progresses through $G_2$ towards mitosis. Topoisomerase IIa is known to be vulnerable to perturbation by oxidative stress during the precise process of cutting and joining DNA strands [4].

**Methods** Gamma-radiation-induced chromatid breaks are scored for chromatid breaks in colcemid-blocked chromosome spreads of metaphase HL60 and mitoxantrone-resistant variants: MX1 and MX2 cells with reduced topoisomerase II expression. Topoisomerase IIa expression levels were measured using western blotting. SiRNA was used to knock down expression in normal exponentially growing human cells that are irradiated with a low dose of γ-rays and scored for the presence of chromatid breaks. The chromatid break frequency and topoisomerase IIa expression (ELISA assay) are being compared in 3-day-stimulated peripheral blood T lymphocytes from a group of breast cancer patients and control individuals exposed to a low dose of γ-radiation.

**Results** We show that chromatid radiosensitivity (based on the frequency of metaphase chromatid breaks in irradiated $G_2$ cells) is significantly lower in a topoisomerase IIa underexpressing variant cell lines [5], and preliminary results show that reducing expression with SiRNA also reduces chromatid radiosensitivity. In a pilot study we are currently comparing the chromatid radiosensitivity and topoisomerase IIa expression in stimulated peripheral blood lymphocytes of a group of Tayside breast cancer patients and a similar number of normal noncancer control individuals.

**Conclusion** Our data support the hypothesis that topoisomerase IIa expression is a determinant of chromatid break frequency in irradiated $G_2$ cells, and thus could be an underlying cause of the observed variability of chromatid radiosensitivity among both sporadic breast cancer cases and normal control individuals.

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## P6

### Chromosome translocations in breast cancer

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*Breast Cancer Res* 2008, **10(Suppl 2):**P6 (doi: 10.1186/bcr 1890)

**Background** Genome rearrangement is a major mechanism of gene alteration in cancer. Chromosome translocations and inversions can result in gene fusion, promoter insertion or gene inactivation. In the past it has been assumed that such rearrangements are not significant players in the common epithelial cancers,

as they are in leukaemias and sarcomas. However, this view is now being challenged. In particular, Tomlins and colleagues found that around 70% of prostate cancers have translocations or inversions of the *ETS* family of transcription factors [1]. In breast cancer, we have shown that the *NRG1/heregulin* gene is translocated in 6% of primary cases [2] and Soda and colleagues described fusions of *ALK* in 7% of lung cancers [3].

**Methods and results** We present a comprehensive analysis by array painting of the chromosome translocations of breast cancer cell lines HCC1806, HCC1187 and ZR-75-30. In array painting, chromosomes are isolated by flow cytometry, amplified and hybridized to DNA microarrays [4]. A total of 200 breakpoints were identified and all were mapped to 1 Mb resolution on BAC arrays, then 40 selected breakpoints, including all balanced breakpoints, were further mapped on tiling-path BAC arrays or to around 2 kb resolution using oligonucleotide arrays. Many more of the translocations were balanced than expected, either reciprocal (eight in total) or balanced for at least one participating chromosome (19 paired breakpoints). Many breakpoints were at genes that are plausible targets of oncogenic translocation, including *CTCF* and *P300*. Two gene fusions were also demonstrated, *TAX1BP1-AHCY* and *RIF1-PKD1L1*.

**Conclusion** Our data establish that array painting is a very effective way to map substantial numbers of translocation breakpoints and support the emerging view that chromosome rearrangements that fuse, activate or otherwise alter genes at their breakpoints may play an important role in common epithelial cancers.

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## P7

### ZNF366 is a novel corepressor for estrogen receptor alpha that mediates its effects through interaction with CtBP

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Regulation of gene expression by the estrogen receptor (ER) requires the coordinated recruitment and dissociation of transcriptional coactivator complexes and concomitant chromatin remodelling and histone modification. In addition to the well-characterised recruitment of coactivator proteins, a number of corepressor proteins can also be recruited to the liganded ER, including RIP140 and L-CoR.

We have recently identified a new ER interacting protein, ZNF366, which is recruited to the liganded ER, through interactions involving the zinc finger domains of both proteins. We show that repression of ER-regulated genes by ZNF366 involves recruitment of the well-described corepressor CtBP. This interaction is mediated by two sequence motifs in ZNF366, conforming to the consensus CtBP-binding motif (PXDLS). Mutation of these motifs in ZNF366 reduces, but does not abolish, the corepressor activity of ZNF366. Additionally, ZNF366 interacts with RIP140, raising the possibility that RIP140 and ZNF366 may act synergistically in regulating ER activity [1].

Finally, we show that although ZNF366 is expressed in normal breast epithelial cells, its expression is not detected in breast cancer cells. This raises the possibility that regulation of ER activity by ZNF366 may be important in breast cancer development.

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## P8

### A novel role for C-terminal binding proteins in the regulation of mitotic fidelity in breast cancer cells

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C-terminal binding proteins (CtBPs) (CtBP1 and CtBP2) are dual-function proteins that act in the nucleus as transcriptional co-repressors and in the cytoplasm as regulators of mitotic Golgi fissioning. They have been implicated in the process of cellular transformation through their physical and functional interactions with the viral oncoproteins adenovirus E1A, and EBNA3C. Studies in which the expression or function of CtBPs has been suppressed in mammalian cells have independently identified both a role in suppressing apoptosis, through their regulation of transcription of proapoptotic genes, and a requirement for cell-cycle progression, dependent on their role in the Golgi. Here we have undertaken a holistic analysis of the phenotypic consequences of ablating CtBP expression in breast cancer-derived cell lines. We find that loss of CtBPs suppresses the proliferation of these lines through a combination of induction of apoptosis, reduction in cell-cycle progression into mitosis, and aberrations in transit through mitosis itself. This third phenotype includes errors in mitotic chromosome segregation, activation of, but failure to sustain, the spindle assembly checkpoint, diminished localisation of spindle checkpoint proteins at kinetochores, and a high rate of failure to complete cytokinesis. These represent novel roles for CtBPs in the regulation of critical stages of the cell division cycle.

## P9

### RASSF2 can suppress the growth of breast cancer cell lines and is epigenetically inactivated in breast tumours

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**Background** RASSF2 is located at 20p13, a region frequently lost in human cancers. RASSF2 is a recently identified member of the ras association domain of family tumour suppressor genes, and many other members of this family are inactivated in human tumours by promoter methylation.
**Methods** Methylation-specific PCR and combined bisulphite and restriction analysis were used to analyse the methylation status of the RASSF2 promoter CpG island in a series of breast tumours and cell lines. Bioinformatic approaches were used to study RASSF2 and a highly conserved putative bipartite nuclear localisation signal (NLS) was identified. Colony formation, growth in soft agar and growth in immunocompromised mouse assays were used to assess the tumour suppressive activities of RASSF2.
**Results** RASSF2 was frequently methylated in breast tumour cell lines, 65% (13/20), and in primary breast tumours, 38% (15/40). In the 10 samples for which corresponding normal DNA was available this methylation was tumour specific. RASSF2 expression could be switched back on in methylated breast tumour cell lines after treatment with 5-aza-2dC. Endogenous RASSF2 localised to the nucleus and mutation of the putative nuclear localisation signal abolished the nuclear localisation. RASSF2 suppressed breast tumour cell growth *in vitro* and *in vivo*, while the ability of NLS-mutant RASSF2 to suppress growth was much diminished.
**Conclusion** These data indicate that RASSF2 is frequently methylated in breast tumours, and thus RASSF2 is a novel methylation marker that has the potential to be developed into a valuable epigenetic marker for screening. We also demonstrate that RASSF2 acts as a tumour suppressor gene and that it contains a functional NLS that is important for its tumour suppressor gene function.
**Acknowledgement** Supported by Breast Cancer Campaign.

## P10

### Role of poly(ADPribosyl)ation of CTCF in cancer and normal breast cells

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*Breast Cancer Res* 2008, **10(Suppl 2):**P10 (doi: 10.1186/bcr 1894)

**Background** CTCF is a conserved, ubiquitous and multifunctional transcription factor with features of a tumour suppressor. We have previously reported that CTCF function is modulated by post-translational poly(ADPribosyl)ation [1,2]. Poly(ADPribosyl)ation of CTCF protein results in two isoforms: a highly poly(ADPribosyl)ated form (called CTCF180) and a hypopoly(ADPribosyl)ated form (called CTCF130). In this study we assessed the presence of both CTCF isoforms in normal and cancer breast tissues and investigated their function using immortalised cell lines.
**Methods** CTCF expression was analysed in breast tissues and breast cancer cell lines by western blotting, immunohistochemistry and immunofluorescence, using antibodies that specifically recognise different CTCF isoforms. Functional investigations of CTCF isoforms in cell culture included induction of apoptosis, senescence and cell-cycle arrest using various chemical treatments and analysis of cells by flow cytometry.
**Results** We discovered, using a large panel of breast tumours and paired peripheral tissues, that only the CTCF180 isoform was present in normal breast tissues, whereas CTCF130 was exclusively detected in breast tumour tissues and immortalised cell lines. Immunohistochemical staining revealed that 91% of the breast tumours contained CTCF130. In addition, correlations were found between the levels of CTCF130 and tumour grade, lymph node metastases and neoadjuvant chemotherapy treatment. In breast cancer cell lines, induction of cell death by apoptosis and senescence resulted in a transition from the CTCF130 to the CTCF180 isoform. This shift was not observed following cell-cycle arrest.
**Conclusion** The present study demonstrates that CTCF180 is characteristic for normal breast tissues, whereas CTCF130 is specific for breast tumours and breast cancer cell lines. The CTCF130 isoform may therefore be used as a specific biological marker for breast tumourigenesis. Our data indicate that loss of CTCF poly(ADPribosyl)ation may be involved in breast tumour development. Poly(ADPribosyl)ation of CTCF, on the other hand, correlates with induction of cell death in breast cancer cell lines.
**Acknowledgements** Supported by Breast Cancer Campaign, the Medical Research Council, and the University of Essex.
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## P11

### *NRG1* is frequently silenced by methylation in breast cancers and is a strong candidate for the 8p tumour suppressor gene

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**Background** It has long been suspected that there is an important breast cancer tumour suppressor gene on the short arm of chromosome 8, 8p, and our array CGH data suggest that it may be close to *NRG1* [1]. *NRG1* encodes growth factors that bind to tyrosine kinases ErbB3 and ErbB4, and can both stimulate cell proliferation and apoptosis. *NRG1* is also quite frequently broken by chromosome translocations [2].
**Methods and results** By quantitiative PCR, *NRG1* expression was repressed or abolished in many breast cancer cell lines and tumours as compared with normal breast. Methylation analysis by sequencing or pyrosequencing bisulphite-treated DNA showed striking DNA methylation at a CpG island in *NRG1*, which is correlated with an absence of *NRG1* transcripts. Treatment of

cancer cell lines with 5-aza-2-deoxycytidine reactivated the expression of *NRG1* by 7 to 100 times. *NRG1* was also methylated in tumour tissue samples while it was not in uncultured normal breast epithelium. Knocking down *NRG1* expression by siRNA led to an increase in net cell proliferation.

**Conclusion** *NRG1* could be the 8p tumour suppressor gene. It is located in the right place. It is silenced by methylation or other mechanisms in many breast cancer cell lines and tumours. Functionally, *NRG1* expression is antiproliferative – shown both by our siRNA experiments and older work that showed expression to be proapoptotic to breast cancer cell line MCF7 [3].

**Acknowledgements** Supported by Breast Cancer Campaign and also Cancer Research UK and the Ludwig Institute for Cancer Research.

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## P12

## p53β isoform modulates differentially p53 transcriptional activity in response to stress

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We recently established that the p53 gene expresses nine different p53 protein isoforms. The p53 isoforms bind preferentially to some p53-responsive promoters and modulate differentially p53 transcriptional activity [1]. We characterized further p53β activity. p53β is differentially recruited to p21 and bax promoters in the absence or in the presence of DNA-damaging drugs.

p53β enhances p53 transcriptional activity on the p21 promoter in a dose-dependent manner in the absence of cellular stress but inhibits p53 transcriptional activity on the p21 promoter in the presence of DNA-damaging agents. On the contrary, p53β has no effect on p53 transcriptional activity on the bax promoter in the absence of stress but enhances p53 transcriptional activity on the bax promoter in response to stress without increasing the p53 protein level.

Our data indicate that p53β is involved in the choice of p53 target gene expression in response to cellular signals, switching cell fate outcome from $G_1$ arrest/DNA repair to cell death.

The present finding supports our hypothesis that differential expression of the p53 isoforms in primary breast tumours may help to link p53 status to biological properties and drug sensitivity.

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## P13

## Inhibitor of apoptosis proteins as a therapeutic target in breast cancer

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**Background** Apoptosis is the process of programmed cell death by which damaged or unhealthy cells are normally destroyed. Cancer cells are able to avoid apoptosis and thereby survive inappropriately. Inhibitor of apoptosis proteins (IAPs) are a family of proteins that block apoptosis in normal cells, by binding to active caspases, the proteases that mediate cell death. There are eight human IAPs, including NAIP, XIAP, cIAP1, cIAP2, livin, survivin and apollon. An upregulation of IAPs could cause resistance to apoptosis. Targeting IAPs in cancer therapy may therefore improve the clinical effectiveness of apoptosis-inducing chemotherapeutics. A number of studies have shown that XIAP and survivin are upregulated in cancer, and inhibiting these IAPs increased the apoptotic response induced by some chemotherapeutics. We aim, first, to examine the expression profile of all IAPs in breast cancer and, second, to determine whether inhibiting IAPs will enhance the apoptotic response to traditional chemotherapeutics and newly developed targeted therapies, such as Herceptin.

**Methods** IAP levels were detected in patient and cell line samples by immunoblotting with validated antibodies using the Li-Cor Odyssey system (Li-Cor Biosciences, Lincoln, NE, USA). IAPs were inhibited using siRNA or cell-permeable mimics of endogenous inhibitors. Control cells and cells with XIAP knocked down or inhibited were exposed to TNF-related apoptosis inducing ligand (10 ng/ml), Herceptin (100 μg/ml), Iressa (10 μM), or Lapatinib (100 nM) for 48 hours. Apoptosis was scored by examining nuclear morphology (DAPI) or active caspase 3 staining. Proliferation was examined by Ki67 staining.

**Results** We have found that IAPs are widely upregulated in breast cancer. In particular cIAP2, XIAP and survivin were more prevalent in breast cancer cells than normal breast epithelium. Knock down of XIAP or inhibition with small molecule inhibitors resulted in an increased apoptotic response to TNF-related apoptosis inducing ligand, in both sensitive and resistant cell lines. Knocking down XIAP also increased the apoptotic response to a number of growth factor receptor-targeted therapies such as Herceptin, Iressa and Lapatinib.

**Conclusion** Inhibiting IAPs in combination with both chemotherapeutic agents and targeted therapies, such as Herceptin and Lapatinib, which act as receptor antagonists, will improve clinical outcome.

## P14

## Cellular localization of the proto-oncogenic p53 inhibitor AGR2 protein in breast cancer

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*Breast Cancer Res* 2008, **10(Suppl 2):**P14 (doi: 10.1186/bcr 1898)

**Background** Proteomic technologies verified AGR-2 as a protein family overexpressed in human cancers, including breast, prostate and oesophagus cancers, with the ability to inhibit the tumour suppressor protein p53 [1]. The *AGR-2* gene is a hormone responsive gene with an unexpected induction by the anticancer drug tamoxifen highlighting the proto-oncogenic role of this protein. The *hAGR-2* gene was first described in the MCF-7 breast

carcinoma cell line, and was found to be coexpressed with the estrogen receptor (ER), in ER-positive cell lines [2,3]. Moreover, it was recently revealed that AGR-2 is secreted from androgen-inducible cell lines in prostate cancer cell lines [4].

**Methods** Localization studies of AGR-2 were performed using fluorescence microscopy in order to determine in which compartment the protein functions. Yeast two-hybrid analysis has identified potential nuclear and cytoplasmic binding proteins for AGR-2, essential for the upstream or downstream regulation of the AGR-2 pathway.

**Results** Anterior gradient 2 encodes one protein that gives rise to two forms: the full-length and the mature. Full-length AGR2wt, which bears the leader sequence, localizes to the ER and the Golgi compartment whereas the mature protein requires the C-terminal KTEL sequence for strong nuclear localization. Deletion of the KTEL, putative ER retention, sequence does not alter the localization of the wt full-length form to a large extent but has a strong effect on the localization shift of the mature form. Subcellular fractionation data verified the difference in the localization patterns of the wt forms and their mutants. Moreover, the localization of the protein and each of the mutants differs significantly in various cell lines, suggesting a multipotent role of the protein when it comes to activation pathways and localization patterns within the cell. Furthermore, we present data showing models of how the AGR-2 family might function as a drug-resistance survival factor in cancer as well as a p53 inhibitor.

**Conclusion** All of the above suggest a multipotent role of *AGR-2* when it comes to trafficking, cellular localization and activation or inhibition pathways in cancer. The localization of the protein can therefore determine the level of p53 inhibition.

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## P15

### Investigating h-Prune activation of Wnt signalling in breast cancer

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We have been investigating a novel link between two independent processes linked to breast cancer: Wnt signalling and h-Prune overexpression. The canonical Wnt signalling pathway was activated in 40% to 60% of human breast cancers through mechanisms that are not understood. Similarly, the phosphodiesterase h-Prune was overexpressed or amplified in 54% of breast cancers and was linked to breast tumour progression through unknown mechanisms.

We have shown that overexpression of xenopus Prune induced formation of a secondary axis in a standard assay to identify activators of the Wnt signalling pathway. In HEK293 cells, xenopus Prune overexpression induced a 300-fold increase in Wnt/TCF-dependent transcription. Whilst human prune does not appear to be able to activate Wnt signalling as potently as its xenopus homologue, it does synergise with other activators of the pathway to increase TCF-dependent transcription.

Here we show whether there is a correlation between over-expression of h-Prune and active Wnt signalling in breast cancer, and whether the synergistic responses described are mediated through the enzymatic activity of prune, or through binding to GSK-3.

## P16

### Coactivation of estrogen receptor alpha by the DEAD-box RNA helicases p68 and p72 and its role in breast cancer

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We have previously demonstrated that the DEAD-box RNA helicase p68 is an important regulator of gene expression [1,2], whilst other groups have shown that p68 interacts with and coactivates estrogen receptor alpha (ERα) [3,4]. The main focus of our project is to investigate the molecular mechanism of ERα coactivation by p68 and to examine the potential consequences for breast cancer development.

We have established that the interaction of p68 and ERα requires the DNA binding domain of ERα and the C terminus of p68. Importantly, this region of p68 lies outside the conserved helicase core and was previously shown by us to be essential for transcriptional regulation by p68. Additionally, coactivation of ERα by p68 requires the ligand binding/AF2 region of ERα and is consistent with the model that p68 is recruited to ERα-responsive promoters in response to estrogen [4]. We have also shown that p72, a helicase that is very highly related to p68 and that had previously been suggested to act in an analogous fashion to p68 [3], poorly coactivates ERα in standard transcriptional coactivation assays, using ER-responsive promoters. This is underscored by our finding that overexpression of p68, but not of p72, in cell lines results in stimulation of expression of physiological target genes of ERα.

Interestingly, siRNA-mediated knockdown of endogenous p68 has little effect on the expression of ERα target genes. This observation is consistent with the idea that p68 has little effect on ERα function physiologically, but that the elevated p68 levels found in tumours may stimulate ERα-mediated gene expression in a pathological context. Strikingly, however, in contrast to p68, knockdown of endogenous p72 results in a marked inhibition of both baseline and estrogen stimulated-expression of these genes. These findings suggest, firstly, that p72 is important physiologically for ERα activity in the cell and, secondly, that p68 and p72 may be acting in an opposing rather than analogous fashion (as had been previously suggested [3]). Moreover, our preliminary data suggest that overexpression of p68 in cells may additionally coactivate ERα in an estrogen-independent manner, a finding that may have implications in the development of resistance to endocrine therapies.

We are currently developing inducible p68/p72 overexpression and siRNA cell lines with a view to examining the effect of augmenting or suppressing p68/p72 expression in mouse

xenograft models. Additionally we are screening a large panel of breast cancers to examine p68 and p72 expression/localisation in the context of ERα expression.

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## P17

## TSC22 in mammary gland development and breast cancer

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Mammary gland involution is characterised by a high degree of apoptosis. By identifying genes that are upregulated at this developmental stage, we aimed to discover key factors that are involved in the induction of mammary epithelial cell death and therefore present potential tumour suppressors for breast cancer. Among 96 genes recently identified as specifically upregulated early during involution were the transforming growth factor beta (TGFβ)-stimulated clone 22 homologue (TSC-22/TGF$\beta_1$-induced transcript 4) and TGF$\beta_3$ [1]. TGF$\beta_3$ has recently been shown to be necessary for induction of apoptosis during mammary gland involution, while TSC-22 overexpression can lead to cell death. We have therefore tested whether TSC-22 mRNA expression can be induced by TGF$\beta_3$ and whether it is involved in or necessary for TGFβ-induced apoptosis. We further show that TSC-22 can enhance TGF$\beta_3$-induced Smad response and epithelial cell death. In addition, overexpression of TSC-22 alone can induce a Smad response and apoptosis in mammary epithelial cell cultures, which is independent of p53. Further, we have performed tests to study the necessity for Smad proteins during TSC-22-induced apoptosis, and to establish the intracellular localisation of TSC-22.

A pilot study on a small cohort of archival breast cancer cases, representing all stages of malignant progression, shows that TSC-22 protein was reduced or undetectable in 60% of breast carcinomas when compared with adjacent normal breast tissue, suggesting that TSC-22 could indeed be a potential novel tumour suppressor gene. We shall present data showing that methylation of the TSC-22 promoter is not involved in the reduction of TSC-22 protein in breast cancer.

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## P18

## Accurate prediction of *BRCA1* and *BRCA2* heterozygous genotypes using expression profiling of lymphocytes after irradiation-induced DNA damage

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**Background** Germline mutations in *BRCA1* and *BRCA2* genes predispose women to an increased risk of breast/ovarian cancer. Both genes have important roles in DNA damage repair and are implicated in gene expression regulation. We have previously shown that normal fibroblasts from mutation carriers can be distinguished from noncarriers following radiation-induced DNA damage. In this new study we used lymphocytes to determine whether these also show differential response to induced DNA damage and whether expression profiling using microarray technology could be used to accurately predict the *BRCA* genotype.

**Methods** Short-term lymphocyte cultures were established from fresh blood samples from 20 *BRCA1* and 20 *BRCA2* mutation carriers and from 10 negative controls (individuals tested negative for the mutation present in the family). Lymphocytes were subjected to 8 Gy ionizing irradiation to induce DNA damage and RNA was extracted 1 hour post γ-irradiation. For expression profiling, genome-wide (30 K) spotted cDNA microarrays manufactured by the Cancer Research UK Microarray Facility were used. We then applied the support vector machine (SVM) classifier with statistical feature selection to determine the best feature set for predicting *BRCA1* and *BRCA2* heterozygous genotypes. We also investigated the prediction accuracy using a nonprobabilistic classifier (SVM) and a probabilistic classifier (Gaussian process classifier).

**Results and conclusion** We achieved high accuracy (92% to 96%) in predicting the mutation carrier status. We shall present the detailed outcome of using the SVM classifier and the Gaussian process classifier in the task of distinguishing between the three classes, *BRCA1* and *BRCA2* mutation carriers and noncarriers, and evaluate whether this microarray technology can be used to facilitate the clinical detection and classification of mutations.

## P19

### Investigation into the molecular mechanism of the antiapoptotic functions of CTCF in breast cancer cells using a proteomics approach

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**Background** CCCTC binding protein (CTCF) is a highly conserved and ubiquitous transcription factor with versatile functions. It is involved in transcriptional regulation, chromatin insulation and epigenetic control [1]. Although CTCF has features of a tumour suppressor gene, it is overexpressed in breast cancer cells; this phenomenon is associated with the resistance of these cells to apoptosis [2]. The aim of the present study is to investigate the molecular mechanisms of the CTCF-dependent resistance of breast cancer cells to apoptosis.
**Methods** A proteomics approach was used to generate protein profiles of breast cancer cells, ZR75.1, with normal and reduced levels of CTCF. In the latter cells CTCF was knocked-down using siRNA and iRNA. Cell extracts were analysed using two-dimensional PAGE, and differentially expressed proteins were identified by matrix-assisted laser desorption/ionization time-of-flight or liquid chromatography/mass spectrometry/mass spectrometry.
**Results** More than 20 putative candidates have so far been obtained; they belong to various protein families involved in the control of signalling, metabolic, apoptotic, stress response and mammary gland specific regulatory pathways. One of the candidates, the proapoptotic protein Bax, was further validated as a target for negative regulation by CTCF. We demonstrated that expression of Bax correlated inversely with CTCF levels. Furthermore, Bax promoter was negatively regulated by CTCF in reporter assays. Two putative CTCF binding sites were identified within the promoter of *Bax* gene; contact nucleotides were determined by footprinting and methylation interference assays.
**Conclusion** Our data suggest that high levels of CTCF may cause repression of Bax and inhibition of apoptosis. Lower levels of CTCF lead to activation of Bax, resulting in apoptosis. Selective reduction of CTCF can therefore be an attractive option in the development of antibreast cancer therapies.
**Acknowledgements** Supported by Breast Cancer Campaign and CONACyT (National Council of Science and Technology, Mexico).
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## P20

### Inhibition of apoptosis by Notch signalling in breast epithelial cells

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**Background** Aberrant Notch signalling has been shown to be involved in many cancers. We have recently observed accumulation of the Notch intracellular domain (NICD) in breast cancer cell lines and tissue samples in comparison with normal cell lines and tissues. Moreover, Notch activation has been shown to inhibit apoptosis induced by chemotherapeutics that activate the p53 pathway. We are thus investigating the role of Notch signalling in breast cancer by studying the molecular mechanisms that underlie its suppression of apoptosis.
**Methods** Notch signalling was activated in the normal breast epithelial cells MCF10A by expression of NICD or a fusion protein CBF1-VP16. To inhibit Notch signalling in MCF7, BT474 and Hs578t cancer cells we expressed NUMB, a natural inhibitor of the pathway or a dominant-negative Mastermind protein. Signalling through apoptotic pathways and extent of cell death were monitored by western blot analysis and chromatin condensation by Hoechst staining, respectively. Apoptosis was induced by melphalan treatment (100 μM). Pretreatment with SH6 (10 μM) or SP600125 (10 μM) was used to inhibit AKT pathways or JNK activity, respectively. Nutlin-3 (10 μM) was used to inhibit p53–MDM2 interaction.
**Results** Activation of Notch signalling in MCF10A cells caused resistance to p53-dependent apoptosis induced by DNA damage. Conversely, inhibiting Notch signalling in MCF7, BT474 or Hs578t cancer cell lines led to a sensitization to apoptosis. We further showed that AKT was phosphorylated on serine 473 and that the AKT targets ASK1 and MDM2 were phosphorylated on serine 83 and 166, respectively, in Notch-activated MCF10A cells. Furthermore, AKT inhibition by treatment with SH6 restored sensitivity of NICD-expressing or CBF1-VP16-expressing cells to DNA-damage-induced apoptosis, showing that AKT activation is sufficient to confer resistance to apoptosis. We thus investigated the role of AKT-mediated MDM2 phosphorylation. Inhibition of p53–MDM2 interaction by treatment with Nultin-3 restored neither NOXA or PUMA accumulation nor sensitivity of NICD-expressing or CBF-1-VP16-expressing MCF10A cells following DNA-damaging agent treatment. We next showed that, following DNA-damaging agent treatment, Notch activation prevented JNK phosphorylation and PUMA and NOXA accumulation. Furthermore, JNK activation in NICD-expressing or CBF1-VP16-expressing cells is sufficient to induce cell death, and inhibition of JNK signalling by treatment with SP600125 is sufficient to prevent cell death in normal MCF10A cells.
**Conclusion** Notch activation of the AKT pathway inhibits DNA damage-induced apoptosis by inhibition of JNK via ASK1 phosphorylation.

## P21

### TopBP1 contains transcriptional regulatory domains and regulates gene pathways involved in breast cancer

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TopBP1 is a nuclear protein with eight BRCT domains and is involved in many aspects of nucleic acid metabolism: it is involved in the initiation of DNA replication in the Xenopus *in vitro* replication system by assisting loading of polymerase onto the replication complex; it is a substrate for ATM/ATR and is essential for the ATR DNA damage signalling pathway, and is also probably involved in the actual DNA repair process; it acts as a transcriptional cofactor for E2F1 where it regulates the apoptotic function of this protein. In addition, the yeast homologues of TopBP1, Dbp11 (*Saccharomyces cerevisiae*) and Cut5 (*Schizosaccharomyces pombe*), are also involved in replication and repair processes. TopBP1 also shares functions with *BRCA1*; both are involved in regulating an intact $G_2$/M checkpoint, they colocalise to sites of DNA damage, they share sequence homology (even outside the BRCT domains), they are substrates for ATM/ATR, and they can regulate expression of the c-*myc* gene. All of these properties of TopBP1 led to us investigating whether TopBP1 plays a role in breast cancer.

There is a polymorphism in TopBP1 that gives an increased risk of breast cancer [1], and work from our laboratory has demonstrated that TopBP1 is aberrantly expressed in a significant number of human breast cancers [2]. Clearly the role of TopBP1 in replication and genome maintenance would mean that disturbance of expression could result in genomic instability contributing towards cancer. Our studies have focused on an additional aspect of TopBP1 that could contribute to the transformed phenotype; gene regulation. We have identified several chromatin modification domains on TopBP1 that could contribute not only to transcriptional regulation but also to the replication and repair functions of this protein [3]. Using siRNA knockdown of TopBP1 in MCF7 cells, we identified genes that are regulated by TopBP1. Following knockdown of TopBP1, the short-term growth of the MCF7 cells was not affected. This was surprising as it has been predicted that TopBP1 is essential for DNA replication and our results demonstrate that this is not the case in all cell lines (we have tested other lines in which TopBP1 is essential for S phase). However, even though these cells cycled for several days, they did not survive long term, presumably due to accumulated damage following replication in the absence of TopBP1. Using this MCF7 system we carried out microarray experiments that revealed the absence of TopBP1 alters the expression of genes involved in many cellular pathways implicated in breast cancer, including the oestrogen signalling pathway, and the mitogen-activated protein kinase signalling network. Future work will focus on determining how TopBP1 regulates these pathways and what cellular interacting partners TopBP1 requires for chromatin modification. Such studies will increase our understanding of breast cancer and assist in developing diagnostic and prognostic gene profiling for breast cancer management.

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## P22

### Regulation of cyclin $D_1$ by the BRCA1–BARD1 complex

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**Background** BRCA1 and cyclin $D_1$ are both essential for normal breast development and mutation or aberration of their expression is associated with breast cancer [1,2]. Cyclin $D_1$ is best known as a $G_1$ cyclin where it regulates the $G_1$ to S phase transition by acting as a rate-limiting subunit of CDK4/6 kinase activity. More recently, however, Stacey has demonstrated that cyclin $D_1$ levels in $G_2$/M determine whether a cell continues to proliferate or exits the cell cycle [3]. The majority of BRCA1 in the cell is bound to BARD1 through their N-terminal RING domains. Heterodimerization is essential for the stability and correct localization of the complex and confers ubiquitin ligase activity to BRCA1. The importance of the ligase activity of BRCA1 to breast cancer development is inferred from the fact that N-terminal disease-associated mutations are proposed to reduce ligase activity [4].

**Methods** Protein–protein interactions were demonstrated using yeast-two-hybrid and coimmunoprecipitation. Protein levels were altered through overexpression, siRNA and antisense technology. The effect of proteasome inhibitors and cycloheximide treatment was also examined.

**Results** We initially identified cyclin $D_1$ as a binding partner of BARD1 in a yeast-two-hybrid screen and defined the minimal binding region as the N-terminus of BARD1. This interaction was confirmed *in vivo* by coimmunoprecipitation. The N-terminus of BARD1 also binds BRCA1 and imparts ubiquitin ligase activity to the complex. Covalent modification of proteins with ubiquitin is a common regulatory mechanism in eukaryotic cells. Traditionally, polyubiquitin chains linked through lysine 48 target proteins for degradation by the 26 S proteasome. We have demonstrated that cyclin $D_1$ protein levels are inversely related to BRCA1 and BARD1 levels in several model systems. Furthermore, regulation of cyclin $D_1$ levels occurs through a post-transcriptional mechanism and requires the ligase activity of BRCA1. Interestingly, this phenomenon is cell-cycle regulated, occurring in $G_2$/M.

**Conclusion** We propose that cyclin $D_1$ is a potential substrate for BRCA1 ubiquitination and that this targets cyclin $D_1$ for proteasomal-mediated degradation. Future work will focus on ascertaining the functional consequence of cyclin $D_1$ regulation by the BRCA1–BARD1 complex; in particular, the impact of BRCA1, mediated through regulation of cyclin $D_1$, on the proliferation versus differentiation decision.

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## P23

### Modelling estrogen receptor alpha-positive breast cancer by transformation of normal human mammary epithelial cells

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Two-thirds of breast cancers express estrogen receptor alpha (ERα) and are estrogen-dependent for growth, yet the unavailability of accurate *in vivo* models has long impeded the characterisation of critical events that lead to the development of these luminal subtypes of the disease. Previously, our group successfully created an ERα-positive breast cancer model by quantitative transformation of normal human mammary epithelial cells (HMECs) derived from reduction mammoplasties. HMECs were grown as mammospheres in suspension to enrich for progenitor cells, which were then transformed using lentiviral vectors encoding ERα and *TERT* as well as the polycomb gene *BMI1* and *MYC*, both of which have been implicated in ERα-positive breast cancer. Injection of transformed HMECs into mammary fad pads of NOD/SCID mice resulted in the formation of estrogen-dependent tumours that metastasised to multiple organs [1], confirming the creation of a model that mirrors the characteristics of human estrogen-dependent breast cancer. Somewhat surprisingly, we observed islands of squamous differentiation in the tumours that formed in the NOD/SCID mice, whereas the large majority of human breast tumours are adenocarcinomas. To address this discrepancy, we are currently testing a combination of our established protocol with new HMECs *in vitro* culture conditions that have recently been shown to abrogate the squamous phenotype of the resulting tumours in mice [2]. Our model system is a powerful tool for the *in vivo* characterisation of candidate genes that have been implicated in development of ERα-positive breast cancer. Genes of interest include *TNRC9*, which has recently been identified in genome-wide association studies as a potential novel breast cancer susceptibility gene [3], as well as *TBX3*, which is known to play a role in mammary gland development as well as breast tumourigenesis. We are currently testing these genes in our model using overexpression and knockdown approaches.

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## P24

### Regulation of estrogen receptor beta by 5′ untranslated regions in breast carcinogenesis

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**Background** Estrogen receptor (ER) expression is a key determinant of breast tumour behaviour. While the role of ERα in carcinogenesis is relatively well understood, the role of ERβ, the more recently identified receptor, remains uncertain. This is partly because analyses have been confused by a consistent discrepancy between ERβ expression at mRNA and protein levels [1]. Recently, evidence has accumulated that deregulation of gene-specific *translation* occurs during carcinogenesis in breast and other tissues. Regulation of ERβ translation could therefore be responsible for nonconcordance of its mRNA and protein levels, and could provide an important level of modulation of ER activity during breast cancer development.

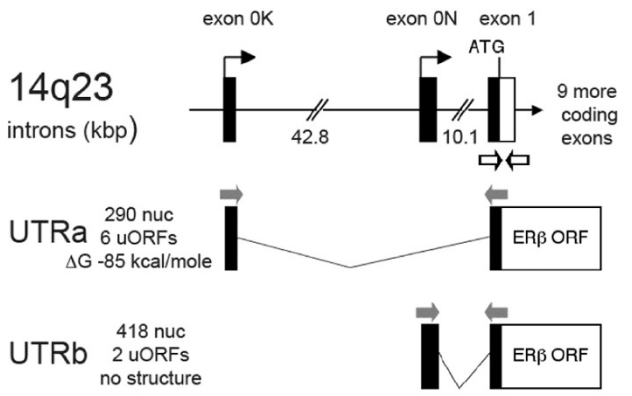
Regulation of translation occurs mainly during initiation. Most initiation occurs by cap-dependent scanning, which requires binding of the initiation complex to the mRNA cap and recruitment of other proteins. These scan along the 5′ untranslated region (UTR) of the mRNA to the reading frame, where they recognise an initiation codon, recruit more factors, and initiate protein synthesis. 5′ UTRs vary greatly in length and sequence with some containing elements that allow regulation of factor recruitment or scanning, and thereby allow regulation of translation of their specific mRNAs [2]. It is thought that deregulation of translation, via 5′-UTR sequences, is responsible for a significant proportion of the expression changes in cancer cells and that this has a role in carcinogenesis.

We identified three alternative 5′ UTRs for ERβ – UTRa (including upstream exon 0K), UTRa long (UTRa containing an additional 5′ sequence) and UTRb (including upstream exon 0N) – from the literature [3] and EST databases (Figure 1). Our hypothesis is that these alternative 5′ UTRs allow differential post-transcriptional regulation of ERβ expression, thereby providing critical regulation of ER function.

**Methods** We investigated the properties of these three ERβ 5′ UTRs using established reporter assays [4]; each 5′ UTR was cloned upstream of a GFP reporter. Breast cell lines (MCF7, MDA-MB-453, MDA-MB-231, BT-20 and HB2) were transiently transfected with either an unmodified GFP reporter as a control (this is identical to experimental vectors except for its non-specialised 5′ UTR), or with equal copy numbers of specific 5′-UTR reporters. Effects of each 5′ UTR on translation were assessed by measurement of relative GFP protein and mRNA expression from each plasmid using flow cytometry and quantitative PCR, respectively. Semi-quantitative PCR was also used to analyse ERβ 5′ UTRa and UTRb expression in matched normal/tumour breast tissues.

**Results** Our results are the first to show that these alternative 5′ UTRs do, in fact, allow the differential regulation of ERβ translation. The UTRa and UTRa long 5′ UTRs strongly inhibited translation of the GFP reporter whilst UTRb had little effect. In addition, our preliminary data suggest that these alternative 5′ UTRs are differentially expressed between breast normal and tumour tissue. The expression of UTRa mRNA was found to be upregulated in a panel of breast tumours compared with matched normal tissue. This may have important implications in breast cancer development. Work is currently ongoing to investigate the stability of these mRNA messages and to identify important regulatory sequences.

**Figure 1 (abstract P24)**




5′ end of the human ERβ gene (14q23) aligned with mRNAs containing different ERβ 5′ UTRs. UTR exons (filled boxes), transcriptional (black arrows) and translational (ATG) start sites, intron sizes, and primers used for PCR analysis (grey arrows, specific UTRs; open arrows, exon 1) are shown. Sequences strongly suggestive of translational regulation are described: uORFs and stable RNA structure, quantified as change in free energy, ΔG; for comparison, Δ*G* of the nonregulatory β-actin 5′ UTR is only −24 kcal/mol.

**Conclusion** Post-transcriptional regulation plays an important role in determining the level of ERβ protein expression and may therefore have an influence on overall estrogen receptor activity. This may have important implications on our understanding of breast cancer biology and treatment.

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## P25

## Reelin expression in breast tumours is associated with increased survival and is controlled by promoter methylation

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**Background** Reelin is a secreted signalling protein whose major function has so far been described in the developing brain, where it is involved in cell positioning of neuronal progenitor cells. Recently, the *Reelin* promoter has been found to be methylated in pancreatic cancer and this was associated with increased migratory ability [1], whereas in the prostate Reelin expression is associated with high-grade cancers [2].

**Methods** We measured the Reelin expression and promoter methylation status in breast cancer-derived cell lines and in a cohort of 64 breast cancer cases. We further stained sections of normal, benign, and cancerous human breast, as well as two tissue arrays of 168 and 2,200 breast cancer patients, respectively. Reelin staining was analysed with regards to other clinical parameters and survival.

**Results** Promoter methylation status in breast cancer cell lines, as well as in primary cancers, corresponds with reduced expression of Reelin. In the normal breast, Reelin is expressed in the luminal epithelium and myoepithelium, but is lost during breast cancer progression. Reelin expression correlates with increased survival ($P = 0.01$) and negative lymph node status. Treatment of breast cancer cell lines with the demethylating agent decitabine leads to re-expression of Reelin RNA.

**Conclusion** Reelin expression in the breast is associated with increased survival and negative lymph node status, and is controlled at least in part by promoter methylation. *Reelin* is therefore a novel potential tumour suppressor gene in the breast.

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## P26

## Investigation of the roles of novel apoptosis-controlling genes in breast cancer

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**Background** Normal mammary epithelial cells, like all other nucleated cells in the body, have the innate capacity to undergo programmed cell death by apoptosis. This process is controlled by external factors such as hormones and growth factors, as well as by cell–cell contacts and recognition of damaged DNA, and plays an essential role in maintaining stable cell numbers. In breast cancer cells, in common with most other cancer cells, the control of apoptosis is defective, so that the rate of cell death falls below that required to maintain a stable cell population size. The analysis of the molecular control of apoptosis is therefore very important in understanding breast cancer development and in producing novel therapies.

We have successfully used functional expression cloning [1] to identify novel genes playing controlling roles in the apoptosis process. We have used the effects of the genes on cell survival itself to isolate those genes that act at rate-limiting steps in the control of this process, and whose level of activity therefore

determines whether the cell lives or dies. The genes we have identified include *protein phosphatase 4* [2], *Fau* [3], *vATPase E*, *PLAC8* and *GAS5* [4]. Subsequently we have studied the effects of upregulation and downregulation of the activity of these genes on breast cancer cells. We have also analysed the levels of expression of these genes in normal breast epithelium and in breast cancer tissue in order to determine which of the genes are involved in the development of these cancers.

**Methods** Gene expression in breast cancer cell lines was upregulated by transfection of full-length cDNAs in pcDNA3 or pCMVSPORT expression vectors. Downregulation was achieved by transfection of siRNAs (Ambion; Applied Biosystems, Warrington, UK). Real-time quantitative RT-PCR was used to monitor gene expression levels in the breast cancer cell lines, and also in breast cancer and matched normal breast tissue.

**Results** Modulation of expression of several of the candidate genes, particularly *Fau*, altered the sensitivity of breast cancer cell lines to apoptosis. While expression of *PLAC8* was not significantly altered in the breast tumour samples as a whole, several of the other genes, including *Fau* and *vATPase E*, did show significant changes in their levels of expression in breast tumour tissue, when compared with normal matched breast epithelial tissue from the same patients.

**Conclusion** Several of the apoptosis-controlling genes identified by functional expression cloning affect the sensitivity of breast cancer cells to apoptosis, including that caused by DNA-damaging agents. Those genes that show differential expression may play particularly important roles in the development of breast cancer and in determining breast cancer resistance or sensitivity to cytotoxic therapy.

**Acknowledgement** Breast Cancer Campaign funded this project.

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## P27

### D133p53 isoform is a direct p53 target gene that modulates p53 tumour suppressor activity

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## P28

### *De novo* expression of $\alpha_v\beta_6$ integrin by myoepithelial cells in ductal carcinoma *in situ* may be an important marker of disease progression

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Myoepithelial cells (MEC) are essential to the maintenance of normal breast function, and loss of normal MEC function is commonly associated with breast cancer. Most established invasive breast carcinomas develop through an *in situ* phase known as ductal carcinoma *in situ* (DCIS). We have identified upregulation of $\beta_6$ integrin on MEC in a subset of DCIS. Normal MEC exhibit potent tumour suppressor function, but it is not clear whether this is compromised in DCIS. The aim of the present study is to investigate the effect of $\beta_6$ expression on myoepithelial tumour suppressor function.

Immunohistochemical analysis of DCIS of different grades with and without an invasive breast cancer was carried out to determine the $\beta_6$ status. For *in vitro* studies magnetic bead sorting was used to isolate a pure normal-like myoepithelial cell line from the immortalised 1089 cell line (N-1089 MEC). These were used to generate $\beta_6$ overexpressing myoepithelial cells (DCIS-modified MEC) by retroviral transduction. The lines were characterised by immunofluorescence and flow cytometry, and the tumour suppressor function of both lines was compared with primary normal and DCIS MEC in coculture with breast cancer cell lines.

Analysis of a series of primary DCIS tissues ($n > 400$) demonstrated induction of $\beta_6$ in MEC in a subset of cases, predominantly high grade. Upregulation was almost universal in DCIS associated with invasive disease. Initial characterisation of the 1089 line revealed a mixed phenotype from which pure MEC were selected on the basis of $\beta_4$ integrin. This population exhibited all characteristic myoepithelial markers. Coculture assays demonstrated that N-1089 MEC could produce the same tumour suppressor effect as primary MEC, leading to significant reduction in tumour invasion ($P < 0.001$) and proliferation ($P < 0.001$). N-1089 MEC transduced with $\alpha_v\beta_6$ (DCIS-1089 MEC) demonstrated enhanced binding and migration to the $\beta_6$ ligand LAP, and activation of a transforming growth factor beta reporter, indicating that the $\alpha_v\beta_6$ was functional. Whilst primary DCIS MEC showed loss of suppressor function ($P < 0.05$), DCIS-1089 MEC exhibit altered behaviour with a more migratory phenotype then the normal counterpart, but at least some of the tumour-suppressor function was maintained.

We have shown that MEC exhibit an altered phenotype in DCIS with *de novo* expression of $\alpha_v\beta_6$. We have generated normal-like cell lines that exhibit all the characteristics of primary MEC and recapitulate primary MEC tumour suppressor function. Primary DCIS MEC show loss of suppressor function whereas $\beta_6$-overexpressing MEC (which resemble DCIS-like MEC) promote or suppress breast cancer cell invasion in a cell-type-specific manner. These findings suggest that changes in MEC during DCIS may influence disease progression, and these cell lines provide a powerful model to study further the mechanisms involved.

## P29

### Understanding and exploiting changes in O-linked glycosylation in breast cancer

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The differences in glycosylation patterns seen in breast malignancy strongly influence the final structure of membrane and secreted glycoproteins, and these novel tumour-associated glycoforms can modify the behaviour of the malignant cell and its interaction with immune effector cells. Changes in mucin type O-glycosylation occur in breast carcinomas and are the result, at least in part, of

changes in the expression of specific glycosyltransferases [1]. A similar change in the expression of glycosyltransferases resulting in the change of glycans attached to O-linked glycoproteins is seen when normal dendritic cells mature and migrate to the lymph nodes [2]. As 70% to 80% of metastatic breast cancers metastasize via the lymphatics, a particular pattern of O-linked glycans may be required for cells to migrate and/or settle in the lymph nodes.

Changes in O-linked glycosylation have a considerable influence on the structure of mucin glycoproteins that carry hundreds of O-linked glycans. The MUC1 membrane mucin is expressed by over 90% of breast carcinomas and in the change to malignancy truncated O-glycans are added to this mucin. *In vitro* synthesis of MUC1-based glycoproteins and glycopeptides carrying specific tumour-associated glycans has allowed an investigation of how individual glycoforms affect the immune response and interact with immune effector cells [3,4]. It is becoming clear that some glycoforms of MUC1 can induce an immune response while others are immunosuppressive. Understanding how the different tumour-associated glycoforms induce or inhibit the immune response is important for the design of clinical studies using MUC1-based antigens.

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## P30

## Downregulation of 15-hydroxyprostaglandin dehydrogenase in hormone-resistant breast cancer

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**Background** Tamoxifen has been the principal endocrine therapy for estrogen receptor alpha (ERα)-positive breast cancer patients and still remains the therapy of choice in the premenopausal setting. However, resistance and recurrence remain a serious problem. Our previous work has indicated that 15-hydroxyprostaglandin dehydrogenase (15-PGDH) was significantly downregulated in two, independently derived, tamoxifen-resistant (TAMr) MCF-7 derivatives compared with sensitive controls [1]. 15-PGDH is the key enzyme for the biological inactivation of prostaglandins, and has been shown to be a tumour suppressor in breast cancer. However, a role for 15-PGDH downregulation in endocrine resistance has not previously been identified.

**Methods and results** Downregulation of 15-PGDH mRNA and protein in TAMr MCF-7 was confirmed by quantitative RT-PCR and western blotting. To determine the role of 15-PGDH in TAMr, we stably transfected TAMr MCF-7 cells with human 15-PGDH cDNA. Overexpression of 15-PGDH partially restored sensitivity of TAMr cells to 4-hydroxytamoxifen by the MTT assay, demonstrating that 15-PGDH downregulation plays a functional role in the acquisition of TAMr. Treatment of TAMr MCF-7 cells with a DNA methyltransferase inhibitor (5-azacytidine), and a histone deacetylase inhibitor (trichostatin A), led to re-expression of 15-PGDH mRNA (by quantitative RT-PCR), suggesting that 15-PGDH is silenced via epigenetic mechanisms during the acquisition of TAMr. To address whether 15-PGDH downregulation is involved in clinical TAMr, we assembled a tissue microarray comprising 89 relapsed primary human breast cancers and 234 tamoxifen-sensitive controls. We are currently optimizing 15-PGDH immunohistochemistry on our tissue microarrays, and results will be presented.

**Conclusion** Our data suggest that the acquisition of TAMr *in vitro* involves epigenetic silencing of 15-PGDH. Moreover, our data show that 15-PGDH downregulation has a novel, functional role in endocrine resistance.

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## P31

## Plasma MMP1, MMP8 and MMP13 expression in breast cancer: protective role of MMP8 against lymph node metastasis

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Elevated levels of matrix metalloproteinases (MMPs) have been found to associate with poor prognosis in various carcinomas. This study aimed at evaluating plasma levels of the collagenases MMP1, MMP8 and MMP13 as diagnostic and prognostic markers of breast cancer. Using ELISA, plasma levels of MMP1, MMP8 and MMP13 were measured in 42 control individuals and in 208 patients – of which 21 were inflammatory breast cancer patients – and were correlated with standard clinicopathological data. Plasma MMP1 levels were higher in breast cancer patients than in control individuals, while the opposite was true for MMP8. Plasma MMP13 levels could not be detected. We found a negative correlation of plasma MMP1 with tumour size ($P = 0.07$); and a positive association of MMP8 with the premenopausal status ($P = 0.06$), Nottingham Prognostic Index ($P = 0.06$) and Her2 expression ($P = 0.07$). Further, a twofold decrease in MMP1 ($P = 0.025$) and MMP8 ($P = 0.007$) levels was observed in inflammatory breast cancer patients, a very rare and not well understood aggressive

disease. Most interestingly, we observed a peculiar relation between plasma MMP8 levels and lymph node metastasis. We found that both control individuals and patients without lymph node involvement (pN0) have lower plasma MMP8 levels than patients with moderate lymph node involvement (pN1, pN2) ($P = 0.001$); and that they show a trend for higher MMP8 levels as compared with patients with extensive lymph node metastasis (pN3) and a strong predisposition to distant metastasis. In summary, we observed differences in MMP1 and MMP8 plasma levels between distinct breast cancer patient groups. As it is not clear to date whether MMPs in blood and body fluids have a physiological function *per se*, we hypothesize that altered levels in blood reflect local changes in the extracellular microenvironment. As such, a positive association of blood MMP levels with clinical characteristics and tumour features reflects a negative association with tissue MMP levels and *vice versa*. Therefore, our results suggest that both MMP1 and MMP8 in the tumour may contribute to the aggressive phenotype of inflammatory breast carcinomas. Interestingly, our results suggest that tumour MMP8 expression may affect the metastatic behaviour of breast cancer cells with a greater protective effect against lymph node metastasis than against distant metastasis.

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## P32

## Association of MMP8 gene variation with breast cancer prognosis

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Animal and cell line studies indicate an inhibitory effect of matrix metalloproteinase 8 (MMP8) on tumorigenesis and metastasis [1-3]. We investigated whether *MMP8* gene variation was associated with breast cancer metastasis and prognosis in humans. We first studied nine tagging single nucleotide polymorphisms (SNPs) in the *MMP8* gene in 140 clinically and pathologically well-characterized breast cancer patients. Four of the SNPs were found to be associated with lymph node metastasis, the most pronounced being a promoter SNP (rs11225395) with its minor allele (T) associating with reduced susceptibility to lymph node metastasis ($P = 0.02$). This SNP was further evaluated for association with cancer relapse and survival among a cohort of approximately 1,100 breast cancer patients who had been followed for cancer recurrence and mortality for a median of 7.1 years. The T allele was associated with reduced cancer relapse and greater survival, particularly among patients with earlier stage cancer. Among patients of tumour-node-metastasis stage 0-II, the adjusted hazard ratio of disease-free survival was 0.7 (95% CI, 0.5 to 0.9) for patients carrying T allele compared with those homozygous for the C allele ($P = 0.02$). *In vitro* experiments showed that the T allele had higher promoter activity than the C allele in breast cancer cells. Electrophoretic mobility shift assays showed binding of nuclear proteins to the DNA sequence at the SNP site of the T allele but not that of the C allele. The data suggest that *MMP8* gene variation may influence breast cancer prognosis and support the notion that MMP8 has an inhibitory effect on cancer metastasis.

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## P33

## CD44 signalling increases cathepsin K and MT1MMP expression to potentiate breast cancer cell invasion through collagen I

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**Background** Bone metastasis is a frequent and often incurable complication of breast cancer causing severe bone pain, pathological fractures, spinal cord compression and hypercalcaemia. We have focused on establishing the significance of the cell surface hyaluronan receptor CD44 in underpinning the preferential metastasis of breast cancer cells to bone. In prior *in vitro* studies we have demonstrated that depletion of CD44 expression in breast cancer cells attenuates their adhesion to bone marrow endothelial cells (BMECs). Our recent experiments have also determined that the expression of CD44 is elevated in a bone-homing breast cancer subline MDAMB231BO relative to that detected in the parental MDAMB231 breast cancer cell line. Together these experiments suggest a physiological role for this receptor in promoting the entry of breast cancer cells into the bone microenvironment.

**Methods** To further understand the potential significance of CD44 signalling to breast cancer metastasis, we established a tetracycline-regulated CD44 expression system in the minimally invasive, CD44-negative MCF7F cell line. Removal of tetracycline from the growth media resulted in time-dependent increases in CD44 expression in MCF7F cells, promoting increased cell invasion and migration responses in addition to potentiating the adhesion of MCF7F cells to BMECs. Subsequent microarray analysis was conducted using this expression system to identify CD44/HA-regulated genes in breast cancer cells.

**Results** The expression and activation of CD44 was associated with increased expression of a subset of genes implicated in metastasis including proteolytic enzymes, growth factors and cytoskeletal proteins (for example, cortactin). Interestingly, the

cysteine protease cathepsin K and the matrix metalloprotease MT1MMP were identified as CD44/HA-regulated genes. These proteases target collagen I, a major component of the bone matrix whose degradation is a major consequence of osteolytic metastasis of breast cancer. Quantitative real-time PCR, immunoblotting and ELISA-based experiments have demonstrated that the transcript and protein expression of cathepsin K and MT1MMP increase in response to CD44/HA signalling in a panel of CD44-expressing breast cancer cell lines (MDA231, MDA157 and MCF7F). Further experiments conducted using a parental and bone-homing subclone of the MDAMB231 cell line (MDAMB213BO) have shown that the expression of CD44, cathepsin K and MT1MMP is elevated in the MDAMB231BO cells relative to their parental counterparts. Furthermore, CD44/HA signalling was shown to increase cathepsin K and MT1MMP mRNA and protein expression in the MDAMB231BO cells. Consistent with their increased metastatic phenotype, MDAMB231BO cells displayed enhanced invasion on HA-supplemented Matrigel and collagen I and demonstrated enhanced collagenolytic activity as demonstrated using an *in vitro* fluorescence-based assay. RNAi mediated depletion of CD44 and MT1-MMP expression and pharmacological inhibition of cathepsin K attenuated CD44 promoted invasion through a collagen I matrix. We are currently investigating the mechanistic basis underpinning the transcription of these proteases in breast cancer cells, and using the MDA231BO cell line to determine the *in vivo* significance of CD44 expression to osteolytic metastasis of breast cancer.

**Conclusion** Our studies demonstrate that CD44 signalling regulates collagenase activity in breast cancer cells underpinning their invasion through matrix substrates that are enriched within breast tissues and organs to which this disease preferentially metastasises. The long-term objective of our research will be to determine whether CD44 expression and that of its transcriptional targets may be predictive for those breast cancer patients at higher risk of developing skeletal disease and/or may potentially lead to the development of novel and more effective therapeutic strategies to attenuate bone metastasis.

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## P34

## Overexpression of CD44 in acquired tamoxifen-resistant breast cancer cells augments their migratory response to heregulin beta 1

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**Background** Acquired endocrine resistance in breast cancer cells is accompanied by altered growth factor receptor signalling [1] and a highly migratory cell phenotype [2]. Interestingly, in tamoxifen-resistant (TamR) MCF7 cells, our microarray analysis has demonstrated elevated levels of CD44, a transmembrane glycoprotein known to interact with, and modulate the function of, growth factor receptors [3]. Here we have explored the role of CD44 as a modulator of heregulin beta-1-induced migratory signalling in TamR cells.

**Methods** Expression of CD44 (standard and v3 isoforms) were confirmed by RT-PCR and western blotting and their association with erbB family members determined by both immunofluorescence microscopy and immunoprecipitation. Activation of intracellular signalling following heregulin beta 1 treatment (10 ng/ml) in the presence or absence of CD44 (using siRNA-mediated inhibition) was determined by western blotting using phosphospecific antibodies. Cellular migration was determined by seeding cells (control and CD44 siRNA-treated) into fibronectin-coated transwell chambers (8.0 μm pore size) in the presence or absence of heregulin beta 1. After 24 hours, migratory cells were fixed, stained with crystal violet and counted.

**Results** Both standard and v3 isoforms of CD44 were overexpressed in TamR cells at both gene and protein levels (mean fold increase in CD44s protein (TamR versus MCF7): $4.26 \pm 1.2$, $P < 0.05$). Moreover, CD44s and v3 colocalised with Her2 and Her3 receptors at the cell surface and were also detectable in Her2/Her3 cellular immunoprecipitates. Treatment of TamR cells with heregulin resulted in phosphorylation of erbB receptors together with a number of downstream signalling intermediates, including Akt, Src and FAK, and resulted in enhanced cellular migration. Significantly, heregulin-induced intracellular signalling was dramatically reduced in cells in which the expression of CD44 was suppressed (via siRNA), with a corresponding loss of heregulin-induced migratory behaviour (mean fold change in cell migration versus untreated control: $6.7 \pm 1.1$, $P < 0.05$ (heregulin beta 1); $1.8 \pm 0.9$ (CD44 siRNA); $1.47 \pm 0.6$, $P < 0.05$ (heregulin beta 1 + CD44 siRNA)).

**Conclusion** These data demonstrate a role for CD44 as a modulator of erbB receptor function in endocrine-resistant breast cancer cells, where it augments heregulin beta 1 migratory signalling.

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## P35

## Role of CLEVER-1 in breast cancer metastasis

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The role that the novel lymphatic-associated adhesion molecule CLEVER-1 plays in breast cancer metastasis has been examined by assessing its expression in human breast tumour specimens and by conducting *in vitro* experiments to monitor its involvement in regulating cell adhesion to human umbilical vein endothelial cells (HUVEC) and hTERT immortalised lymphatic endothelial cells (LEC).

CLEVER-1 expression was examined in tonsil, lymph node and 148 formalin-fixed paraffin-embedded archival breast carcinoma specimens using standard immunohistochemistry protocols. *In vitro* CLEVER-1 expression was studied, in HUVEC and LEC, via fluorescence-activated cell sorting. Tumour cell (breast MCF-7 and MDA-MB-231, and melanoma SKMEL-30) adhesion and leukocyte adhesion to parental and CLEVER-1 siRNA knockdown endothelial cells was also examined.

The results show that, in tissue specimens, CLEVER-1 is present in blood and lymphatic vessels and in certain leukocyte sub-

populations (macrophage or dendritic cells). Although expression, in tumours, is higher in blood vessels than in lymphatic vessels (62.4% versus 18.2%), only lymphatic expression is associated with lymph node metastasis ($P = 0.027$). CLEVER-1 expression in blood vessels and lymphatic vessels correlates with the density of inflammatory infiltrate ($P < 0.001$ and $P = 0.004$, respectively) and expression in macrophages ($P < 0.001$). *In vitro* results show that although CLEVER-1 is expressed intracellularly in both HUVEC and LEC, only LEC exhibit surface expression. Interestingly, adhesion assays show that tumour cells adhere preferentially to LEC with maximal adhesion exhibited at 30 to 40 minutes. Tumour cells adhere less to CLEVER-1 knockdown LEC than to control LEC. The role of CLEVER-1 in cellular adhesion is being further investigated, using tumour cells and different leukocyte populations, to determine its involvement in adhesion and migration of different cell types across lymphatics.

## P36

## Characterization of a cytoskeletal signaling pathway underpinning CD44-initiated, integrin-mediated adhesion of breast cancer cells to bone marrow endothelium

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**Background** Bone metastasis is a frequent complication of breast cancer. It is estimated that up to 85% of breast cancers will metastasize to the bone. The selective metastasis of breast cancer to the bone is thought to result from the preferential adhesion of breast cancer cells to the bone marrow endothelial cells (BMECs) lining the bone marrow sinusoids. Our studies have shown that CD44 promotes the primary adhesion of breast cancer cells to bone marrow endothelium *in vitro*. The aim of the present study was to further explore the cascade of events underpinning CD44-initiated adhesion.
**Methods** Experiments using parental and bone-homing (BO) clones of the MDA-MB-231 breast cancer cell line established the importance of CD44 to integrin-mediated adhesion to BMECs.
**Results** MDA-MB-231BO cells displayed increased CD44 expression and adhesion to both BMECs and fibronectin, relative to parental cells. MDA-MB-231BO cells also displayed increased expression and activation of the $\beta_1$-integrin subunit. In addition, the bone-homing cells exhibited elevated constitutive phosphorylation of the kinases Src and FAK and the cytoskeletal proteins cortactin and paxillin relative to the parental cells. Stimulation of MDA-MB-231BO cells with the CD44 ligand hyaluronan (HA) induced an increase in the expression of the $\beta_1$-integrin chain, FAK and paxillin; and, furthermore, promoted a rapid increase in the activation status of the $\beta_1$-integrin subunits, Src, cortactin and paxillin in these cells. The HA-induced phosphorylation of paxillin was attenuated by depletion of CD44 and cortactin expression using selective RNAi strategies, suggesting that it is a downstream target of HA-CD44-cortactin signaling. MDA-MB-231BO cell adhesion to fibronectin or to hBMECs was attenuated by RNAi-mediated suppression of CD44, cortactin and paxillin expression or following administration of two neutralizing antibodies that inhibit $\beta_1$-integrin and $\alpha_4\beta_1$-integrin receptor signaling. Antibody-based inhibition of integrin signaling also attenuated the HA-induced phosphorylation of cortactin and paxillin, suggesting that these proteins constitute a signaling cascade activated downstream of a CD44-initiated, integrin-dependent process.
**Conclusion** Our results describe a molecular pathway promoting cytoskeletal reorganization that is activated downstream of a CD44-induced, integrin-dependent event and that is critical to efficient breast cancer cell adhesion to hBMECs.

## P37

## Identification of genes involved in the formation of lymph node metastasis from human tumour xenograft models of breast cancer

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**Background** Lymph node metastasis is associated with considerable morbidity and is linked to poor prognosis in breast cancer. We have developed experimental models of lymphatic metastasis from the human breast carcinoma cell lines GI 101a and MDA-MB-435. Several sublines of cells derived from lymph node metastases *in vivo* have been developed. When injected into mammary fat pads (MFP) of athymic mice, all cell lines produced spontaneous lymph node metastases. These cell lines also generated lymph node metastases (in addition to the expected lung metastases) when injected intravenously. In the latter, the tumour cells need to traverse the pulmonary capillary bed and either show tropism for, or adaptation to, the lymph node environment. These distinct patterns of spread – due respectively to direct (intralymphatic) and indirect (haematogenous) colonisation of nodes – will enable us to explore determinants of both putative passive and active (nodal tropism) mechanisms independently.
**Methods** RNA was extracted from frozen primary tumours and lymph node metastases derived from the different cell lines, after MFP or intravenous injection, and was used to generate gene expression profiles. A supervised learning method from the BRB ArrayTools 3.5.0 software was used to identify the genes that were differentially expressed between the lymph node metastases obtained from the two routes of dissemination, as well as between matched primary tumours and their lymph node metastases.
**Results** Microarray results indicate that it is possible to distinguish between the lymph node metastases and matched primary tumours. Additionally, the nodal metastases derived from the MFP primary site segregate from those derived from the peripheral circulation. These samples cluster together irrespective of the cell line of origin. We have now identified genes upregulated and downregulated in each cluster, and are validating their expression at the protein level.
**Conclusion** The presented results will provide more information about the molecules involved in the generation of lymph node metastases. Furthermore, the identification of genes differentially expressed between metastases originating from MFP and intravenously suggests that at some level distinct molecular mechanisms may be in operation in active and passive modes of dissemination.
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## P38

## Matrix metalloproteinase-8 is a regulator of the clinical aggressiveness of mammary tumours

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Protease genes are involved in multiple steps of cancer progression, including cell growth, migration and angiogenesis. These genes are valuable as prognostic and/or diagnostic markers of disease and are potential therapeutic targets.
We used TaqMan® real-time quantitative PCR to conduct the first detailed quantitative expression profiling of the entire family of metalloprotease and serine protease genes and their inhibitors in breast cancer (over 380 genes). Using a bank of 60 samples (50 cancer and 10 normal mammary tissue) collected at the Norfolk and Norwich University Hospital [1] we have identified a number of genes that show significant disregulation in tumour samples compared with normal breast tissue. Expression correlates either positively or negatively with tumour grade in many genes.
A further cohort of 229 Dutch patients with more extensive clinical history [2] was profiled in a subset of the metalloproteinase genes. Among the genes that showed significant aberrant expression, Matrix metalloproteinase-8 (MMP8) emerged as a candidate to play a protective role during tumour progression. MMP8 was found to have significant prognostic value and was strongly correlated with prolonged survival. MMP8 is prognostic as a continuous variable for relapse-free survival (hazard ratio = 0.76, $P = 0.045$) and for overall survival (hazard ratio = 0.69, $P = 0.025$). Expression of MMP8 also correlated with lymph node involvement, reduced expression equating to greater nodal spread ($P = 0.001$). Expression of MMP8 was independent of tumour grade. These data show that MMP8 is prognostic in breast cancer, and suggest that the function of MMP8 antagonizes metastasis.
**Acknowledgement** Supported by Breast Cancer Campaign.
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## P39

## Actions of IGF-I are differentially regulated by fatty acids in normal and breast cancer epithelial cells

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**Introduction** Obesity will soon be the leading preventable risk factor for many cancers. The insulin-like growth factors (IGFs) have been strongly implicated as important risk factors for many epithelial cancers, including breast cancer, and for mediating the link between nutrition and these cancers. Obesity-related increases in circulating fatty acids cause insulin resistance with consequent morbidity but, despite the considerable overlap between insulin and IGFs, there have been no studies of the effects of fatty acids on IGF activity.
**Objective** To examine the effects of the most abundant circulating fatty acids (oleate – unsaturated; palmitate – saturated) alone and in combination with IGF-I on MCF-10A nonmalignant breast and MCF-7 breast cancer epithelial cells.
**Methods** Following 24 hours in serum-free media, cells were exposed to albumin-bound fatty acids (100 to 400 μM) for 48 hours with or without IGF-I (20 to 25 ng/ml). Cell growth and death were assessed by cell counting and the trypan blue dye exclusion assay, respectively. Data were analysed by ANOVA.
**Results** For MCF10-A and MCF-7 cells, IGF-I increased cell growth ($P < 0.01$ and $P < 0.001$) whereas oleate (100 to 400 μM) alone had no effect. However, IGF-induced growth was differentially affected in combination with oleate: being enhanced in the MCF-10A cells (by 57% at 400 μM; $P < 0.001$) but inhibited in the cancer cells (by 28% at 400 μM; $P < 0.05$).
For both cell lines, palmitate alone only inhibited growth at the highest dose (400 μM), which was coincident with the induction of apoptosis. Palmitate did not affect IGF-induced proliferation in either cell line. The cells were differentially sensitive to palmitate-induced death (at 400 μM a 1.5-fold increase of MCF-7 cells; an eightfold increase of MCF-10A cells). Palmitate-induced death in MCF10-A cells was inhibited by a ceramide synthase inhibitor, fumonisin B1 (0.1 μM) (61%), and by oleate (96% at 400 μM) but was unaffected in the presence of IGF-I.
We are currently investigating the signalling pathways underlying the differential effects of oleate on IGF-induced growth of MCF-10A and MCF-7 cells.
**Conclusion** Palmitate had no effect on IGF-induced cell growth, whereas oleate enhanced that of normal cells but inhibited that of cancer cells. Unlike oleate, palmitate induced apoptosis although cancer cells were relatively resistant to this. This apoptosis was via ceramide production and was inhibited by oleate but not IGF-I. Saturated and unsaturated fatty acids have differential effects on IGF-induced growth and the survival of human breast epithelial cells, supporting the notion that nutrition is a major environmental influence on breast cancer progression.
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## P40

## Insulin-like growth factor binding protein 3 modulates epidermal growth factor (EGF)-induced growth of breast epithelial cells by altering EGF receptor internalization

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**Introduction** Insulin-like growth factor binding protein 3 (IGFBP-3) is the most abundant insulin-like growth factor binding protein in human serum and is able to modulate cell proliferation independently of its ability to bind insulin-like growth factor. Tumour-associated increases in IGFBP-3 levels relate to upregulation of epidermal growth factor receptor (EGFR) and HER-2 with increasing oestrogen independence. Remodelling of the extracellular matrix with increased fibronectin expression in poor prognostic tumours further enhances EGFR levels and signalling.
**Objective** To explore the potential interactions of IGFBP-3 with the EGFR/HER-2 pathways.
**Methods** Normal breast epithelial cells (MCF-10A) and breast cancer cells (T47D) were dosed with EGF (5 ng/ml and 10 ng/ml), IGFBP-3 (100 ng/ml), an EGFR/HER-2 tyrosine kinase inhibitor, (Iressa, 0.25 μm) and a ROCK inhibitor (Y-27632, 5 μM) either alone or in combinations on either plastic, laminin or fibronectin (0.25 μg/ml). Cell growth was evaluated by cell counting and tritiated thymidine incorporation. Internalisation of the EGFR and

HER-2 was assessed by biotinylation and affinity purification using a Pin Point Cell Surface Isolation Kit (Pierce, Northumberland, UK) on whole cell lysates followed by western immunoblotting for the EGFR and HER-2. Statistical significance was determined using ANOVA.

**Results** On plastic and laminin with MCF10A cells, EGF and IGFBP-3 each increased cell proliferation alone (by 55.2%, $P < 0.001$ and 31.7%, $P < 0.01$, respectively), and together there was a synergistic increase of 278% ($P < 0.001$). In addition, the proliferative effect of IGFBP-3 alone, like that of EGF, was completely abrogated in the presence of Iressa. With T47D cells, EGF increased cell proliferation (by 33.9%, $P < 0.001$), IGFBP-3 alone had no effect, but in combination, in contrast to the normal cells, IGFBP-3 completely blocked EGF-induced growth ($P > 0.01$). These actions of IGFBP-3 on EGF-induced growth were reversed when the cells were cultured on fibronectin. Furthermore, we found that the modulation of EGF-induced proliferation by IGFBP-3 was not mediated by changes in the phosphorylation status of EGFR or HER-2. It was, however, associated with modulation of the internalisation of the EGFR and activation of Rho.

**Conclusion** We found that IGFBP-3 had differential, matrix-dependent effects on EGF-mediated proliferation in normal and breast cancer cells, which was achieved through modulation of EGFR internalisation and the activation of Rho. Breast tumour levels of IGFBP-3 may determine their dependence on EGFR/HER-2 activity and their response to therapies targeting these receptors.

## P41

## Anti-oestrogen therapy switches off tumour suppressors and proapoptotic genes in breast cancer and reveals a new therapeutic opportunity

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**Background** Previous studies in the Tenovus Centre have demonstrated that the development of antioestrogen resistance *in vitro* is accompanied by unfavourable changes in the breast cancer phenotype leading to increase tumour cell growth rate. Here evidence is presented to suggest that this is in part due to antihormones causing the epigenetic silencing of oestrogen-induced genes involved in the negative regulation of cell growth. Importantly, we show that reversal of this process using the demethylation agent 5-azacytidine (5AZA) allows oestrogen-induced cell kill by a previously unrecognised mechanism.

**Methods** The breast cancer cell lines used in this study were MCF7, MCF7-derived tamoxifen-resistant variant (TamR) and TamR sublines that had been withdrawn from tamoxifen (TamRwd) for up to 6 months. Cells were challenged by oestradiol (E2), antihormones and 5AZA. Cell growth responses were assessed by anchorage-dependent growth assays and alterations in expression/activity of oestrogen receptor (ER) and ER-regulated genes were analysed by real-time PCR, western blotting and/or immunocytochemistry.

**Results** Compared with the parental MCF7 cells, TamR cells showed a significant upregulated basal rate of growth that was maintained on tamoxifen withdrawal for 6 months. Following the tamoxifen withdrawal, the cells remained ER-positive and showed a slight growth response to E2. In contrast, they showed no growth inhibitory response to tamoxifen. Examination of the methylation status of the promoters of two classically ER-regulated genes switched off in TamR and TamRwd cells, pS2 and progesterone receptor (PR), confirmed their increased methylation and that 5AZA was able to reverse this process, allowing the re-expression of pS2 and PR on E2 treatment. Although pS2 and PR are not thought to play a role in the regulation of cell growth, these data provide proof of principal that gene silencing occurs in TamR cells and that it can be reinstated by 5AZA plus E2. To determine whether tamoxifen was capable of inducing the methylation of ER-regulated genes involved in cell growth, TamRwd cells pretreated with 5AZA were subject to an E2 dose–response challenge. In contrast to TamRwd cells treated with E2, which promoted a growth response, E2 in combination with 5AZA was strongly inhibitory at physiological doses of the steroid ($10^{-9}$ M), with this action being reversed by tamoxifen. An Affymetrix analysis of the TamR cells has revealed multiple E2-regulated genes that are switched off in the resistant cells whose ontology indicates tumour suppressor/proapoptotic functions.

**Conclusion** Our data suggest that antihormone resistance may be associated with the epigenetic silencing of growth inhibitory genes leading to enhanced growth rates. We propose that reinstatement of the expression of such genes using demethylation agents in combination with E2 may provide a previously unrecognised therapeutic opportunity in breast cancer.

## P42

## Zinc transporter HKE4 as a new target in antihormone resistance of breast cancer

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**Background** Oestrogen receptor-positive breast cancers develop resistance to anti-oestrogens by utilising alternative growth factor pathways as observed in our tamoxifen-resistant cell line (TAMR). These include EGFR, IGF1-R and Src signalling as well as increased growth and invasion. Zinc is elevated in breast cancer tissue and has been demonstrated to activate certain growth factor signalling pathways. We have tested the expression level of members of the LIV-1 family of zinc influx transporters and discovered that HKE4 (SLC39A7, ZIP7), previously shown by us capable of increasing the intracellular zinc levels, has increased expression in TAMR. We have therefore investigated whether the development of the more aggressive phenotype observed in our TAMR cells, including activation of these signalling pathways as well as increased growth and invasion, is due to an increase of intracellular zinc and as a direct result of increased expression of HKE4.

**Methods** All nine members of the LIV-1 subfamily of ZIP transporters were measured in our model of tamoxifen-resistant breast cancer using Affymetrix arrays. Zinc-induced activation of growth factor signalling pathway components was investigated by western blot and/or fluorescent microscopy. Short-term (15-min) treatments with 20 μM zinc included ionophore, whereas long-term (hours/days) did not. Recombinant LIV-1 family members with a V5 tag were expressed using pcDNA3.1/V5-His-TOPO vector, and siRNA (Dharmacon smartpools with relevant controls) was used to reduce endogenous expression.

**Results** HKE4 (SLC39A7), a ZIP transporter from the LIV-1 subfamily, was discovered to be elevated in TAMR cells by Affymetrix analysis and confirmed by PCR and western blot. We have observed that our TAMR cells have a twofold increase in intracellular zinc compared with wild-type cells, using the zinc-

specific fluorescent dye Newport Green. Short-term zinc treatment of TAMR cells activates the signalling pathways implicated in antihormone-resistant proliferation and is reduced by both the zinc chelator TPEN and the Src kinase inhibitor SU6556. The same effects are observed after longer term (6 days) zinc treatment with additional increases in cell growth and invasion through Matrigel. Since we have previously demonstrated that HKE4 is capable of increasing intracellular zinc in cells and, more recently, that these TAMR have elevated intracellular zinc levels, we have tested the hypothesis that elevated HKE4 expression is directly responsible for the aggressive phenotype observed in our TAMR cells. Reducing HKE4 levels by siRNA demonstrated a role for this molecule in driving the zinc-induced activation of multiple signalling pathways. In the presence of siRNA for HKE4, the previously observed zinc-induced activation of EGFR, Src, and IGF1-R was eradicated and the EGF-stimulated activation was also decreased. Additionally, we have demonstrated the converse by transfecting recombinant HKE4 into wild-type cells and/or treating them with zinc to observe the activation of these signalling pathways and increases in invasive capability. Interestingly, we have observed a similar role of HKE4 in our model of faslodex-resistant breast cancer.

**Conclusion** The presented results propose that HKE4, a member of the LIV-1 subfamily of ZIP transporters, is directly involved in the activation of the aggressive phenotype observed with the development of antihormone resistance, and as such is a potential new target for the prevention of resistance to antihormones in breast cancer progression.

## P43

## Stromal fibroblasts with nuclear β-catenin are present within breast tumours and increase proliferation and invasion of epithelial breast cancer cells

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**Background** β-catenin, when located within the nucleus, acts as an oncoprotein by activating TCF/LEF transcription factors, which in turn regulate transcription of a wide range of growth and proliferation-associated genes. Nuclear β-catenin is frequently seen in epithelial cancer cells as a result of either inappropriate *Wnt* signalling or inactivating mutations in genes for key β-catenin regulators or for β-catenin itself. Breast tumours are unusual, however, in that nuclear β-catenin is relatively rare in epithelial breast cancer cells. On the other hand, nuclear β-catenin expression has been documented in fibroblasts within breast fibroadenomas and benign phyllodes tumours [1]. Preliminary observations within our laboratory indicated that stromal fibroblasts in and around breast carcinomas also frequently express nuclear β-catenin. Our aim in the present work was to validate this observation, and to determine how fibroblasts with nuclear β-catenin might influence cancer behaviour.

**Methods** We performed immunohistochemistry for β-catenin on whole sections of breast cancers from 200 individual cases. A scoring system based on the number of fibroblasts expressing nuclear β-catenin was devised and fibroblasts around tumour and normal breast tissue were scored. To examine the potential influence of nuclear β-catenin-positive fibroblasts on breast tumour behaviour, we have developed a tissue culture model. With appropriate controls, fibroblasts (MRC5/immortalised primary breast fibroblasts) were transfected to overexpress β-catenin and the influence of these cells on breast cancer cells (MCF7/MDA-MB-231) *in vitro* was determined. First, proliferation rates of breast cancer cells treated with conditioned media from transfected fibroblasts were determined (MTT assays). Secondly, invasion assays were carried out in transwell plates; fibroblasts were β-catenin or control transfected in lower chambers and breast cancer cells were seeded into upper chambers onto membranes coated with extracellular matrix (Matrigel/ECMatrix). Epithelial cells invading through membranes were quantified (cell counting/fluorometric assay using CyQuant GR dye).

**Results and conclusion** We found that fibroblasts expressing nuclear β-catenin are frequent in and around breast tumours, while they are very rare around normal breast. In our tissue culture model, β-catenin-transfected fibroblasts stimulated both proliferation and invasion of breast cancer cells. In conclusion, nuclear β-catenin within stromal fibroblasts may have a potent influence on breast cancer behaviour. This influence further highlights the importance of stromal–epithelial interactions in breast carcinogenesis.

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## P44

## Proapoptotic protein Bid is regulated by phosphorylation during anoikis and the cell cycle

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Adhesion to the extracellular matrix is fundamental in cell survival, proliferation and differentiation. In the absence of correct signals from the extracellular matrix, normal cells die by a form of apoptosis termed anoikis. These mechanisms are abrogated in invasive breast cancer. The BH3-only protein Bid is involved in anoikis. The full-length protein translocates to the mitochondria following cell detachment from the matrix and this is not dependent on Bid cleavage by caspase 8. Understanding the regulation of full-length Bid during apoptosis will help identify ways to manipulate the apoptotic machinery to prevent cell survival during metastasis. Bid can be phosphorylated following a number of stimuli. We have shown that Bid becomes dephosphorylated in epithelial cells undergoing anoikis. We have also identified Bid as being phosphorylated during normal cell cycle. Arresting the cell cycle at $G_1$/S results in accumulation of nonphosphorylated Bid. Conversely, arresting cells during mitosis results in an increase in the phosphorylated form of Bid. Mutant forms of the protein in which potential phosphorylation sites were removed were used to identify serine 66 as a critical site of phosphorylation. Inhibition of the cell cycle kinase cdk1 in fibroblasts blocks Bid phosphorylation and is therefore a potential regulatory kinase acting to control Bid during the cell cycle. Our results indicate a novel site of regulation in Bid at serine 66. This residue is phosphorylated during mitosis and may act to control the sensitivity to apoptosis during this part of the cell cycle.

## P45

### Breast cancer and environmental risk factors: an appraisal of the scientific evidence

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With a few exceptions, the number of new breast cancer cases among women is increasing in almost all western countries. Although lifestyle, life choices, genetics and the diet are shown to contribute to the increase in breast cancer, the sheer number of newly diagnosed cases cannot solely be explained by these factors. The present review aims to evaluate evidence that environmental factors, including chemical exposure, also play a role.

Studies among identical twins have shown that the most important contributor to the causation of breast cancer is the environment not shared by the pair, even under circumstances where the genetic predisposition is very similar. Similarly, in families with a heritable predisposition to breast cancer, time of birth, physical activity and obesity can profoundly influence risk.

There is overwhelming evidence that oestrogens are strong determinants of breast cancer risks. This is not limited to natural oestrogens formed in a woman's body, but extends to synthetic hormones used as pharmaceuticals, such as those used for the alleviation of menopausal symptoms. The demonstration of breast cancer risks from oestrogen-only and, more pronounced, from combined oestrogen–progesterone regimens is a case in point. Very recent decreases in breast cancer incidence in the USA and in parts of Germany could even be linked to a dropping off of hormone therapy use.

To date, studies carried out to examine whether certain environmental chemicals are implicated in breast cancer could neither prove nor rule out a possible link. But to avoid wrongly dismissing a role for chemicals in breast cancer, two issues must be addressed. First, the available studies have largely focused on single chemicals and have ignored the possibility that large numbers of agents may act in concert. Recent evidence from Spain strongly suggests that cumulative exposure to oestrogenic chemicals is associated with breast cancer risks. Second, instead of looking at exposures later in a woman's life, when the breast tissue is less vulnerable, critical periods of vulnerability during puberty and development in the womb must be considered. Very recent studies demonstrating breast cancer risks from exposure to the pesticide DDT during puberty and from exposure to the oestrogenic anti-miscarriage drug DES further underline the importance of chemical exposure in breast cancer.

Taken together, there is a case for abandoning the view of breast cancer as solely a lifestyle and genetic disease. It is necessary to take account of the role of environmental factors, especially chemical exposures. With UK breast cancer incidence at an all time high, risk reduction will not be achievable without considering preventable causes, such as exposure to chemicals.

## P46

### Assessment of angiogenesis in the hyperplasia preinvasive, invasive breast carcinoma sequence

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**Background** Tissue factor (TF), the primary initiator of coagulation, has been shown to stimulate angiogenesis, which is crucial for the development and metastasis of solid tumours, in part by upregulating vascular endothelial growth factor A (VEGF). Angiogenesis in invasive breast cancer is well documented, but little is known about the role of angiogenesis in premalignant breast disease, or when the angiogenic switch occurs during the development of breast malignancy. This study therefore quantifies angiogenesis, VEGF and TF in the hyperplasia, preinvasive, invasive breast carcinoma sequence.

**Method** One hundred and eighty-seven serial sections of normal human breast ($n = 12$), benign hyperplastic breast (usual ductal hyperplasia; $n = 35$), premalignant hyperplastic breast (atypical ductal hyperplasia; $n = 31$), preinvasive cancer (ductal carcinoma *in situ*; low/intermediate grade, $n = 23$; high grade, $n = 43$) and invasive breast cancer specimens ($n = 43$) were immunohistochemically stained for CD31 (pan endothelial cell (EC) marker), endoglin (proliferating EC marker), VEGF and TF. The microvessel density (MVD), a surrogate marker for angiogenesis, was quantified using Chalkley grid analysis. VEGF staining was assessed semi-quantitatively and TF expression was graded as present or absent.

**Results** CD31 staining was observed in ECs in all of the breast specimens observed. There was a significant increase in MVD between normal and hyperplastic/preinvasive breast cancer tissue ($P < 0.005$) and between preinvasive and invasive carcinomas ($P < 0.0005$), which was associated with a significant increase in VEGF expression in breast epithelial ($P < 0.0005$) and tumour cells, respectively ($P < 0.0005$). The significant increase in MVD observed between preinvasive and invasive cancers was also associated with a significant increase in TF expression in invasive tumour cells ($P < 0.0005$). In contrast to CD31 staining, endoglin was not expressed in normal breast, but was expressed by ECs in 11% of usual ductal hyperplasia cases, 13% of atypical ductal hyperplasia cases, 17% and 26% of ductal carcinoma *in situ* cases (low/intermediate grade and high grade, respectively) and 81% of invasive breast cancer specimens. A significant increase in the number of proliferating ECs was seen in invasive cancers compared with all the classes of breast tissue examined ($P < 0.0005$). Moreover, the significant increase in proliferating ECs seen between preinvasive and invasive carcinomas was associated with a significant increase in VEGF and TF expression in invasive tumour cells ($P < 0.0005$). There was evidence for a close association between VEGF and TF in tumour cells of invasive cancers ($P = 0.007$) and between VEGF and TF in ECs ($P < 0.0005$), suggesting a role for both in angiogenesis.

**Conclusion** These data indicate that angiogenesis is initiated at the earliest stages of dysplasia and increases rapidly between preinvasive and invasive cancer. VEGF and TF expression patterns suggest these factors play a role in this process.

## P47

### Altered myoepithelial cell expression and function in cancer-containing breasts

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We have previously identified that the noninvolved tissue from breasts that contains a cancer (NTCCB) differs from age-matched normal breast from women without cancer, having lower apoptotic indices [1] and altered expression of epidermal growth factor receptor [2] and $\beta_4$ integrin [3]. Both of the latter are proteins expressed by myoepithelial cells. The myoepithelial cell is now recognised as being important in the regulation of growth, apoptosis and differentiation of luminal epithelial cells. Our hypothesis is that altered myoepithelial cell function could lead to reduced apoptosis and therefore a lower ability of the breast to remove cells with DNA damage, predisposing to cancer development.

To investigate the expression of myoepithelial proteins, immunohistochemistry was used to examine two series of NTCCB and equal numbers of age-matched normal breast controls, with a total of 180 tissues assessed. FGF2, IGF1, oestrogen receptor beta, p63, 14-3-3σ, glucocorticoid receptor and maspin were investigated. There was a significant difference in the expression of FGF2 ($P = 0.02$), with NTCCB having greater staining in both series of tissues. p63 was significantly different ($P = 0.008$) in one series but not the other. None of the other proteins showed a significant difference between the NTCCB and controls.

Myoepithelial cells are isolated from reduction mammoplasties and noninvolved tissue from mastectomies using positive selection [4]. Purity and changes in expression with passage have been checked by RT-PCR and immunocytochemistry for myoepithelial and luminal markers. Expression of FGF2 is being examined by quantitative PCR and western blotting. A limited study of the effects of conditioned media from myoepithelial cells from NTCCB and controls on breast cancer cell line growth and apoptosis has shown reduced induction of apoptosis by NTCCB, and these studies are being extended.

Myoepithelial cells from cancer-containing breast are different, particularly in expression of FGF2, which is being investigated further.

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## P48

### Association of gene variants in the TGF-beta signalling pathways with invasive breast cancer risk

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Many studies of normal cells *in vitro*, of transgenic mouse models and of somatic mutations in human cancers have provided evidence that the cytokine transforming growth factor beta (TGFβ) acts as a suppressor of primary tumour initiation. However, studies of transformed cells *in vitro* and of mouse models have also implicated TGFβ as a promoter of the later stages of tumour development. A hypothesis that has been proposed to account for this dual action is that TGFβ acts as a tumour suppressor through the ubiquitous ALK5 receptor signalling via SMAD2 and SMAD3 to inhibit proliferation of primary tumour cells, but acts subsequently through the endothelial-specific ALK1 receptor via SMAD1 and SMAD5 to promote angiogenesis, which is required for tumour progression [1].

In a recent meta-analysis we showed that a single nucleotide polymorphism (SNP) generating a leucine to proline substitution in the signal peptide of the TGFB1 protein is associated with an increase in the risk of invasive breast cancer (OR per additional proline allele = 1.08 (95% CI = 1.04 to 1.11), $P_{trend} = 2.8 \times 10^{-5}$) [2]. We have also reported that this SNP increases the amount of TGFB1 protein secreted *in vitro* by threefold [3]. These data suggest that higher levels of TGFB1 may promote the invasive breast tumour phenotype. To determine the effect of host TGFB1 levels in an *in vivo* model, *Tgfb1*$^{+/-}$ *and Tgfb1*$^{+/+}$ mice have been compared in which the mice carry one or two *TGFB1* alleles with the ancestral SNP form encoding proline in the signal peptide. These studies have revealed major effects of TGFB1 in controlling the site of metastatic seeding and the number of metastases that develop.

The *TGFB1* SNP association with breast cancer suggested that other genes in the TGFβ signalling pathways might be associated with altered risk. We have conducted association studies with SNPs in 16 further genes encoding proteins directly implicated in TGFβ signalling. *LTBP1*, *LTBP2*, *LTBP4*, *TGFB1*, *TGFB2*, *TGFB3*, *ALK1*, *ALK5*, *TGFBR2*, *Endoglin*, *SMAD1*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD5*, *SMAD6* and *SMAD7* were analysed. A comprehensive SNP tagging approach was used to select variants for genotyping in a staged study design using up to 6,900 cases and 6,900 controls, all collected from the East Anglia region of the UK (>98% of northwestern European ancestry). From 1,254 common SNPs (minor allele frequency >0.05) in these genes identified from the International HapMap project data, we defined and genotyped 354 tagging SNPs in the East Anglia cases and controls. Statistically significant associations were followed up by genotyping in a Polish set of 2,215 cases and 2,374 controls. Meta-analysis of these results identified associations with cancer susceptibility of a variant in *ALK5* (OR per additional rare G allele = 0.88 (95% CI = 0.81 to 0.95), $P_{trend} = 0.001$) and in TGFBR2 (OR per additional rare G allele = 0.96 (95% CI = 0.92 to 1.00), $P_{trend} = 0.039$). Data from two genome-wide studies have been examined to search further for associations in these genes. The haplotype risks and interactions between two or more loci have been investigated and survival analyses have been conducted.

From this comprehensive study we have identified tagging SNPs in two TGFβ receptor genes that are significantly associated with risk of invasive breast cancer. Identification of the causative SNPs

within the LD blocks tagged by the two SNPs and their effects on signalling via the ALK1 and ALK5 pathways remain to be determined.

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## P49

### From association to cause: fine mapping of the *TNRC9* gene region, a novel susceptibility locus identified in the first genome-wide association study for breast cancer

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We identified five novel breast cancer susceptibility loci in a recent Genome Wide Association Study [1] using 227,876 single nucleotide polymorphisms (SNPs) and up to 50,000 female breast cancer cases and controls from 22 studies.

One of these loci, tagged by SNP rs3803662, lies in a large linkage disequilibrium (LD) block on chromosome 16q12. This region encompasses the largely uncharacterised *TNRC9* gene (also known as *TOX3/CAGF9*) as well as a hypothetical gene *LOC643714*. This association is robust and has recently been independently verified [2]. The aim of the present study is to map the associated locus and ultimately identify the causal variant(s) responsible for the increased risk of breast cancer.

There are 101 common variants in the 165 kb LD block covering the entire footprints of both genes catalogued by the International HapMap Project. These are efficiently tagged by 19 tagging SNPs (tagSNPs) that were genotyped in 2,270 breast cancer cases and 2,280 controls from the East Anglian region of the UK. Using these, we were able to exclude the coding region of *TNRC9* and reduce the associated region to a 133 kb LD block including both the 5′ end of the *TNRC9* gene and the 3′ end of *LOC643714*.

This block was re-sequenced in 45 European subjects. Three hundred and forty-four SNPS were found, of which 170 were not previously recorded and 175 were common (minor allele frequency >0.05). Twenty-two of these SNPs are strongly correlated ($r^2 > 0.9$) with the best tagSNP and have been genotyped, where possible, in an East Anglian case–control study set of increased size. Thus there are 23 potential causative variants, which are distributed across both genes.

In an attempt to reduce this set of candidate causative SNPs and to further narrow the region of interest, they are being genotyped in breast cancer case–control sets from Asian and African-American populations. These populations exhibit greater haplotype diversity than the more closely related East Anglians, thus providing greater power to separate the causative variant(s) from the other candidates.

To complement this work, a further study to determine the functional properties of the gene region in human breast cells has been initiated.

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## P50

### Cytochrome P450 modulates the therapeutic actions of tamoxifen, as evidenced in novel breast cancer models

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*Breast Cancer Res* 2008, **10(Suppl 2):**P50 (doi: 10.1186/bcr 1934)

Regulation of cytochrome P450s in breast cancer and their role for tumour growth and anticancer chemotherapy are studied. Mammary cancer can develop for many reasons, one of which is the exposure to environmental carcinogens and/or steroid hormones. The cytochrome P450 enzyme family not only catalyses the metabolism of a wide range of carcinogens but is also involved in metabolism of steroids. This process alters their steroidogenic properties, a mechanism important for mammary carcinogenesis.

At the centre of this research stand cytochrome P450 1B1 (CYP1B1) and cytochrome P450 CYP1A1. Unlike many other P450s, these isoforms are expressed extrahepatically. CYP1B1 protein is found to be overexpressed in tumours compared with the corresponding healthy tissues. Special regulatory mechanisms are likely to cause this difference.

In the present study we employed TaqMan analysis, immunoblotting and reporter assays to investigate the expression patterns of CYP1B1 and CYP1A1 in a panel of breast cancer cell lines derived from different stages of mammary carcinomas. Furthermore, we investigated the expression of these P450s in cell lines derived from primary human mammary epithelial cells that have been transfected with various combinations of oncogenes and telomerase. In the transformed human mammary epithelial cells we found that the expression of CYP1B1, CYP1A1 and their inducibility by TCDD was differentially affected by the different oncogenes. Presently, we investigate the regulatory mechanisms that cause this response.

In a second investigation, we analysed the relevance of P450 expression for mammary-tumour development and tumour therapy. For this purpose we have developed MCF-7-derived cell lines in which the expression of CYP1A1 and CYP1B1 could be switched on/off by treatment with low doses of doxicycline. We demonstrated that expression of these P450s altered the effects of estrogens and antiestrogens on cell cycle and apoptotic markers. The MCF-7-derived cell lines were grown in xenografts. P450 expression was induced by doxicycline in the drinking water. We were able to demonstrate that P450 expression in our xenograft-model was tightly regulated by tetracycline. In future, animals will be treated with or without tamoxifen. Subsequently, the effects of P450 expression on tumour growth, angiogenesis and apoptosis will be measured.

It is anticipated that the results of these investigations will greatly enhance our understanding about the aetiology of breast cancer and may provide strategies to improve treatment.

## P51

### Multicentre study of CASP8 polymorphisms in breast cancer

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*Breast Cancer Res* 2008, **10(Suppl 2):**P51 (doi: 10.1186/bcr 1935)

One approach to improve our understanding of the aetiology of breast cancer is to identify the genes involved in inherited susceptibility. These range from the rare high-penetrance mutations of the *BRCA1* and *BRCA2* genes to common low-penetrance variants, which are just beginning to be identified by means of whole-genome and candidate gene association studies. Owing to their small effect, these common variants are difficult to identify, requiring studies with large sample sizes. The Breast Cancer Association Consortium recently identified a single nucleotide polymorphism (SNP) in the *CASP8* gene that is associated with a reduction in risk of breast cancer (rs1045485; D302H; $P_{trend} = 1.1 \times 10^{-7}$) in a large multicentre cohort [1].

To further investigate the association between the *CASP8* gene and breast cancer, we genotyped 15 haplotype-tagging SNPs across a 60 kb region spanning *CASP8* in 1,200 cases and 1,200 controls from Sheffield. Two further SNPs demonstrated a significant association with breast cancer; rs6435074 ($P_{trend} = 0.042$) and rs6723097 ($P_{trend} = 0.024$). These markers were therefore genotyped in two additional case–control cohorts based in Utah and Germany. The rs6723097 SNP displayed the strongest association with odds ratios of 1.15 (95% CI = 1.02 to 1.29) and 1.35 (95% CI = 1.15 to 1.59), for the heterozygous and rare homozygous genotypes respectively, compared with the common homozygous genotype ($P_{trend} = 0.0002$).

At present there is no functional explanation for the observed associations. Therefore it is possible that the associated SNPs are in linkage disequilibrium with other causative variants; haplotype analysis and further sequencing of the region will be needed to identify these.

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## P52

### Abnormal expression of p53 isoforms can be associated with poor survival in primary breast tumours

**J-C Bourdon**

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Abstract not available at time of publication.

## P53

### The effect of intermittent versus chronic energy restriction on breast cancer risk biomarkers in premenopausal women: a randomised pilot trial

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*Breast Cancer Res* 2008, **10(Suppl 2):**P53 (doi: 10.1186/bcr 1937)

**Background** Postmenopausal breast cancer risk increases twofold in women who gain significant amounts of weight [1] and there is evidence that energy restriction may reduce risk [2]. Animal studies indicate that intermittent energy restriction (IER) reduces risk and may be superior to continuous energy restriction (CER) [3]. We have shown that CER reduces breast cancer risk biomarkers in women but is hard to maintain. We hypothesise that IER may be superior to CER in reducing biomarkers of breast cancer risk and may also be more acceptable to women.

**Methods** One hundred and eight premenopausal women, mean age 40.0 years (SD = 4.0), mean adult weight gain 20.1 kg (SD = 11.0), were randomised to either CER (75% estimated energy requirements: ~1,500 kcal 7 days/week) or IER (75% estimated energy requirements: 650 kcal for 2 days and ~1,800 kcal 5 days/week) over 6 months. The study endpoints are weight and body composition (waist/hip circumference, fat free and total fat mass by bioelectrical impedence), measures of insulin sensitivity (HOMA, SHBG, testosterone), potential breast cancer growth factors (IGF axis, leptin adiponectin), inflammatory markers (C-reactive protein and sialic acid) and oxidative stress markers (serum isoprostane). The relative acceptability of IER and CER will be assessed using a quality of life questionnaire (RAND SF-36) and scales of behaviour change and adherence.

**Results** Nineteen participants (17.6%) have withdrawn from the study (IER = 12, CER = 7; main reasons: stress = 4, pregnancy = 3, change in employment = 3, could not stick to diet = 3). Baseline to 6-month results for weight and body composition are reported in Table 1.

**Table 1 (abstract P53)**

**Six-month results**

| | IER ($n = 42$) | CER ($n = 47$) | $P$ value |
|---|---|---|---|
| Baseline weight (kg) | 81.5 (1.97) | 84.4 (2.35) | 0.39 |
| Weight loss (kg) | 7.8 (0.77) | 6.4 (0.64) | 0.18 |
| Percentage weight loss | 9.4 (0.85) | 7.5 (0.69) | 0.09 |
| Fat loss (kg) | 6.0 (0.68) | 4.9 (0.56) | 0.24 |
| Waist decrease (cm) | 7.6 (0.82) | 5.7 (0.54) | 0.05 |
| Percentage of weight lost that is fat[a] | 75.6 (68.7 to 87.5) | 79.1 (60.4 to 95.6) | 0.69 |

Data presented as mean (SE), $P$ values for $t$ test; or as [a]median (interquartile range), $P$ value for Mann–Whitney test.

**Conclusion** Significant decreases in weight, fat and waist occurred in both groups over 6 months, with the IER group doing slightly better. Greater proportions of the IER group achieved 5% weight loss (IER 79% cf. CER 66%, $P = 0.19$) and 10% weight loss (IER 43% cf. CER 28%, $P = 0.13$). We await results for

biochemistry and relative acceptability, which will be presented at the meeting.

**Acknowledgement** Study funded by Breast Cancer Campaign, World Cancer Research Fund and Genesis.

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## P54

### Living with genetic risk of breast cancer: what have we learned?

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Since genetic testing became a possibility for breast cancer predisposition in the mid-1990s, research attention has focused on the impact of predictive genetic testing for people who are told they are at significantly increased risk of developing breast cancer. The present study will review research evidence for the impact of testing in terms of distress experienced and risk management strategies adopted to manage risk of developing breast cancer [1,2]. In addition to the psychosocial implications and impact on risk management behaviour, research has uncovered dilemmas that people face in talking to their family members. This presentation will highlight some of the dilemmas that genetic testing and associated research has raised for families who are living with a family history of breast cancer [3,4]. With the evidence base that now exists, the challenge for the future is to develop interventions to support people undergoing genetic testing. In Southampton we are developing an intervention to support discussions within families about genetic testing and associated risks.

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## P55

### Urinary and serum biomarkers of phytoestrogen exposure are not associated with breast cancer risk in the European Prospective into Cancer Norfolk study

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*Breast Cancer Res* 2008, **10(Suppl 2):**P55 (doi: 10.1186/bcr 1939)

**Background** Phytoestrogens are a group of compounds found in plants that structurally resemble the hormone estradiol, and thus have the potential to act as estrogen agonists or antagonists. Their potential effects may alter the risk of breast cancer, but only a limited range of phytoestrogens has been examined in prospective cohort studies.

**Methods** Serum and urine samples from 237 incident breast cancer cases and 952 controls (aged 45 to 75 years) in the European Prospective into Cancer (EPIC) Norfolk cohort were analyzed for seven phytoestrogens (daidzein, enterodiol, enterolactone, genistein, glycitein, *o*-desmethylangolensin, and equol) using liquid chromatography/mass spectrometry. Data on diet, demographics, anthropometrics, and medical history were collected upon recruitment. All models were adjusted for weight, fat and energy intake, family history of breast cancer, social class, analytical batch, and factors related to estrogen exposure.

**Results** With a few exceptions, urinary or serum phytoestrogens were not associated with breast cancer risk in the EPIC Norfolk cohort. Breast cancer risk was marginally increased with higher levels of total urinary isoflavones (OR = 1.08 (95% CI = 1.00 to 1.16), $P = 0.055$); this association was stronger when restricted to premenopausal and perimenopausal women (OR = 1.30 (95% CI = 1.04 to 1.64), $P = 0.022$). Among the 105 women with estrogen receptor-positive tumours, the risk of breast cancer was increased with higher levels of urinary equol (OR = 1.07 (95% CI = 1.01 to 1.12), $P = 0.013$).

**Conclusion** There was limited evidence of an association between phytoestrogens and breast cancer risk in the EPIC Norfolk cohort. Further study is required to determine whether the observations from the present study are replicated in other populations with similarly low relative intake of phytoestrogens.

## P56

### Adherence to hormone therapy in a chemoprevention randomised trial

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**Background** Nonadherence to oral medication exists amongst women with breast cancer [1] and those participating in randomised clinical trials [2]. There is also evidence to suggest that nonadherence is more prevalent in chemoprevention trials than in adjuvant trials, with between 20% and 46% of patients in chemoprevention trials not adhering to medication [3]. The purpose of this study is to examine adherence amongst a

subgroup of women participating in the International Breast Cancer Intervention Study (IBIS II) cognitive subprotocol. IBIS II is a randomised double-blind chemoprevention clinical trial of anastrozole versus placebo in postmenopausal women at high risk of breast cancer.

**Methods** Two hundred and seven women participating in the cognitive subprotocol of the IBIS II trial are having cognitive and quality-of-life assessments conducted at three time points (prior to receiving the trial tablets, at 6 months and at 24 months post randomisation). Following the final assessment, a short semi-structured interview is conducted to elicit information regarding trial medication-taking behaviours.

**Results** Fifty-three out of 207 women who participated in the IBIS II cognitive substudy had dropped out by 24 months (primarily due to side effects) and adherence data on 124 women who had a final assessment have been collected and are reported here. Seventy-one per cent (89 participants) were taking allopathic medication aside from the trial tablets, and 47% (58/124) were also taking supplements, for example multivitamins, ginkgo biloba, omega 3, and glucosamine. The total number of tablets taken a day ranged from 1 to 14, with a mean of 4.8 tablets per day. Only 15 women said they had ever experienced difficulty swallowing tablets and 10 of those were taking medication aside from the trial tablets. When participants were asked to indicate whether they had ever forgotten to take their trial tablets 50% (62 participants) said yes, but 37% (46 participants) stated only rarely. When asked whether participants ever chose not to take their trial tablets, for whatever reason, only 6/124 (5%) reported occasions when they had done so. Reasons included going away for holiday, not wanting to mix it with painkillers, and stomach upsets. When asked whether taking the trial tablets interfered with their daily life, the majority (90%, 111 participants) said never.

**Conclusion** Adherence data from the IBIS II trial participants contrast with those found in the first IBIS trial (tamoxifen versus placebo). Early indications are that, contrary to previous findings, women receiving an aromatase inhibitor to prevent breast cancer appear on self-report to have little problem with daily tablet taking during the first 2 years of this 5-year clinical trial. Reasons for the differences may be due to sampling; women participating in the cognitive substudy may be a more motivated group, and it should be noted that almost one-third of women who dropped out did so because of vasomotor symptoms. Further monitoring of this group over a longer period is warranted.

**Acknowledgement** Cancer Research UK funded this research.

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## P57

## Health inequalities in breast cancer screening

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*Breast Cancer Res* 2008, **10(Suppl 2):**P57 (doi: 10.1186/bcr 1941)

**Objective** To establish the significant factors that impact upon the likelihood of women attending breast cancer screening. These factors include ethnicity, ethnicity and socioeconomic status, the season of the appointment and the travelling distance for each woman.

**Method** All women screened in the Borough of Oldham in 2006 were investigated ($n = 5,490$). Ethnicity was attributed by analysing their names. Socioeconomic status was designated through their area's average house price. The distance to the screening van was measured from their postcode.

**Results** There was a significant difference between Asian (43.7%) and non-Asian (73.7%) attendance ($P < 0.05$). The difference remained significant when socioeconomic status was accounted for ($P < 0.05$). Both Asian and non Asian women showed a reduced uptake in poorer areas. Asian women were less likely than non-Asian women to attend breast screening irrespective of their socioeconomic area. Significantly more women attended during autumn ($P < 0.05$). The travelling distance to the screening van had no effect upon attendance ($P = 0.38$).

**Conclusion** Since the Forrest Report, improvements in diagnosing and treating breast cancer have advanced while improvements in uptake of screening have not. The present study shows that inequalities still exist within the breast cancer screening system. Increasing levels of immigration is resulting in a more diverse nature of our population, thus these inequalities are set to increase. One fundamental objective is to abolish these inequalities. There must be a substantial increase in the uptake rate in both non-Asian and Asian women in all socioeconomic areas so the benefits of better treatment can be accessed. Well structured and funded qualitative research is required to establish why such high levels of nonattendance exist. With the government lengthening the ages women are eligible for screening to 47 to 73 years old, an extra 200,000 women per year are eligible for screening. Unfortunately as has been shown, eligibility does not correlate with attendance – resulting in increasing administration costs and wastage of valuable resources.

## P58

## MCPH1, a potential predictor for response to cancer chemotherapy

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*Breast Cancer Res* 2008, **10(Suppl 2):**P58 (doi: 10.1186/bcr 1942)

**Background** We previously identified MCPH1, a DNA damage response protein involved in the regulation of the breast cancer tumour suppressor gene *BRCA1*, as the defective protein in one form of microcephaly [1]. We found that reduced expression of MCPH1 causes premature chromosome condensation (PCC) [2]. PCC is a hallmark of mammalian cells that begin mitosis before completing DNA replication. The *MCPH1* locus (8p22–p23) is frequently deleted in many tumour types and this is associated with a poor prognosis and a reduced response to chemotherapy in breast cancer [3]. Many chemotherapeutic agents such as taxanes (for example, Taxol) require a functional spindle checkpoint for the induction of apoptosis in cancer cells.

**Methods** Using time-lapse imaging we have studied mitotic progression in *MCPH1*-deficient cells. The presence of a functional spindle assembly checkpoint was tested for using two different spindle poisons – for example, Taxol and nocodazole – in *MCPH1*-deficient cells. Immunohistochemistry using a MCPH1 antibody was performed on 54 breast cancer samples and was correlated with pathology data.

**Results** We have identified a number of mitotic defects including slower mitotic progression displaying aberrant chromosomal congression and micronuclei formation in *MCPH1*-deficient cells. *MCPH1*-deficient cells displayed a reduced mitotic arrest in response to spindle poisons, indicating impairment of the spindle checkpoint. Our immunohistochemistry data have identified reduced MCPH1 expression in 32% (17/54) of breast cancers, particularly in higher grade tumours.
**Conclusion** The mitotic phenotype suggests that loss of MCPH1 function in tumours could cause mitotic errors resulting in aneuploidy development. Our data indicate MCPH1 plays a role in resistance to chemotherapeutic agents such as Taxol through its involvement in the spindle checkpoint and apoptosis. We therefore hypothesise that, while germline defects in MCPH1 cause microcephaly, somatic defects may cause aneuploidy development and resistance to chemotherapy in breast cancer.
**Acknowledgement** Supported by Yorkshire Cancer Research.
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## P59

### Quantitative proteomics reveals proteins associated with radiotherapy resistance in breast cancer cells

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*Breast Cancer Res* 2008, **10(Suppl 2):**P59 (doi: 10.1186/bcr 1943)

**Background** Resistance to radiotherapy may be a significant factor in the development of local recurrence following surgical resection and radiotherapy. We aimed to develop a novel *in vitro* model of radioresistance using a breast cancer cell line and to subsequently identify molecular biomarkers that may be associated with the radioresistant phenotype. We utilised a quantitative proteomics technique (iTRAQ; Applied Biosystems, Warrington, UK) based on matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF)/TOF mass spectrometry to identify differentially expressed proteins.
**Methods** We established three novel breast cancer cell sublines that were significantly resistant to radiotherapy when compared with the parental cells. The radioresistant sublines were created by irradiating cells in fractionated doses of 2 Gy up to a total dose of 40 Gy. Sufficient time was allowed for the cells to recover between subsequent irradiations. A dose–response curve was assessed at the end of treatment to demonstrate a statistically significant increase in radioresistance for each novel cell subline when compared with parental cells. One radioresistant/parental cell pair was first analysed using in-solution digestion and liquid chromatographic separation with protein identification by MALDI-TOF/TOF (LC-MALDI analysis) on an Applied Biosystems 4800 Plus instrument (Applied Biosystems, Warrington, UK). Quantitative iTRAQ was then performed on the same instrument for all three radioresistant/parental cell pairs.
**Results** A total of 586 and 652 proteins were identified in T47D and T47D**RR** cells, respectively, by LC-MALDI. Those proteins identified in both cell lines and any redundant entries were removed to reveal those proteins that were unique to each cell line. In total, 244 unique proteins were identified in T47D cells and 311 unique proteins were identified in T47D**RR** cells. Comparison of the three pairs of radioresistant/parental cell samples by iTRAQ revealed a number of differentially expressed proteins. Using a standard ≥2-fold change in expression, these iTRAQ analyses revealed significant changes in the expression of 51 proteins in one or more of the radio-resistant derivatives. Further confirmation by immunoblotting is underway. Currently the decrease in expression of 26S proteasome associated subunits has been confirmed by this method.
**Conclusion** LC-MALDI and iTRAQ analysis has revealed a large number of candidate proteins that may be associated with a radioresistant phenotype. These now require further confirmatory studies. These mass spectrometry-based techniques offer a powerful proteomic approach to identify candidate biomarkers that may be involved in radioresistance.

## P60

### Identification of proteins associated with radiotherapy resistance in breast cancer cells: a combined proteomic and microarray screening approach

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*Breast Cancer Res* 2008, **10(Suppl 2):**P60 (doi: 10.1186/bcr 1944)

**Background** Radiotherapy is one of the major modalities in breast cancer treatment. However, resistance to radiotherapy may be a significant factor in the development of local recurrence following surgical resection and radiotherapy. In addition, if patients with radioresistant breast cancers can be identified, harmful side effects from exposure to unnecessary ionizing radiation could be prevented. We aimed to develop novel *in vitro* models of radioresistance using breast cancer cell lines and to subsequently identify molecular biomarkers that may be associated with the radioresistant phenotype. We used a combined proteomic (two-dimensional gel electrophoresis/mass spectrometry) and transcriptomic (expression microarrays) screening approach.
**Methods** We established three novel breast cancer cell sublines that were significantly resistant to radiotherapy when compared with the relevant parental cells (MCF-7, T47D, MDA-MB-231). Radioresistant sublines were created by irradiating cells in fractionated doses of 2 Gy up to a total dose of 40 Gy. Sufficient time was allowed for the cells to recover between subsequent irradiations. A dose–response curve was assessed at the end of treatment to demonstrate a statistically significant increase in radioresistance for the novel cell sublines when compared with parental cells. Each radioresistant/parental cell pair was analysed using two-dimensional gel electrophoresis. The protein profiles were compared and differentially expressed proteins were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Immunoblotting was used to confirm the

identities of a subset of proteins. A 3k cancer-related oligonucleotide microarray was also used to identify targets that were differentially expressed between each novel radioresistant derivative and its parental cell line. Real-time quantitative PCR was used to confirm the difference in expression of a subset of genes that demonstrated significant (at least twofold) differential expression.

**Results** Using the proteomic approach, 47 differentially expressed proteins were identified from one or more cell line pair. These proteins include glutathione S transferase mu 3 (GSTM3), proteasome activator subunit 1 (PSME1), PSME2, PSMA7, L-plastin, cytokeratin 17, TRAP-1 and aldolase A. The differential expression of GSTM3, PSMA7, L-plastin, cytokeratin 17, TRAP-1 and aldolase A have so far also been confirmed by western blotting. Using expression microarray analysis, the expression of 69 genes was found to be significantly altered in one or more radiotherapy-resistant cell sublines. Real-time quantitative PCR expression was also used to confirm the differential expression of *GSTM3*, *PSME1* and *PSME2.*

**Conclusion** The development of these novel radiotherapy-resistant breast cancer cell sublines and a combined proteomic/transcriptomic complementary approach has identified candidate biomarkers that may be associated with radiotherapy resistance. In particular, proteasome activator subunits and GSTM3 appear to be of interest from both screening approaches. Further validation, functional and clinical evaluation is required, but this complementary screening approach has identified candidate biomarkers that may be involved in radioresistance and may reveal novel therapeutic targets in breast cancer.

## P61

### Proteomic screening of 725 antibodies simultaneously using antibody microarray technology to identify proteins associated with radiotherapy resistance in breast cancer cells

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*Breast Cancer Res* 2008, **10(Suppl 2):**P61 (doi: 10.1186/bcr 1945)

**Background** Resistance to radiotherapy may be a significant factor in the development of local recurrence following surgical resection and radiotherapy. In addition, if patients with radioresistant breast cancers can be identified, harmful side effects from exposure to unnecessary ionizing radiation could be prevented. We aimed to develop a novel *in vitro* model of radioresistance using a breast cancer cell line and to subsequently identify molecular biomarkers that may be associated with the radioresistant phenotype. Antibody microarrays offer a complementary approach for proteomic analysis in conjunction with standard screening methods such as two-dimensional gel electrophoresis/mass spectrometry. We have previously utilised the Panorama Cell Signalling Antibody Microarray Kit (Sigma-Aldrich, Poole, UK) consisting of 224 antibodies [1]. In the present study we assessed a novel high-density 725-antibody microarray to screen for proteins associated with radioresistance.

**Methods** We established a novel breast cancer cell subline that was significantly resistant to radiotherapy when compared with the parental cells (T47D). The radioresistant subline was created by irradiating cells in fractionated doses of 2 Gy up to a total dose of 40 Gy. Sufficient time was allowed for the cells to recover between subsequent irradiations. A dose–response curve was assessed at the end of treatment to demonstrate a statistically significant increase in radioresistance for the novel cell subline when compared with parental cells. The radioresistant/parental cell pair was analysed using the Panorama Antibody Microarray XPRESS Profiler725 Kit (Sigma-Aldrich). The microarray comprised 725 different antibodies on nitrocellulose-coated microscope slides. The antibodies were selected from a wide variety of pathways, including apoptotic and cell signalling pathways.

**Results** Utilising a Cy3/Cy5 labelling strategy, the antibody microarray approach yielded a number of possible targets for further study. These include zyxin, growth factor independence 1 and lysine-specific demethylase 1, which were differentially expressed between the radioresistant subline and parental cells. Immunoblotting has confirmed the identities and differential expression of some candidate protein targets.

**Conclusion** The use of a novel high-density antibody microarray has successfully identified a number of protein targets that may be associated with a radioresistant phenotype. These proteins require further study to validate the results. High-density antibody microarrays potentially offer a powerful new proteomic technique to allow the global analysis of many proteins simultaneously. These could be invaluable in the identification of candidate biomarkers that may be involved in radioresistance and may reveal novel therapeutic targets in breast cancer.

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## P62

### Development of anti-MUC1 DNA aptamers for the imaging and radiotherapy of breast cancer

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*Breast Cancer Res* 2008, **10(Suppl 2):**P62 (doi: 10.1186/bcr 1946)

**Background** Aptamers have shown great potential as novel targeted radiopharmaceutical entities for the diagnosis and imaging of disease. They offer reduced immunogenicity, good tumour penetration, rapid uptake and clearance compared with their monoclonal antibody counterparts. In previous work we have reported the labelling of such aptamers against breast-cancer-related biomarkers with radionuclide ligands.

**Methods** We have now conjugated previously selected aptamers against the protein core of the MUC1 glycoprotein tumour marker with chelating agents and labelled them with $^{99m}$Tc, for the diagnostic imaging of breast cancer. The conjugation is achieved using standard peptide coupling reactions between an amino modification on the aptamer and the carboxylic group on the ligands. Labelling with $^{99m}$Tc used tin chloride as the reducing agent, and analysis was by HPLC where both the UV and the gamma emission was monitored. Radiolabelled aptamer conjugates were separated from free, unconjugated $^{99m}$Tc using microcon filters. For the analysis of the pharmacokinetic properties of the aptamer–radionucleotide conjugate we used gamma-camera imaging in MCF-7 breast cancer tumour model systems.

**Results** We coupled the aptamer with the highest affinity for the MUC1 glycoprotein to different ligands (MAG2 or meso-2,3-dimercaptosuccinic acid) and labelled it with active $^{99m}$Tc to obtain stable complexes that were used in pharmacokinetic studies. This allows us to compare the properties of a single conjugate with a biaptamer conjugate, as two of the DMSA–aptamer conjugates

can coordinate the metal core. An efficient and convenient labelling of the aptamer with short half-life radioisotopes was achieved as the last step of the synthesis (postconjugation labelling). The labelled aptamers were separated from free $^{99m}$Tc using microcon filter separation and were monitored by HPLC at all stages, to ensure that only radiolabelled aptamers were injected and imaged for their pharmacokinetic properties.

**Conclusion** The aptamer–chelator conjugates have strong $^{99m}$Tc binding properties and the resulting complexes are highly stable *in vivo* both in terms of nuclease degradation and leaching of the metal. The presence of more than one molecule of aptamer per complex alters the binding and pharmacokinetic properties of the radiolabelled products, allowing the complex to remain longer in circulation and thus offering improved tumour imaging properties, without affecting the tumour penetration of the aptamer. Furthermore, different ligands affect accumulation of the aptamer in different organs, as they alter the lipophilic properties of the conjugate. These results aim to open new possibilities for the diagnostic imaging of, and potentially the targeted radiotherapy of, breast cancer.

**Acknowledgement** Breast Cancer Campaign provided financial support.

## P63

### Prognostic significance of steroid receptor co-regulators in breast cancer: co-repressor NCOR2/SMRT is an independent indicator of poor outcome

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*Breast Cancer Res* 2008, **10(Suppl 2):**P63 (doi: 10.1186/bcr 1947)

**Background** Advances in the understanding of the molecular basis of breast cancer has necessitated a definition of more sensitive and specific indicators of prognosis that are central to the underlying cancer biology and that reflect the complicated and heterogeneous nature of the disease. The present study investigates the pattern of expression of the steroid receptor coregulators NCOA1/SRC1, NCOA3/RAC3, NCOR2/SMRT, and CBP/p300 in breast cancer. The aims were to identify whether their expression was related to patient outcome, their relationships to known prognostic factors and to provide a basis for further research to investigate the mechanistic significance of such associations.

**Method** The protein levels of steroid receptor coregulators were assessed using immunohistochemistry in a large well-characterised series of breast carcinomas prepared as tissue microarrays. Relationships between these targets, other clinicopathological variables and patients' outcome were examined.

**Result** The most important finding was that NCOR2/SMRT was an independent prognostic indicator of overall patient survival and the disease-free interval and was significantly correlated with distant metastases and local recurrence, whereas tumours expressing NCOA1/SRC1 had significantly longer overall patient survival and disease-free interval. There were also significant correlations between coregulator expression of NCOA1/SRC1, CBP/p300 and NCOA3/RAC3 that were associated with lower tumour grade. NCOA1/SRC1 was also correlated with smaller tumour size. Furthermore, the coactivators had a significant association with steroid receptors, particularly estrogen receptor alpha.

**Conclusion** The corepressor NCOR2/SMRT is associated with poor patient outcome, independent of other prognostic factors. In contrast, steroid receptor coactivator expression is generally associated with a good prognosis. Further investigations are needed to establish the mechanisms of these links between the steroid receptor coregulator system and patient outcome.

**Acknowledgement** Funded by Breast Cancer Campaign (2005Nov08).

## P64

### High-throughput optical proteomics and breast cancer patient profiling: novel applications to individualise prognosis and treatment

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*Breast Cancer Res* 2008, **10(Suppl 2):**P64 (doi: 10.1186/bcr 1948)

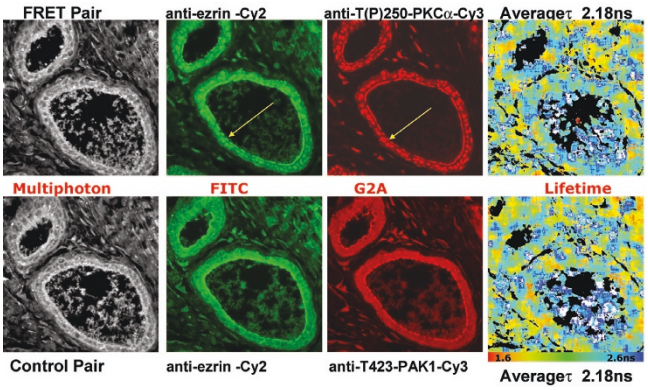
**Background** Breast cancers that appear similar by stage and grade are not identical in terms of outcome for each patient affected. Heterogeneity would be better understood using genomic/proteomic profiles to predict for relapse. Risk estimation could be truly individualised and treatment personalised. Proteomics goes beyond possession of an abberrant gene by assessing post-translational modifications such as phosphorylation and measuring protein–protein interactions. Optical proteomics uses fluorescence lifetime imaging microscopy (FLIM) to quantify associations between signalling proteins in tissues beyond the spatial resolution of light microscopy by measuring Förster resonance energy transfer (FRET). These technologies improve understanding of how extracellular signals are sensed by cancer cells and transduced to trigger invasion. Protein kinase C alpha (PKCα) is a signalling protein that can oppose apoptosis. The actin-binding protein ezrin provides a direct link between the cytoskeleton and plasma membrane, necessary for cell migration and metastasis. Ezrin–PKCα interaction has been demonstrated in breast cancer cell line experiments [1].

**Methods** Fluorophore-conjugated antibodies to PKCα and ezrin were applied to breast cancer tissue microarrays (TMA), obtained from a well annotated tissue bank with a rich complement of clinical data. Each TMA was created from 84 invasive breast carcinoma samples, formalin fixed and paraffin embedded. Immunofluorescence enables imaging of both proteins simultaneously at two different wavelengths from the same section of tissue. As intensity is proportional to concentration, proteins can be accurately quantified. FLIM analysis was performed. Where anti-ezrin-Cy2 and anti-PKCα-Cy3 are located within nanometre proximity intracellularly, measurable energy transfer occurs (FRET). Controls were matched tumour areas of noninteracting proteins.

**Results** We imaged six TMAs (histopathological grades I to III) in triplicate to generate epifluorescence images and FRET/FLIM data. We have demonstrated a wide spectrum of distribution for both ezrin and PKCα in human breast cancer tissue. We have reported on the activation state of ezrin and the colocalisation of both proteins (Figure 1). We have measured FRET between anti-ezrin-Cy2 and anti-PKCα-Cy3. The present study is the first to demonstrate ezrin–PKCα interaction in human breast tissue (Figure 2). In a subset, the FLIM assay was complemented by an independent intensity method. Tissue data are further corroborated by parallel assays performed on cultured cancer cells. All parameters are undergoing multivariate analysis and further statistical comparison with respect to time to relapse of disease.

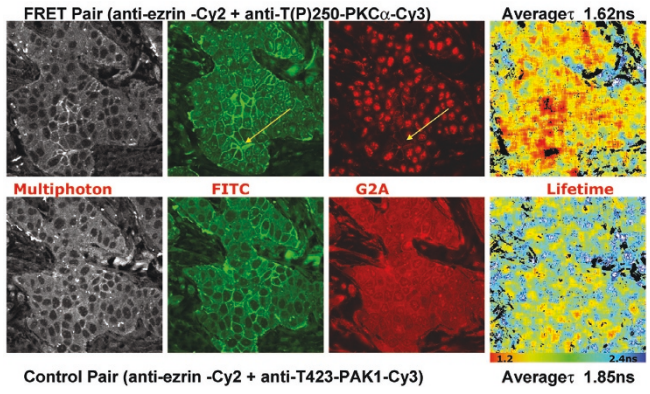
**Conclusion** The present study has established several optics-based parameters to be used in a multivariate correlation with breast cancer patient outcome. The goal is to derive multiple high-

**Figure 1 (abstract P64)**




Ezrin and PKCα colocalised in normal breast ducts. Ezrin–PKCα colocalisation is demonstrated but with a long fluorescent lifetime and a blue pixel-by-pixel fluorescence lifetime (τ) map. The fluorescent lifetime of Cy2 does not reduce. There is no interaction between the proteins. Controls were matched tumour areas in the same tissue core (3 to 5 μm deeper in the TMA block) stained for noninteracting proteins.

**Figure 2 (abstract P64)**




Ezrin and PKCα colocalised in grade II invasive breast cancer. The fluorescent lifetime has shortened and a the pixel-by-pixel fluorescence lifetime (τ) map shows areas of red. There is interaction between the proteins in these areas when compared with the control. In this section of invasive ductal breast cancer, ezrin–PKCα interaction is demonstrated as FRET has occurred.

throughput optical proteomic markers that could be applied to tumour tissue at first diagnosis to better predict risk for individual patients. This project aims to translate advanced optical proteomic science into real-life benefit, assisting patients and physicians in the difficult decisions regarding treatment.

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## P65

## Expression analysis of novel biomarkers for breast cancer

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*Breast Cancer Res* 2008, **10(Suppl 2):**P65 (doi: 10.1186/bcr 1949)

**Background** Cancer Research UK (2007) [1] stated that the most common cancer for women in the United Kingdom is breast cancer. In 2004, about 20% of all breast cancer cases diagnosed would lead to death [2]. The accepted prognostic factors fail to establish accurately the outcome for breast cancer patients as a large proportion of those diagnosed with invasive breast carcinomas are given aggressive treatments even though many of them are unlikely to develop a life-threatening cancer even without therapies. Over the past decade, many genetic and molecular pathways have been associated with breast cancer. To progress towards personalized therapies, there is a need for novel biomarkers for diagnosis, for the detection of metastasis and as targets for new selective immunotherapies. The *BUC* genes (Breast UniGene Cluster) are novel breast-associated genes identified on the basis of their specific expression spectrum, which includes testis, normal breast and breast cancer tissue. During *in silico* analysis of the *BUC* gene sequence, we discovered that the *BUC11* gene sequence shares significant similarity with the gene sequence of an unpublished gene that codes for a predicted protein (source data obtained from the NCBI website [3]).

**Methods** siRNA was designed for specific *BUC11* silencing. *BUC11* siRNA efficacy was first tested using real-time RT-PCR following transfection and mRNA isolation. The transfection of breast cancer cell line MDA231 was carried out using INTERFERin siRNA Transfection reagent (Autogen Bioclear, Calne, UK). The experiment was performed in duplicate wells. Each experiment comprised cells with *BUC11* gene-specific siRNA, cells with negative control siRNA, cells with INTERFERin alone and cells alone. On day 2, $^{3}$H-thymidine (Sigma-Aldrich, Gillingham, UK) was added to the cells. On day 3, cell suspensions were transferred to a filter plate, Microscint solution (Packard, Meriden, CT, USA) was added and the reading of the plate was performed. The procedure was repeated on day 7 and on day 10. To quantify gene expression at the mRNA level in breast tumours, conventional RT-PCR as well as real-time quantitative RT-PCR were carried out. Samples used in this study come from various invasive and noninvasive histological subtypes of breast cancer, different malignancies (for example, melanoma, testis cancer, mesothelioma) and normal tissues.

**Results** Regarding *BUC11* gene knockdown, 72 hours following transfection, 89.7% of specific inhibition of *BUC11* mRNA expression was observed (real-time RT-PCR results). Three days following transfection of MDA231 with *BUC11* siRNA, cell proliferation was inhibited by 98%. This result is still observed 7 days following transfection. However, the inhibition of proliferation is no longer observed 10 days following transfection, which is not surprising due to the transient nature of transfection. The *BUC11* gene was expressed in 90% of the breast cancer tissues tested. *BUC11* mRNA was not (or at very low levels) expressed in the normal tissues tested (heart, liver, prostate, brain, uterus, spleen, skeletal muscle, lung, kidney, placenta, trachea, thyroid, spinal cord, salivary gland, thymus and peripheral blood mononuclear cell) except for normal testis and normal breast tissues. *BUC11* mRNA was expressed in varying levels in the breast cancer samples tested. *BUC11* mRNA was expressed at

similar levels in the normal testis and testicular cancer tissues tested. *BUC11* mRNA was only expressed in the breast cancer cell lines T47D and MDA231. Furthermore, *BUC11* mRNA appears to be overexpressed in breast tumour compared with the normal counterpart in the early stages of the disease and down-regulated in more advanced aggressive breast cancers. Finally, *BUC11* mRNA was not expressed in any of the other cancer samples tested (oesophageal, mesothelioma, melanoma, gastric and kidney).

**Conclusion** *BUC11* could potentially be a good candidate for the diagnosis and prognosis of breast cancer due to the correlation of *BUC11* gene expression with the stage of breast cancer. siRNA silencing of *BUC11* led to the inhibition of the proliferation of MDA231 breast cancer cells. This suggests that *BUC11* might have a role in the proliferation of cancer cells in the breast. The tissue specificity of the *BUC11* expression profile provides a rationale to consider *BUC11* as a tissue-specific gene involved in the differentiation of breast and testis tissues. If the restricted expression spectrum is confirmed in a larger cohort of samples, *BUC11* could be useful to detect micrometastasis in the lymph nodes or peripheral blood of breast cancer patients. Finally, *BUC11* gene is not expressed in vital organs; thus it could potentially be a good target for vaccine strategies.

**Acknowledgement** Funded by the John and Lucille van Geest Foundation.

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## P66

## Identification and role of migration stimulating factor isoforms in breast carcinomas

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**Background** Migration stimulating factor (MSF) is a 70 kDa truncated isoform of fibronectin containing a unique intron-derived 10 amino acid C-terminal sequence not present in any previously described full-length (250 to 280 kDa) fibronectin isoform. Unlike fibronectin, MSF is not an extracellular matrix component, but a soluble factor showing potent bioactivities relevant to cancer development, such as stimulation of cell migration, matrix remodelling and angiogenesis. MSF motogenic activity is mediated by its constituent IGD (isoleucine–glycine–aspartate) motifs. Two isoforms of MSF have been cloned. These differ by a 15 amino acid deletion and are referred to as MSF+aa (AJ535086) and MSF–aa (AJ276395). The term MSF will be used to denote both isoforms. We have identified an inhibitor of MSF+aa (MSFI) that is present in serum and in approximately 50% to 65% of breast carcinomas. MSF+aa and MSF–aa differ in their functional interaction with this inhibitor: MSF+aa is inhibited by MSFI, whereas MSF–aa is not. Both isoforms contain the same MSF-specific sequence and IGD functional domains; consequently, they are equally active in the absence of MSFI [1-4] (K. Kankova, S.J. Jones, I.R. Ellis, M.M. Florence, S.L. Schor, A.M. Schor, unpublished data, 2008).

**Objective** To examine the expression of MSF isoforms and their possible role in breast tumours.

**Methods** Three types of monoclonal and polyclonal antibodies were raised and characterised: identification antibodies (VSI) that recognise the MSF-unique sequence; function-neutralising antibodies (pepQ) that recognise the IGD functional domain; and antibodies (TYN) that recognise MSF–aa but not MSF+aa. MSF expression in paraffin-embedded archival specimens of breast tumours was examined by immunohistochemistry using VSI and TYN antibodies. The expression of MSF mRNA and protein by four breast carcinoma cell lines (MCF-7, T47D, MDA-MB435 and MDA-MB-231) and their response to rhMSF was determined by molecular, biochemical and functional assays.

**Results** Immunostaining with VSI and TYN antibodies indicated that approximately 80% of breast carcinomas ($n = 85$) overexpressed total MSF and MSF–aa. High expression was associated with poor prognosis. One of the breast carcinoma cell lines (MCF-7) did not produce MSF. The remaining three lines secreted bioactive MSF into their conditioned medium. Analysis of the type of MSF produced indicates that T47D, MDA-MB435 and MDA-MB-231 cells produce both MSF+aa and MSF–aa; the latter representing approximately 50% to 60% of total MSF. rhMSF stimulated the invasion of tumour cells through three-dimensional gels of type I collagen or through collagen-coated membranes. Conversely, cell invasion by MSF-producing tumour cells was effectively abolished by MSF-function-neutralising antibodies (pepQ).

**Conclusion** MSF isoforms are present in most breast tumours and are secreted by breast tumour cell lines. MSF stimulates tumour cell invasion in an autocrine and paracrine manner, modulated by the type of isoform expressed and by the presence of MSFI.

**Acknowledgements** The authors thank Breast Cancer Campaign, Cancer Research UK, Tayside Area Oncology Fund and the Anonymous Charitable Trust for financial support.

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## P67

## Development of functional assays for BRCA1 missense mutations

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*Breast Cancer Res* 2008, **10(Suppl 2):**P67 (doi: 10.1186/bcr 1951)

**Background** The breast cancer susceptibility gene, *BRCA1*, is mutated in a high percentage of hereditary breast and ovarian

cancers. It is a large gene containing 5,592 nucleotides, and since its discovery over 1,500 distinct mutations have been identified throughout the entire coding region. While genetic screening can be informative it is frustratingly ambiguous, as a complete spectrum of mutation types are presented and, while those that result in the introduction of a premature stop codon or a frame shift can be predicted to adversely affect protein function, there is considerable uncertainty regarding the functional outcome of the majority of the missense mutations. Evaluating the functional significance of such mutations is challenging due to the difficulties in purifying such a large protein. The identification of functional domains in BRCA1 will therefore be critical to the development of functional assays to evaluate their pathogenicity. Prior to our studies only two domains had been identified – the N-terminal RING domain and the C-terminal BRCT domain – and while the structures of both these domains provide a platform from which the structural consequences of missense mutations can be predicted, they only account for 16% of the total protein and hundreds of mutations of unknown pathogenicity remain to be characterised. Our work aims to identify domains in BRCA1 that can be used to determine the functional outcome of missense mutations.

**Methods** BRCA1 (230 to 534), identified as a soluble fragment [1], was overexpressed in *Escherichia coli* BL21 (DE3) codon plus and purified to homogeneity from crude cell extracts by ion exchange and $Ni^{2+}$-nitrilotriacetate affinity chromatography. The purified fragment was digested with trypsin at an enzyme to substrate ratio of 1:500 and a resistant domain identified using a combination of N-terminal sequencing and MALDI mass spectrometry (Bruker REFLEX III). The domain identified as amino acids 340 to 554 was purified and characterised as described previously [2]. The DNA binding affinity and selectivity were determined by surface plasmon resonance and gel retardation assays. Heteronuclear single quantum coherence NMR spectra of free and bound protein were recorded on a Varian INOVA 600 MHz spectrometer. Site-directed mutagenesis was carried out using the Quik change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), and the ability of the resultant proteins to bind to DNA was assessed. All mutant proteins were purified by a simple one-step procedure using nickel chelate affinity chromatography.

**Results and discussion** A host of reports implicate a role for BRCA1 in the repair of damaged DNA, in particular that of double-strand breaks by homologous recombination. The formation of DNA crossovers (four-way junctions) is a central feature of this repair process, and the ability of BRCA1 to specifically recognise these structures is an important part of its function, as it potentially targets it to sites of DNA repair. Using a combination of limited proteolysis and mass spectrometry we have identified and characterised a domain in BRCA1 that binds specifically to these DNA structures [1,2]. This region is comprised of amino acids 340 to 554 and contains one polymorphic and 42 unclassified missense variants. Analysis of the residues involved in DNA binding by NMR spectroscopy reveals that three of four arginine residues (R507, R506, R504 and R496) are potentially involved in binding; which three remains to be identified but R507, R506 and R496 are known to be mutated in some cancer patients (R507I, R504H, R496C and H). It is therefore tempting to speculate that the three involved in DNA binding are R507, R506 and R496. To determine whether this is the case, and indeed the value of using the DNA binding activity as a functional screen, each of these have been changed to their respective mutations, and also to glutamic acid and alanine, by Quik change site-directed mutagenesis. R506 has also been changed to E and A. All mutant proteins contain a C-terminal hexahistidine fusion, which has allowed a simple one-step purification procedure using nickel chelate affinity chromatography to be developed. The ability of each mutant domain to bind to four-way junction DNA is currently under investigation. These studies will allow us to determine whether the DNA binding activity of BRCA1 can be used as a potential functional assay for BRCA1 missense mutations.

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## P68

## Chemotherapy-induced modulation of [$^{18}$F]Fluoro-2-deoxy-D-glucose incorporation at the tumour cell level

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*Breast Cancer Res* 2008, **10(Suppl 2):**P68 (doi: 10.1186/bcr 1952)

**Background** Changes in the incorporation of [$^{18}$F]Fluoro-2-deoxy-D-glucose (FDG) determined using positron emission tomography (PET) is a highly sensitive technique for the early detection of tumour response to therapy [1]. Using serial FDG-PET scans, tumours responding to therapy generally show a decrease in FDG incorporation compared with pretreatment. How FDG incorporation at the tumour cell level is modified by treatment and the mechanisms involved is poorly understood and is the subject of the present study.

**Methods** [$^{18}$F]FDG incorporation, glucose transport, hexokinase activity and ATP content were determined in breast tumour (MCF-7, T47D), colorectal (SW620, HCT-8) and gastric (AGS) tumour cell lines during response to typical and novel agents utilised in the treatment of the respective tumour types. Treatment doses causing 50% growth inhibition ($IC_{50}$ determined by MTT assay) over a 72-hour period were utilized.

**Results** All 72-hour treatment/cell line combinations (breast tumour cells – MCF-7 and T47D – with tamoxifen, doxorubicin or docetaxel; colorectal cells – SW620 with 5FU, oxaliplatin or irinotecan – and HCT8 with irinotecan, cetuximab or both; gastric tumour cells – AGS – with cisplatin, 5FU or epirubicin) resulted in decreased FDG incorporation compared with untreated cells. Decreased FDG incorporation most closely reflected changes in glucose transport by the HCT-8 and AGS cells, ATP content by the breast tumour cells and hexokinase activity by the SW620 cells. Apoptosis was evident in epirubicin-treated AGS cells, which showed the greatest decrease in FDG incorporation, but was also apparent in irinotecan-treated cells, which showed declines in FDG incorporation comparable with treatments without apoptotic fractions.

**Conclusion** FDG incorporation in tumour cells responding to a wide variety of chemotherapy agents is decreased compared with untreated cells, suggesting that the decrease in FDG incorporation observed in the clinical setting reflects changes at the tumour cell level. The rate-limiting step varies with the tumour cell line and can be glucose transport, hexokinase activity or ATP content. Large decreases in FDG incorporation may in some tumours be indicative of the presence of apoptotic fractions.

**Acknowledgement** Funding of this work by grants from the Association for International Cancer Research and Breast Cancer Campaign is gratefully acknowledged.

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## P69

### Identification and definition of novel clinical phenotypes of breast cancer through consensus derived from automated clustering methods

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*Breast Cancer Res* 2008, **10(Suppl 2):**P69 (doi: 10.1186/bcr 1953)

**Background** Breast cancer is a heterogeneous disease for which several forms have recently been identified on the basis of their gene expression characteristics [1]. We have previously demonstrated that protein expression characteristics can be used to identify comparable classes [2]. In the present study we extend this approach using improved biostatistical methods to confirm the validity of such an approach and to further define the key criteria for class membership.
**Methods** Expression of 25 proteins, with known relevance to breast cancer, have been assessed in a series of 1,076 patients. This large dataset has been examined by four alternative computational data clustering techniques. Concordance between techniques was used to elucidate core classes of patients that could be well characterised.
**Results** A total of 663/1,076 (62%) patients were assigned to six different core classes, while 413 (38%) patients were of indeterminate or mixed class. Three of these core classes correspond to well known clinical phenotypes (luminal A, luminal B and HER2). Two classes correspond to the well known basal phenotype, but exhibit a novel differentiation into two subgroups. The last class appears to characterise a novel luminal subgroup.
**Conclusion** The present study serves to confirm that key clinical phenotypes of breast cancer can be identified. It has identified that both the luminal and basal breast cancer phenotypes appear to be heterogeneous and contain distinct subgroups. Of importance is the observation that only 62% of breast cancer cases in this cohort have been assigned to the determined phenotypes, while the remaining 38% of cases express mixed or indeterminate characteristics. This latter observation, although previously recognised, has not been emphasised in the past. It has important clinical implications should either cDNA expression or protein expression assays be used for stratification of patients into treatment groups either in clinical trails or for routine clinical management. The clinical phenotypes determined in this study are a new luminal group, luminal N, the new basal subgroups, basal X and basal Y, and the previously well-established luminal A, luminal B and HER2 groups.
**Acknowledgements** Funded by Breast Cancer Campaign (2005Nov08), BIOPATTERN FP6 Network of Excellence (FP6-IST-508803) and the BIOPTRAIN FP6 Marie-Curie EST Fellowship (FP6-007597).
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## P70

### Detection of gene amplification in matched tumour and plasma DNA from breast cancer patients by quantitative PCR

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**Background** The aim of the present study was to determine whether tumour-specific gene amplification is detectable in circulating cell-free DNA isolated from the plasma of breast cancer patients. Four loci, known to show amplification in breast cancers, were chosen for investigation of paired plasma and tumour DNA: Her-2 and C35 on chromosome 17, and FGFR1 and RAB11f1P1, which map to chromosome 8. Both gene pairs are frequently, but not exclusively, coamplified in breast tumours.
**Methods** Lymphocyte, tumour and plasma DNA from 21 unselected breast cancer cases were analysed for amplification by a real-time quantitative PCR assay. Primers and a minor groove binder (MGB) TaqMan probe were targeted to the four genes and to an unamplified reference gene selected from each of the two intervals (CNTNAP1 and UNC5ND, respectively). A normal lymphocyte DNA sample was included in each experiment to examine interassay reproducibility and as an experimental calibrator. A ratio above 2.0 was regarded as positive for gene amplification [1].
**Results** Amplification was detected in tumour and plasma DNA at all four loci, with frequencies ranging from 19.1% to 52.3%. Five of 21 cases showed concordant amplification of Her-2 in plasma and tumour DNA, compared with four, one and two of 21 cases for C35, FGFR1 and RAB11f1P1 amplification, respectively. No amplification was seen in the lymphocyte DNA samples.
**Conclusion** Taken together, these data confirm that tumour-specific amplification, including Her-2 amplification, is detectable in circulating cell-free DNA isolated from the plasma of breast cancer patients. This suggests the possibility of developing a rapid and simple blood screening tool for identification of gene amplification in breast cancer cases.
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## P71

### Prospective Study of Outcome in Sporadic versus Hereditary Breast Cancer: pros and cons of running a cohort study

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*Breast Cancer Res* 2008, **10(Suppl 2):**P71 (doi: 10.1186/bcr 1955)

**Background** The Prospective Study of Outcome in Sporadic versus Hereditary Breast Cancer (POSH) first started recruiting in

June 2001 and will stop recruiting in December 2007. Follow-up is funded until December 2009. There are 2,665 participants recruited to the study by 10 October 2007. All participants were diagnosed with a first primary invasive breast cancer since January 2000 and were ≤40 years old at diagnosis. Eligibility criteria also allow inclusion of those with known *BRCA* gene diagnosed at ≤50 years of age.

**Method** Recruitment has been mainly through oncology centres across the UK and was greatly facilitated by adoption into the National Cancer Research Network in November 2002. Data collection points are at diagnosis, 6 months, 12 months and annually to 5 years.

**Results** *Pros*

- Patients like the study, as it usually only involves patient contact at the point of consent and at the first annual follow-up. Very few decline to participate.
- Clinical trials practitioners like the study as it is easy to recruit to, and fits with routine follow-up regimes.
- The study gives enormous potential for translational studies improving the understanding of not only genetic aspects but the basic pathobiology of young-age breast cancer.

*Cons*

- It can be difficult to obtain funding for cohort studies.
- Some clinical trials practitioners dislike the follow-up forms and the quantity of data requested.
- Postal strikes have an impact on recruitment timing and can lead to additional patient appointments for blood donation.
- Changes in ethics regulations over a 6-year period have resulted in inconsistent and confused responses to protocol amendments with consequent delays.
- The UK Cancer Research Network point system changed, with treatment trials given higher points.

**Conclusion** The mechanics of running this study and some early summary data about the cohort will be presented and will be of interest to researchers already involved in the study or who may be considering starting a similar study or with a specific interest in collaborative studies around young-onset breast cancer.

**Acknowledgements** Sponsored by Cancer Research UK, National Cancer Research Network, Cancer Research UK Clinical Centre, Southampton and Wessex Cancer Trust.

## P72

### Lymphovascular invasion in breast cancer: improved methods of detection and clinical significance

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*Breast Cancer Res* 2008, **10(Suppl 2):**P72 (doi: 10.1186/bcr 1956)

**Background** The presence of vascular invasion (VI), encompassing both lymphovascular invasion (LVI) and blood vascular invasion (BVI), in breast cancer has been found to be a poor prognostic factor. It is not clear, however, which type plays the major role in metastasis.

**Methods** To distinguish between LVI and BVI, sections from 177 consecutive paraffin-embedded specimens of primary breast cancers, with known long-term follow-up, were immunohistochemically stained with two blood vascular markers (CD34 and CD31) and with a lymphatic marker (podoplanin/D2-40). BVI and LVI were identified and the results correlated with clinicopathological criteria and patient survival.

**Results** VI was detected in 56/177 specimens (32%); 54 (96%) were LVI and two (4%) were BVI. The presence of LVI was significantly associated with the presence of LN metastasis, development of distant metastasis, regional recurrence, and a worse disease-free interval and overall survival. In multivariate analysis, LVI retained a significant association with decreased disease-free interval and overall survival. When assessment of LVI using H&E was compared using the lymphatic marker, VI was missed in 30/177 (16.9%) and was falsely positive in 12/177 (6.8%) using H&E.

**Conclusion** VI in breast cancer is predominantly of lymph vessels and is a powerful independent prognostic factor. The use of immunohistochemical staining with podoplanin/D2-40 increases the accuracy of identification.

## P73

### TARGIT: an international trial of intraoperative versus external beam radiotherapy

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*Breast Cancer Res* 2008, **10(Suppl 2):**P73 (doi: 10.1186/bcr 1957)

**Background** Over the past 30 years, there has been a dramatic change in the local management of breast cancer, with radical operations being replaced by more conservative surgical procedures, together with the widespread use of radiotherapy. This shift has been prompted by results from randomised clinical trials that have clearly demonstrated breast-conserving surgery followed by radiotherapy is equivalent to more radical procedures in terms of local control and overall survival [1]. However, although the surgery is now conservative, the radiotherapy remains radical and may be overtreatment. Evidence for this comes from large studies of breast-conserving therapy where more than 90% of early breast recurrences were found to occur at the site of the original primary tumour site, whether or not radiotherapy was given and/or the margins were involved [2,3]. The development of a novel radiotherapy device enabled the launch of an international randomised controlled trial designed to compare intraoperative versus conventional external beam radiotherapy in women with early breast cancer [4].

**Methods** Intrabeam® (Carl Zeiss, Germany) is a miniature electron beam-driven X-ray source that provides low-energy X-rays directly into the area of interest immediately after excision of the tumour, to provide intraoperative radiotherapy accurately targeted to the tissues with the highest risk of local recurrence. Following treatment delivery in the operating theatre, women can then proceed to have chemotherapy and/or adjuvant hormonal therapy as required. An equivalence trial, the main outcome objective is risk of local relapse within the treated breast. Secondary objectives are to compare the treatment arms with respect to the site of relapse within the breast; relapse-free survival and overall survival; and local toxicity/morbidity.

**Results** With centres in eight countries, TARGIT is nearly halfway to the accrual goal of 2,232 patients. Follow-up information is gathered through a web-based data entry system. Separate protocols are being written to address cosmetic outcome; patient satisfaction and quality of life; health economics and cost–benefit; and patient preference.

**Conclusion** This technique could have enormous implications for both cost and availability of breast cancer treatment. TARGIT is currently open to accrual of patients and centres. For further details please visit www.targittrial.org

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## P74

## Cambridge Breast Intensity Modulated Radiotherapy Trial: dosimetry results for 1,139 patients

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**Introduction** Two-dimensional radiotherapy (RT) breast plans can lead to substantial dose inhomogeneity, which may cause increased normal tissue toxicity. We report the dosimetry results of our National Cancer Research Network-adopted randomised trial comparing standard two-dimensional RT with intensity-modulated radiotherapy (IMRT).
**Methods** Following 3D imaging, a standard plan was produced for all patients. Plans were classified as having significant dose inhomogeneity if they exceeded the upper limit of International Commission on Radiation Units and Measurements Report 50 (>107% of prescribed dose). Those patients with satisfactory dose homogeneity were treated with standard RT. Patients with significant dose inhomogeneity were randomised to standard breast RT or IMRT. The randomised group were replanned with forward-planned IMRT.
**Results** A total of 1,145 patients were recruited from March 2003 to July 2007. One patient was randomised in error and therefore excluded. Eight hundred and fourteen out of 1,139 (71%) had significant dose inhomogeneity with standard 2D RT, and were randomised to IMRT or control; 325/1,139 (29%) had acceptable dose homogeneity, and were treated with standard 2D RT. The mean improvement in volumes >107% for IMRT plans was 34 $cm^3$ ($P < 0.0001$, 95% CI = 26 to 42 $cm^3$). The mean improvement in volumes <95% for IMRT plans was 48 $cm^3$ ($P = 0.0001$, 95% CI = 34 to 62 $cm^3$).
The mean difference in breast volume between randomised and nonrandomised patients was 596 $cm^3$ ($P < 0.0001$, 95% CI = 530 to 662 $cm^3$). We aim to report the acute and interim late side effects in spring 2008, if the data are released by the Independent Data Monitoring Committee.
**Conclusion** IMRT improves radiotherapy planning, and patients with larger breasts are more likely to require this treatment. However, as there was considerable overlap in the range of breast volumes between the randomised groups, size alone cannot predict the need for IMRT. This trial will quantify the clinical benefit of breast IMRT, in a patient group who consume 30% of RT resources. It will also provide DNA samples linked with high-quality clinical outcome data, for a translational study investigating individual patient variation in normal tissue toxicity. This will bring us closer to the ultimate aim of individualised RT based on a patient's genetic profile.
**Acknowledgement** JSW, the trial radiographer, is funded by a grant from Breast Cancer Campaign.

## P75

## Bevacizumab resistance in breast cancer: are neuropilins the key?

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**Background** During breast cancer growth and development, angiogenesis is triggered by the interaction between vascular endothelial growth factor (VEGF) and its receptors VEGF-R1 and VEGF-R2. In breast cancer, alternative VEGF receptors, the neuropilins (Np1 and Np2), are often upregulated and serve to augment the effects of VEGF-R1/VEGF-R2 binding and provide alternative signalling pathways. Recently, a humanized antibody, Bevacizumab (Bz), which prevents VEGF binding to VEGF-R1/VEGF-R2, in combination with chemotherapy demonstrated initial efficacy (increased progression-free survival) in breast cancer phase III clinical trials. Eventually, however, the tumours evade treatment control. This may be because neuropilins are not blocked by Bz and provide an alternative VEGF signalling pathway in breast cancer. Therefore the present study aims to evaluate the potential of enhancing efficacy of Bz treatment by simultaneously blocking VEGF–neuropilin binding.
**Methods** Western blot analysis of VEGF receptors in human endothelial cells (HUVEC and HuDMEC) and breast cancer cell lines (MDA-MB-436, MCF-7 and T47D) assessed relative expressions of VEGF-R1, VEGF-R2, Np1 and Np2 in these cells. Microvascular endothelial tubule formation assays were then performed *in vitro* where cells were incubated on Matrigel in the presence of VEGF for 24 hours, and then Bz (1 to 4 mg/ml) and/or anti-Np (50 to 100 ng/ml) antibody were added to the wells. The number of branch points were quantified in three fields of view/well in three separate experiments.
**Results** Western blot analysis revealed Np1 and Np2 expression in both breast cancer and endothelial cell lines, whereas VEGF-R1 is expressed in MCF-7, MDA-MB-436 and endothelial cells and VEGF-R2 is only expressed by endothelial cells. HuDMEC stimulated by VEGF formed tubules *in vitro*, and the number of branch points/field of view for VEGF-stimulated controls (43 ± 6) versus Bz (25 ± 3) versus anti-NP antibody (26 ± 4) versus Bz + anti-Np antibody (17 ± 3) demonstrated a significant ($P < 0.001$) reduction in tubule formation.
**Conclusion** These data show that anti-Np antibodies increase the inhibitory effect of Bz and suggest that efficacy of breast cancer treatment with Bz may be enhanced by addition of a neuropilin blocking agent.

## P76

### Brk expression may affect the differentiation status of breast cancer cells

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The breast tumour kinase Brk (PTK6) is found in over two-thirds of breast cancer cell lines and tumours but is not expressed in normal mammary cells. Brk has previously been shown to play a role in regulating proliferation in breast tumour cells [1]. However, *in vivo*, the site of Brk expression in normal tissues is restricted to nonproliferating cells that are undergoing terminal differentiation such as those in the gut or the skin [2,3]. This led us to hypothesise that Brk expression in breast tumours could be reflective of a differentiation phenotype, especially as a previous study had shown that involucrin, a marker of terminal keratinocyte differentiation, was expressed in a subset of tumours [4]. We therefore examined involucrin expression in breast tumour cells lines and patient biopsy samples. In addition we investigated whether inducers of differentiation in keratinocytes such as prolonged culture in suspension or vitamin D3 treatment could also affect differentiation of breast tumour cells.

We found that the expression of Brk in cultured cell lines correlated with involucrin expression. In addition the change in Brk expression, as a result of culture conditions, was accompanied by a change in involucrin levels. Moreover, treatment with vitamin D3 resulted in a decrease in cell numbers in the Brk-positive cell lines relative to the control treatments. The Brk-negative cell line was unaffected by vitamin D3 treatment.

These data suggest that Brk and involucrin may be coregulated and that inducers of differentiation such as vitamin D3 could be considered potential therapeutic strategies.

**Acknowledgements** Funded by Breast Cancer Campaign and Brunel University.

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## P77

### C35 overexpression defines subsets of human breast cancer and its immunoreceptor tyrosine-based activation motif represents a novel treatment target

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C35 is a protein overexpressed in invasive breast cancer. The *C35* gene is located on chromosome 17, next to ERBB2/HER2. *C35* encodes a canonical immunoreceptor tyrosine-based activation motif (ITAM) sequence. ITAM-containing proteins have key signalling roles in the hematopoietic system and in oncogenic retroviruses. The ITAM interacts with Syk kinase, which mediates downstream signalling events.

C35-overexpressing breast tumours were found to be of two subsets. In one subset, C35 is coexpressed with HER2. The second subset is found within the basal-like carcinoma group. In order to evaluate the therapeutic potential of targeting C35 ITAM/Syk signalling, we utilised 3D cell cultures. Transformed cell lines act in a manner resembling their *in vivo* behaviour when grown in 3D cultures, on reconstituted basement membrane. Using this method, C35-expressing cells formed enlarged structures in both an ITAM-dependent and Syk-dependent manner. Furthermore, BT474 cells coexpressing C35 and HER2 formed more normal 3D structures when treated with a combination of Herceptin and Syk inhibitors.

## P78

### Why do most c-erbB-2/HER-2-positive breast cancer patients fail to respond to Herceptin?

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*Breast Cancer Res* 2008, **10(Suppl 2):**P78 (doi: 10.1186/bcr 1962)

**Background** Herceptin is active in a subset of patients over-expressing the epidermal growth factor receptor (EGFR) c-erbB-2 (HER2) but it is not possible to predict which individuals will respond. Several molecular hypotheses have been proposed for how Herceptin causes tumour regression: one is that the antibody binds to HER2 and causes it to be internalised into breast cancers cells, where it is either degraded or locates to a compartment in which it can no longer signal (or signal in the same way).

The present research aims to explore possible molecular and cellular mechanisms involved in resistance of Herceptin. We are also interested in identifying whether inhibiting other pathways (such as signalling via HER3) would increase the number of patients who show a response. We have created a plasmid containing c-erbB-2 fused to Yellow Fluorescent Protein (c-erbB-2-YFP) and an epidermal growth factor receptor fused to Green Fluorescent Protein (EGFR-GFP). The correct sequence was obtained for both of these and we showed that they react with specific antibodies using western blotting. We have established a system in which we can express c-erbB2-YFP with or without coexpression of the EGFR labelled (or not) with GFP and add Herceptin chemically coupled to the red fluorescent compound Alexa Fluor 568 to see if there is an effect in cell trafficking. We have made a monoclonal antibody called SGP1 that recognises the extracellular domain of HER3 receptor [1] and we would like to see whether addition of a HER3-specific monoclonal antibody to Herceptin will increase its anticancer activity. If so, SGP1 antibody

could be humanised and then both coadministrated with Herceptin in clinical trials.

**Methods** Both constructs c-erbB-2-YFP and EGFR-GFP were used to transiently transfect COS-7 cells to determine their biosynthesis and transport to the cell surface. Time-course studies using low-light fluorescent microscopy have shown that both receptors are found on the surface of cells between 18 and 24 hours post transfection. We have chemically labelled Herceptin immunoglobulin (Genentech Inc., South San Francisco, CA, USA) with Alexa Fluor 568 (Invitrogen Molecular Probes, Inc., CA, USA) and have shown that this binds only to cells expressing the c-erbB2-YFP receptor. The specificity is notably high in relation to previous experience with similar antibodies as no signal has been seen on any untransfected cells of several types. Treatment of cells at 37°C with Herceptin-568 for increasing time periods up to 48 hours was performed. Most of the effects of the drug on receptor localisation were seen in the first four hours and so in future experiments we employed this timeframe.

**Results** These studies indicate that Herceptin applied to cells expressing only c-erbB-YFP induces receptor internalisation into a compartment apparently just under the surface of the plasma cell membrane, supporting the observations of Austin and colleagues [2] who explored this by electron microscopy. Addition of Herceptin-568 to cells expressing the EGFR gave no binding as expected. However, cotransfection of c-erbB-2 (unlabelled) with EGFR tagged to GFP gave the unexpected result that the EGFR was internalised over about 1 hour (significantly slower than the effect of adding EGFR-Alexafluor). Preliminary results to determine the effect of the antibody SGP1 on the c-erbB-3 receptor have shown induced phosphorylation of a 60 kDa protein that is probably Shc, which already has been identified as one of the main second messenger proteins recruited by HER3. However, further studies are needed to fully characterise this protein.

**Conclusion** The results from the present work have shown that both constructs can be expressed in mammalian cells and that receptor trafficking can be observed and evaluated using two-colour digital fluorescent microscopy. In addition we have fluorescently labelled Herceptin, and its ability to bind c-erbB-2 is retained. We showed that cotransfection with c-erbB-2-YFP and EGFR labelled (or not) with GFP and addition of the labelled Herceptin is affected by the presence of EGFR. Our preliminary results using monoclonal antibody SGP1 have shown that the presence of HER3 receptor can affect the extent of downregulation. It may be that multiple targeting of the HER-family receptors will help to increase the number of patients that respond to the therapy.

**Acknowledgement** Supported by Breast Cancer Campaign.

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## P79

### Identification of components of the ubiquitin system as targets for therapeutic intervention

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Abstract not available at time of publication.

## P80

### Pretreatment of breast cancer cells with doxorubicin facilitates the subsequent uptake of zoledronic acid

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*Breast Cancer Res* 2008, **10(Suppl 2):**P80 (doi: 10.1186/bcr 1964)

**Background** Breast cancer patients commonly receive a combination of different therapies; however, our understanding of how such combined treatments work is incomplete. We have previously shown that sequential administration of the cytotoxic agent doxorubicin (dox) (Pharmachemie BV, Haarlem, The Netherlands) followed by the antiresorptive agent zoledronic acid (zol) (Novartis Pharma, Basel, Switzerland) synergistically increased tumour cell apoptosis *in vitro*, and also increased tumour cell apoptosis, decreased tumour cell proliferation and reduced subcutaneous in breast tumour growth *in vivo*. In contrast, pretreating the cells with zol before dox or adding both drugs simultaneously did not cause synergy. The aim of the present study was to determine the mechanism by which sequential administration of dox followed by zol exerts the increased antitumour effects.

**Methods** All experiments were carried out using MDA-MB-436 breast cancer cells, or MDA-MB-436 cells expressing green fluorescent protein (MDA-G8). Effects of dox on cell membrane integrity were monitored following propidium iodide (PI) or 7-amino-actinomycin D (7AAD) staining. Effects of single or sequential treatment with dox and zol were assessed using Annexin (apoptosis antibody), TMRE (mitochondrial membrane potential dye) and 7AAD (permeable membrane dye) staining by flow cytometry. Uptake of zol was assessed following western blotting using an antibody to the unprenylated form of Rap1a.

**Results** Following administration of 1 nM dox for 24 hours, 95% of the MDA-G8 showed uptake of both PI and 7AAD. The cells showed no sign of apoptosis and remained viable. Administration of 25 μM zol for 1 hour to cells pretreated with dox for 24 hours resulted in increased cell death compared with that caused by treatment with either dox or zol alone. Accumulation of unprenylated Rap1a was detected following treatment for 1 hour with lower doses of zol (8 μM) in the dox then zol treated cells, compared with cells treated with zol alone (12 μM). These data imply that pretreatment with dox facilitated the uptake of zol.

**Conclusion** Treatment of MDA-MB-436 cells with 1 nM dox disrupts cell membrane integrity without reducing cell viability. Administration of dox 24 hours prior to zol facilitates the uptake of zol in MDA-MB-436 breast cancer cells.

**Acknowledgement** Breast Cancer Campaign funded this work.

## P81

### Mechanisms of apoptosis and cell-cycle arrest in subcutaneous breast tumours treated sequentially with doxorubicin followed by zoledronic acid

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*Breast Cancer Res* 2008, **10(Suppl 2):**P81 (doi: 10.1186/bcr 1965)

**Background** Breast cancer patients commonly receive a combination of different therapies; for patients with late-stage breast cancer involving metastasis to the bone, a chemotherapeutic agent is usually given in combination with the antiresorptive drug zoledronic acid (zol) (Novartis Pharma, Basel, Switzerland). We have previously reported that administration of

doxorubicin (dox) (Pharachemie BV, Haarlem, The Netherlands) 24 hours prior to zol inhibits subcutaneous breast tumour growth, inhibits tumour cell proliferation and increases apoptosis *in vivo*. The aims of the present study were to determine the mechanisms by which dox and zol exert their synergistic antitumour effects.

**Methods** MDA-MB-436-GFP (MDA-G8) cells ($0.5 \times 10^8$) were inoculated into the right flank of female MF1 nude mice ($n = 3$/array). Mice were treated once per week for 6 weeks with saline, 2 mg/kg dox, 100 μg/kg zol or dox followed 24 hours later by zol. Animals were sacrificed 24 hours following final treatment and one half of each tumour was stored in RNAlater and the other half in protein lysis buffer. RNA was extracted using a SuperArray ArrayGrade™ total RNA extraction kit (Tebu-bio, Peterborough, UK) and biotin-labelled riboprobes were subsequently produced using a SuperArray TrueLabelling-AMP™ 2.0 kit (Tebu-bio). Four micrograms of biotin-labelled RNA from each group was hybridised overnight at 60°C to separate GEArray cell-cycle and apoptosis pathway-specific microarrays (Superarray.com; Tebu-bio). Gene expression was analysed using GEAsuite software (Superarray.com; Tebu-bio) and gene maps were produced using Pathway Architecture software (Stratagene, CA, USA). Genes were considered relevant if they showed a twofold or greater change in gene expression compared with control and they showed direct interactions on the gene map. Expression of relevant genes was confirmed by quantitative PCR and protein expression assessed by western blot.

**Results** Molecular analysis of subcutaneous MDA-G8 tumours showed no effect on tumour cell cycle or apoptosis following administration of 100 μg/kg zol. Conversely, 2 mg/kg dox caused a cell-cycle block at $G_1$-S and $G_2$-M with a downregulation of cyclin E/CDK2 and cyclin B/CDC2; dox alone did not affect apoptosis. When dox was administered 24 hours prior to zol, however, the cell cycle was further suppressed, compared with dox alone, there was a downregulation of cyclin E1, cyclin B, cyclin $D_1$ and cyclin $D_3$ as well as their related cyclin-dependent kinases CDK2, CDC2, CDK4 and CDK7. Furthermore, tumours treated sequentially with dox then zol showed an induction in the apoptotic pathway with an upregulation in Bax, a downregulation in Bc12 and an increase in caspase 3 cleavage.

**Conclusion** In subcutaneous MDA-G8 tumours: administration of zol does not effect the apoptotic cell cycle pathways, administration of zol disrupts the cell cycle but has no effect on apoptosis, and sequential administration of dox followed by zol results in cell-cycle inhibition and induction of apoptosis.

**Acknowledgement** Breast Cancer Campaign funded this work.

## P82

## An exploration of the management of menopausal symptoms for women with a diagnosis of breast cancer: lay and professional experiences and expectations

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*Breast Cancer Res* 2008, **10(Suppl 2):**P82 (doi: 10.1186/bcr 1966)

**Background** Many breast cancer survivors are menopausal either at diagnosis or as a result of a premature therapy-induced menopause, complaining frequently of climacteric symptoms [1]. The menopause is widely seen as part of the natural ageing process; however, for many women who have had treatment for breast cancer; it can be viewed as a further complication, which can significantly impact on their quality of life as they recover from cancer treatment [2]. The increased symptoms often coincide with a time of transition from the completion of intensive treatment to follow-up care when there can be a perceived decrease in levels of support [3]. The limited evidence to guide practice both pharmacologically and nonpharmacologically within breast cancer has created a confused environment, for both clinicians and patients [4]. The aim of the present study was therefore to explore the experiences and expectations of both women with breast cancer and the health professionals, in relation to the management of menopausal symptoms in a clinical setting.

**Method** A qualitative exploratory study using focus groups and in-depth individual interviews was carried out to collect data from 14 female patients with breast cancer and from 18 health professionals who worked predominately with breast cancer within a large cancer centre. The data were coded and organised using QSR Nvivo 7 Software and were analysed thematically.

**Results** Three main themes emerged across both groups; namely, recognising the inevitability, building relationships and moving forward. The data presented an insight into the complexities of menopausal symptoms that are experienced by women with breast cancer within the context of their diagnosis, treatment and ongoing care, and the contrasting perceptions of the health professionals who manage their care.

**Conclusion** While the findings have highlighted the complex nature of menopausal symptoms for women with breast cancer, it has also identified the difficulty of isolating these symptoms from the whole experience associated with breast cancer from diagnosis and beyond. There is a need to assess and manage women both individually and within a multidisciplinary context, particularly as women continue to see different healthcare professionals following completion of treatment. This would allow complex issues that span across the premenopausal, perimenopausal, or postmenopausal stages, to be identified and resolved effectively.

**Acknowledgement** Funded by the Centre for Integrated Healthcare, Edinburgh.

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## P83

## Investigation of immunoregulatory mechanisms relating to poor surgical wound healing and breast cancer recurrence

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*Breast Cancer Res* 2008, **10(Suppl 2):**P83 (doi: 10.1186/bcr 1967)

**Background** The factors leading to breast cancer recurrence are incompletely understood. We recently carried out a retrospective

study of treatment and outcome for 1,065 breast cancer patients for which we examined factors correlating with cancer recurrence. We found that infection of surgical wounds after surgery for primary disease positively correlated with cancer recurrence [1]. Patients with wound complications were almost threefold more likely to have systemic recurrences than those without over the follow-up period ($P < 0.0001$). The aim of our current study is to determine mechanisms responsible for this correlation. Our approach is based on two possible theories. First, patients may have an underlying immune dysfunction that predisposes them to developing both wound complications and also recurrence. This may be as a result of the tumour itself suppressing the activity of immune regulatory cells including dendritic cells, T cells and NK cells via increased levels of some critical cytokines (for example, vascular endothelial growth factor, IL-10, IL-6). Secondly, factors released at sites of wound complications may have direct influences on the remaining occult tumour cells, thereby increasing the likelihood of metastases. This model is supported by observations that cytokines released at sites of infection as part of inflammatory/immune responses are capable of enhancing growth and survival of tumour cells [2]. Also there is evidence that bacterial components may stimulate metastatic growth, most probably via cytokine mediators [3].

**Methods and results** Patients with primary operable breast cancer are recruited prospectively. Blood samples are collected from patients preoperatively, 4 hours and 16 hours postoperatively and again at 2 weeks, 3 months and 6 months postoperatively. A variety of investigations are carried out on each sample to establish the immune status of the patient at that time point, and to identify potential mediators of crosstalk between the immune system, the wound and any occult tumour cells. These investigations include: full blood count; detailed immune cell phenotyping (absolute numbers/frequency of B-cell, T-cell and NK-cell subtypes using multicolour flow cytometry); and cytokine profiling (using fluid-phase cytometric multiplex immunoassays for 27 critical cytokines and growth factors). Patients are followed up and monitored for postoperative wound complications and evidence of recurrence. We shall determine whether patients that develop wound complications and/or metastases show immune defects from the outset, and whether systemic changes in immune regulators during wound complications reflect the development of metastases. Recruitment is ongoing with samples from >100 patients expected to be collected and processed by the end of 2007. Preliminary data concerning immune status and wound complications will be presented.

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## P84

## Discrepancies and challenge of ductal carcinoma *in situ* for health professionals

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*Breast Cancer Res* 2008, **10(Suppl 2):**P84 (doi: 10.1186/bcr 1968)

**Background** Ductal carcinoma *in situ* (DCIS) is a noninvasive breast cancer. While debate persists about its most appropriate treatment, women diagnosed with DCIS are faced with a paradox; although they are often reassured that the condition has been caught early and is not life-threatening, they undergo similar treatments (including mastectomy) to invasive breast cancer patients [1]. Ongoing research by the authors exploring the psychosocial impact of DCIS has found that women hold diverse beliefs about the condition [2]. This work, along with previous research, suggests that DCIS patients can be confused about the condition and that the terminology used by health professionals and the treatment recommendations given to patients may enhance this misunderstanding [3]. Health professionals' attitudes about DCIS may also vary, which in turn could impact on patient care, satisfaction and risk perceptions of the condition. Previous research suggests that discrepancies between patient and health professional perceptions of invasive breast cancer can disrupt communication and compromise care [4]. Therefore, the present study aimed to explore health professionals' perceptions of DCIS, including the terminology they use with patients and the challenges the condition presents in their work.

**Method** Two hundred and ninety-three UK health professionals (for example, surgeons, breast care nurses, radiologists, oncologists and radiographers) involved with the diagnosis and treatment of DCIS patients completed an online survey including demographic information and items relating to the terminology used to describe DCIS, risk and perceptions. A number of open-ended questions, providing qualitative data, were also included.

**Results** Findings suggest that professionals have diverse beliefs about DCIS; 35.2% perceived it as breast cancer, whereas 44% viewed it as a precancer. Oncologists were significantly more likely to view it as not breast cancer ($\chi^2 = 14.83$, df = 6, $P = 0.022$). Participants were asked to rate the risk associated with DCIS for patients' overall long-term health. The results suggest that breast care nurses, surgeons and oncologists considered it to be less serious than radiologists, radiographers and pathologists. Overall, however, 80% rated DCIS as a low (39.6%) or intermediate risk (41.3%), but despite this relatively positive prognosis 46.8% of health professionals found explaining DCIS to patients more difficult than invasive breast cancer. The qualitative findings indicate that explaining the condition and the lack of consistent terminology between health professionals was a key challenge.

**Conclusion** The findings suggest that there is considerable variation in both health professionals' perceptions of DCIS and the terminology they use. This is likely to have a substantial impact on patients' experiences and perceptions, which is the focus of ongoing research by the authors. The nature and impact of these variations warrant further exploration and debate with both health professional and patient groups in order to inform the provision of appropriate care and information to meet the needs of DCIS patients.

**Acknowledgement** Funded by a PhD studentship grant from Breast Cancer Campaign.

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## P85

### Exploring the breast cancer experiences, needs and preferences of women aged 70 years and over: a study in progress

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*Breast Cancer Res* 2008, **10(Suppl 2):**P85 (doi: 10.1186/bcr 1969)

**Background** Tens of thousands of women are diagnosed with breast cancer each year in the UK. Because the risk of developing the disease increases with age, more than one-third of cases are diagnosed in women aged 70 years and over. In addition, improvements in treatment have meant that many women, diagnosed before the age of 70, are surviving into older age. Despite this, the breast cancer experiences of women in the age group of 70 years plus have been largely neglected [1], alongside their information and support needs and preferences. So whilst it is recognised that women in this age group are less likely to use existing information and support opportunities [2], it is not known why this is so.

This presentation will report on a study that is using one-to-one interviews and focus groups to explore the breast cancer experiences of women aged 70 years and over, particularly when other health conditions are also present, and their information and support needs and preferences. Preliminary findings will be offered, alongside some developing recommendations for information and support mechanisms that are informed by, and better meet, the needs and preferences of women with breast cancer aged 70 years and over.

**Acknowledgement** Funded by Macmillan Cancer Support.

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## P86

### Exploring the acceptability of, and preferences for, an ongoing support network for known BRCA1 and BRCA2 mutation carriers

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*Breast Cancer Res* 2008, **10(Suppl 2):**P86 (doi: 10.1186/bcr 1970)

**Background** There is increasing concern that the longer-term psychological and information needs of individuals found to carry genetic mutations predisposing them to an increased risk of developing breast/ovarian cancer are not being met. The present study sought to explore preferences for an ongoing support network for mutation carriers.

**Methods** Sixteen female BRCA1/2 mutation carriers within the All Wales Medical Genetics Service attended one of three focus groups. A topic guide was used to explore patients' current and ongoing information and psychological support needs, and preferences for the content, nature and format of support group or network. Data were transcribed and thematically analysed.

**Results** The results reflected a diverse range of experiences amongst participants. Many patients reported adequate support from the genetic service both upon receipt of test results and in the longer term, whilst others were not aware that ongoing support was available. Many participants believed they and family members would benefit from increased psychological support and information. General consensus was reached that a support network, incorporating elements including a traditional support group alongside matching schemes, web-based chat forums and professional-led workshops, would be the best approach. It was felt important that such an initiative should have professional input.

**Conclusion** The data will inform the development of an appropriate support network for mutation carriers to help them adapt to living with their genetic risk and cope with their worries for themselves and family. The results of the study will also inform the development of similar support networks for other at-risk patients.

## P87

### The QUEST study: a multicentre randomised trial to assess the impact of the type and timing of breast reconstruction on quality of life following mastectomy

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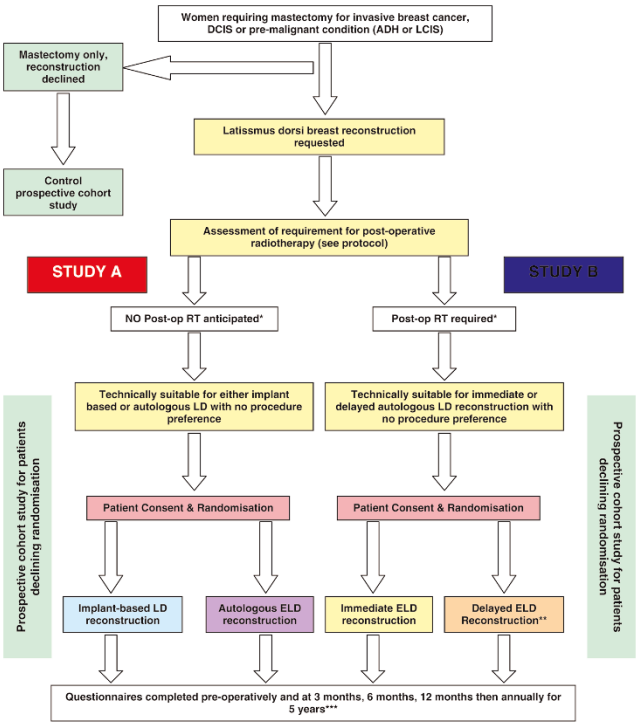
*Breast Cancer Res* 2008, **10(Suppl 2):**P87 (doi: 10.1186/bcr 1971)

**Background** Breast reconstruction is performed to improve the quality of life and body image for women facing mastectomy. Whilst significant anecdotal evidence and surgical dogma exists regarding the optimal reconstructive practice, a comprehensive MEDLINE literature review has revealed a paucity of well designed, statistically powered studies to address the impact of either the type or the timing of breast reconstruction on these key patient-reported outcomes. There is little high-quality evidence to support the benefit of immediate versus delayed breast reconstruction, for example, or to suggest the superiority of autologous over implant-assisted reconstructions in terms of improvements in quality of life or body image, particularly in the context of postoperative radiotherapy (RT). There is also very limited evaluation of the impact of latissimus dorsi (LD) breast reconstruction, although this is the procedure most commonly offered by oncoplastic breast surgeons in the UK. There is currently, therefore, very little to guide patients or their surgeons in making important decisions regarding their reconstructive options.

**Study aim** The Quality of Life following Mastectomy and Breast Reconstruction (QUEST) study aims to apply rigorous scientific methodology to definitively address causality and to address key reconstructive questions, thus ultimately providing patients and their surgeons with high-quality evidence as the basis for truly informed consent.

**Study design** The QUEST study is the first multicentre statistically powered randomised controlled trial to assess the impact of the type and timing of the most commonly offered form of breast reconstruction on quality of life following mastectomy. The study consists of two parallel randomised controlled trials, with study entry determined by a preoperative assessment of the requirement for postoperative RT (see Figure 1). Women unlikely to require postoperative RT will be randomised to either autologous or implant-assisted LD breast reconstruction, while those requiring RT will be randomised to either immediate or delayed extended LD procedures. These approaches are consistent with current accepted reconstructive practice. The use of a randomised methodology in the context of patient-centred procedures of this kind has been much criticised on the basis of additional patient stress and the view that patients who consent to randomisation are

**Figure 1 (abstract P87)**




The QUEST study flow chart. ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma *in situ*; ELD, extended latissimus dorsi; LCIS, lobular carcinoma *in situ*. *Prospective patients will undergo preoperative evaluation of the breast (mammogram/ultrasound scan/MRI and core biopsy) and axilla (ultrasound scan and fine-needle aspiration of suspicious nodes or preoperative sentinel lymph node biopsy) to determine whether they are likely to require postoperative radiotherapy (RT) according to each centre's local RT guidelines (for example, multifocality, grade 3, lymphovascular invasion and nodal involvement). **Delayed reconstructions must be performed within 12 months of randomisation. ***Patients in the delayed arm will complete baseline questionnaires prerandomisation and at 3 months and 6 months post mastectomy, then prereconstruction (usually approximately 12 months postoperative). Following reconstruction, follow-up will be as per the other arms – 3 months, 6 months, 12 months and annually for 5 years.

likely to differ significantly from the population as a whole, thus limiting the generalisability of the results. The QUEST study, however, addresses this issue by recruiting patients declining or not eligible for randomisation to a prospective cohort study, thus creating a comprehensive cohort and allowing the impact of the decision-making process to be assessed.

**Patients** Women requiring mastectomy for invasive breast cancer, ductal carcinoma *in situ*, atypical ductal hyperplasia, lobular carcinoma *in situ* or other premalignant condition who request LD breast reconstruction are eligible for inclusion in the study.

**Sample size** A power calculation has suggested that 150 patients will be required in each study arm to detect an effect size of 0.4 at the 5% level with 90% confidence. As a randomised trial in breast reconstruction is a challenging prospect, the main study will be preceded by a 12-month feasibility study of the same design to assess patient recruitment to each study arm.

**Conclusion** As the survival rate from breast cancer increases, quality of life becomes an increasingly significant outcome. This is an exciting, innovative and challenging project, but it is only through the use of rigorous scientific methodology that definitive evidence can be produced. Women facing mastectomy deserve truly informed decision-making regarding their reconstructive options. This is the QUEST.

## P88

## 'The sooner the better' or 'too much too soon'? A pilot prospective longitudinal study to evaluate quality of life and body image following immediate latissimus dorsi breast reconstruction

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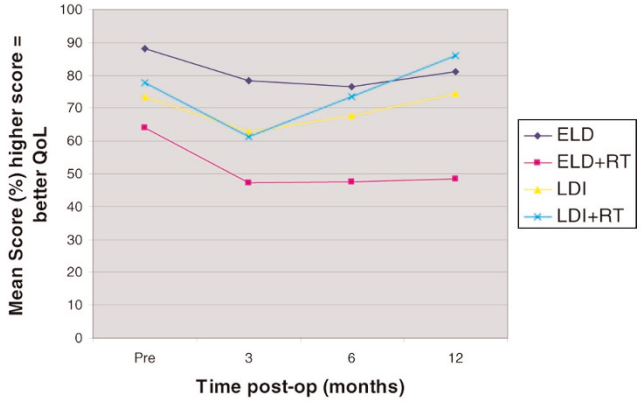
*Breast Cancer Res* 2008, **10(Suppl 2):**P88 (doi: 10.1186/bcr 1972)

**Background** Immediate breast reconstruction (BR) is currently advocated by the National Institute of Clinical Excellence on the basis of improved psychosocial outcomes with benefit of a single operative intervention. Tissue-based procedures are largely preferred due to cosmetic superiority, particularly in the context of postoperative radiotherapy (RT). The impact of this approach on quality of life (QoL), however, has never been fully evaluated. We present a pilot prospective longitudinal study that questions this practice.

**Methods** Patients undergoing immediate latissimus dorsi (LD) BR, the most commonly offered reconstructive procedure in the UK, completed validated QoL questionnaires (EORTC C30+BR23/FACT B+4) together with the Body Image Scale preoperatively and at 3 months, 6 months, 12 months and 24 months post surgery. QoL and body image were compared in women undergoing extended (ELD) and implant-assisted (LDI) procedures ± postoperative RT.

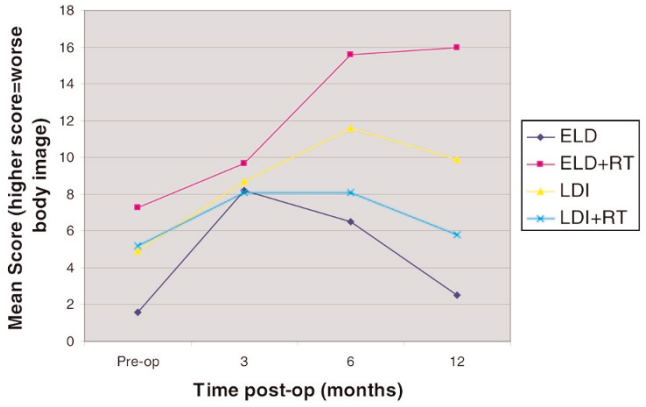
**Results** Sixty-two women underwent 46 (74%) implant-assisted and 16 (26%) extended LD BR with RT in 13 (28%) and seven (44%) cases, respectively. One hundred and ninety-four questionnaires were completed with a median follow-up of 6 months (range 3–24 months). The QoL in all groups declined initially before improving, with patients undergoing implant-assisted procedures reporting a more rapid return to baseline levels of QoL than those in the extended group (Figure 1). Body image was superior in the

**Figure 1 (abstract P88)**




Variation in Global EORTC scores with time.

**Figure 2 (abstract P88)**




Variation in body image scores with time.

extended group when compared with LDIs at 12 months, but QoL was comparable (Figure 2). Irradiation of ELD but not LDI reconstructions produced dramatic and persistent deteriorations in both QoL and body image.

**Conclusion** Body image is superior following tissue-based reconstruction, but this difference is not reflected by superior QoL. Combining ELD with RT, however, has a profound effect on both body image and QoL. Surgeons should consider patient-reported outcomes as well as cosmesis when recommending surgical options.

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## P89

## Diagnosed with breast cancer whilst on a family history screening programme: an exploratory qualitative study

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**Background** Early mammographic screening (under the age of 50) is offered to many women in the UK who are at moderate or high risk of developing breast cancer because of their family history of the disease [1]. While studies are underway to establish the clinical effectiveness of early mammographic screening [2], relatively little is understood about the impact of early and regular surveillance on the psychological wellbeing of women [3], and even less about the impact of being diagnosed with breast cancer while on a screening programme. This qualitative study explores the emotional effect that the diagnosis of breast cancer had on women, and the value they placed on having joined the family history screening programme, both pre and post diagnosis.

**Methods** In-depth interviews were undertaken with 12 women in the UK, aged 35 to 50, diagnosed with a screen-detected breast cancer while on a mammographic surveillance programme because of their family history of breast cancer. The interviews include explorations of women's motivations for joining the early screening programme, their views about the value of mammography, and the process of and their reactions to their cancer detection.

**Results** The interviews revealed different convictions of the likelihood of developing breast cancer, but all women gained a strong sense of reassurance from the possibility of the early detection of a cancer through undergoing regular mammography. A number of women relied solely on mammography to detect abnormalities, often reluctant to examine their breasts due to the fear of finding a symptom. Reactions to the diagnosis of a cancer ranged from relief to intense shock. While all women were very positive about having undergone mammography, not all wanted to continue with screening. For some, prophylactic mastectomy was preferable, to reduce future cancer risk, and to alleviate anxieties about the detection of another cancer at each subsequent screen. Our study shows that for this group of women, detection of their cancer was ultimately a positive experience. They perceived surveillance to have achieved its goal of detecting a cancer at a stage when treatment was likely to be effective, and the future described was often one free of the fear of cancer that they had carried with them for many years.

**Clinical implications** Not all women diagnosed with breast cancer will have a pronounced negative reaction to their diagnosis; the period during which they are under threat of developing the disease may be a time of psychological preparation, thus enabling an easier adjustment to the diagnosis. Women may seek bilateral mastectomy as their treatment of choice, although their cancer my warrant a less radical approach. Surgeons need to be aware of the fears associated with future screening. Identification of women who are averse to self-examination may allow the development of strategies to overcome this avoidance. Women who have experienced the process of diagnosis and treatment may be in an ideal position to provide a mentoring system to women on the family history screening programme who are very distressed at the possibility of being diagnosed with breast cancer. Their perceptions of being able to cope should a breast cancer be detected may be improved through such contact.

**Acknowledgement** Research funding from Cancer Research UK.

***Management Group** Stephen Duffy, Wolfson College of Preventive Medicine, London; Hilary Fielder, Screening Services, Velindre NHS Trust, Wales; Jonathon Gray, Institute of Medical Genetics, University Hospital Wales; James Mackay, Institute of Child Health, London; and Douglas Macmillan, Professorial Unit of Surgery, University of Nottingham.

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## P90

## 'More positive about mammography' – reactions of women to a false positive recall: a qualitative study of women at risk of familial breast cancer

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**Background** Annual mammographic screening from the age of 40 is recommended for women in the UK whose family history places

them at a lifetime risk of developing breast cancer of ≥1:6 [1]. While the clinical benefits of screening younger women at increased risk are not established, emerging evidence suggests screening may lead to increased survival [2]. However, little is understood of the emotional impact of screening on women with a family history. This is particularly important in view of the increased likelihood of recall for further tests in women under 50 years old compared with those over 50 years old [3]. A recent questionnaire study of the psychological impact of mammographic screening on women under 50 years old with a family history of breast cancer showed that, contrary to expectations, women who were recalled for further tests prior to an all-clear result reported significantly more positive feelings post result about screening than women not recalled [4]. This complementary qualitative study explored the value women placed on having joined a programme of regular screening, and sought to understand the reactions of women who had received an initial all-clear result and who had received an all-clear result following further tests.

**Methods** In-depth interviews were performed with 58 women, aged 35 to 50, undergoing mammographic surveillance due to their family history of breast cancer, and who had taken part in the questionnaire survey. Women with initial all-clear results (36 women) and women with all-clear results after further testing (22 women) were recruited. Interview topic areas included experiences of breast cancer within the family, motivations for joining the programme, likelihood of developing breast cancer, views of mammography, emotional responses to the screening process and results, and views about the overall value of participating in the programme. All interviews were transcribed verbatim and analysed thematically.

**Results** Participating in the programme reflected a strong desire within the women to be proactive about their risk of breast cancer, particularly if delay in diagnosis was a factor in their relatives' disease. Regardless of their individual experiences of cancer within the family, faith in the ability of mammography to detect a cancer at an early stage gave reassurance that a cancer diagnosis could lead to a positive outcome. Many women placed a much greater faith in mammography than in their own ability to detect an abnormality, particularly at the very early stages of a symptom developing. A high degree of reassurance and relief was described by women receiving an initial all-clear result, although for a small number this relief was slightly tempered by doubts about the accuracy of their result. Of the women recalled for further tests, most experienced immediate distress; for some, this remained throughout the process of further testing. The subsequent all-clear result was often followed by an increased feeling of security and reassurance, and the women appeared to place an even greater faith in screening than those receiving an initial clear result. Far from being a negative component of screening, recall was interpreted as proof that mammography worked. Recall for a nonmalignant symptom, or for an unclear mammogram, enhanced belief in the detection of any future malignancy. Women's concerns about being at risk of developing breast cancer appear to be alleviated by participating in an annual surveillance programme. Irrespective of their screening result, their stories demonstrated the significance of mammography in enabling them to establish a sense of being in control of their family history.

**Clinical implications** These findings highlight the emotional benefits to many women of participating in a family history screening programme. Counselling women prior to joining could include detailed discussions of the effectiveness of mammography, which may need to be reiterated with screening results. The importance of remaining breast aware between screens should be reinforced. These findings are important in the context of the introduction of a national screening programme for women under 50 years old with a family history of breast cancer, and the increased likelihood of recall in this group compared with women over the age of 50.

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## P91

## What is the psychological impact of mammographic screening on younger women with a family history of breast cancer? Findings from a prospective cohort study (PIMMS)

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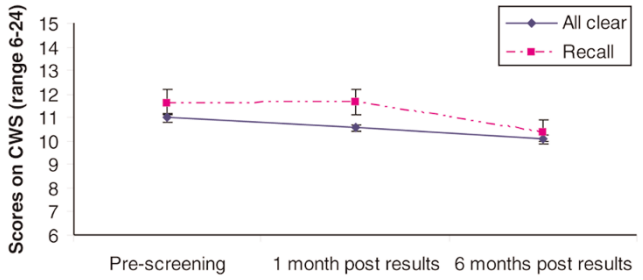
**Background** It is not yet known whether the benefits of regular screening for women with a family history of breast cancer (FHBC) outweigh the harms. One of the harms associated with having a mammogram is recall for further tests such as additional imaging and biopsies [1]. This has been shown to cause significant anxiety in the short term, and possibly the long term, in women in routine screening [2]. Given the greater cancer worry in women with a FHBC [3], it is possible they may be particularly adversely affected by a recall. This multicentre, prospective study investigated both the positive and negative psychological effects of regular mammographic screening in women <50 years with a family history of breast cancer [4].

**Methods** Women who received an immediate all-clear result after mammography ($n$ = 1,174) and women who were recalled for further tests prior to receiving an all-clear result (false positive) ($n$ = 112) completed questionnaires: 1 month before mammography, and 1 month and 6 months after receiving final results. The questionnaires included measures of cancer worry, psychological consequences and perceived benefits of breast screening.

**Results** See Figure 1. Women who received an immediate all-clear result experienced a decrease in cancer worry and negative psychological consequences immediately post result, whereas women who were recalled for further tests did not. By 6 months this cancer-specific distress had reduced significantly in both

**Figure 1 (abstract P91)**




Cancer Worry Score (CWS).

groups. Changes in levels of distress were significantly different between the two groups, but in absolute terms the differences were not large. Recalled women reported significantly greater positive psychological consequences of screening immediately post-result, and were also more positive about the benefits of screening compared with women who received an immediate all-clear result.

**Conclusion** For women receiving an immediate all-clear result, participating in annual mammographic screening is psychologically beneficial. Furthermore, women who are recalled for further tests do not appear to be psychologically harmed by screening. Women's positive views about mammography suggest that they view any distress caused by recall as an acceptable part of screening.

**Acknowledgement** Research funding from Cancer Research UK.

***PIMMS Study Management Group** Other members of the management group are: Stephen Duffy, Wolfson College of Preventive Medicine, London; Gareth Evans, Department of Clinical Genetics, St Mary's Hospital, Manchester; Hilary Fielder, Screening Services, Velindre NHS Trust, Wales; Jonathon Gray, Institute of Medical Genetics, University Hospital Wales; James Mackay, Institute of Child Health, London; and Douglas Macmillan, Professorial Unit of Surgery, University of Nottingham.

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## P92

## Food choice and phytoestrogen consumption in women previously treated for postmenopausal breast cancer

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**Background** Phytoestrogens are plant-derived, bioactive substances with a chemical structure similar to that of 17β-oestradiol. Women previously treated for breast cancer may increase their phytoestrogen intake to avoid conventional hormone replacement therapy or because of a belief that they may help avoid recurrence [1,2]. There is no recommended daily intake and there are some concerns about phytoestrogen safety in this group, although the evidence is conflicting and more research is needed [3,4].

**Methods** Three hundred and sixteen women each completed a 4-day food and drink diary (14 of whom also completed a 7-day weighed intake diary 6 weeks previously). The 55 most recently recruited women collected their urine for 24 hours whilst completing their diaries and were interviewed by telephone regarding their food choices since diagnosis.

**Results** A new dietary analysis database was created using peer-reviewed published data and analysing 34 additional foods and beverages for which there were no published results. The urinanalysis results contributed validation data. A summary of the dietary intake data is shown in Table 1. There was a lack of primary analytical data on the phytoestrogen profile of many foods and beverages routinely consumed by this study population. However, food frequency data from the highest quartile show the important contribution of nonsoya foods to high intakes (Table 2). Telephone

**Table 1 (abstract P92)**

**Summary of intake data by receptor status and antioestrogenic drug prescription**

| Total phytoestrogen intake (μg/1,000 kcal) ($n = 316$) | | | $n$ | First quartile | Second quartile | Third quartile | Fourth quartile |
|---|---|---|---|---|---|---|---|
| | | Receptor status | | | | | |
| Mean | 8,388 | ER-negative | 42 | 11 (14%) | 11 (14%) | 13 (16%) | 7 (9%) |
| Range | 126 to 77,703 | ER-positive | 182 | 40 (51%) | 42 (53%) | 48 (61%) | 52 (66%) |
| Quartile ranges | | Not available | 92 | 28 (35%) | 26 (33%) | 18 (23%) | 20 (25%) |
| First quartile | <3,817 | Antioestrogenic drugs | | | | | |
| Second quartile | 3,817 to 6,798 | No prescription | 109 | 30 (38%) | 30 (38%) | 25(32%) | 24 (30%) |
| Third quartile | 6,799 to 10,255 | Tamoxifen or arimidex[a] | 200 | 47 (59%) | 47 (59%) | 52 (65%) | 54 (68%) |
| Fourth quartile | >10,255 | Other/missing | 7 | 2 (3%) | 2 (3%) | 2 (3%) | 1 (2%) |

[a]AstraZeneca, London, UK.

**Table 2 (abstract P92)**

**Main food sources of phytoestrogens**

| From highest quartile | Daidzein | Genistein | Glycitein | Formononetin[a] | Biochanin A[a] | Coumestrol[a] | Matairesinol[a] | Secoisolariciresinol[a] |
|---|---|---|---|---|---|---|---|---|
| Main food group source | Cereal foods | Cereal foods | Soya products | Fruit, vegetables | Legumes, blackcurrants | Fruit, vegetables | Tea (black leaves), cereal foods | Tea (black leaves), fruit, vegetables |

[a]Limited data available on content in some foods.

interviews were completed by 39 subjects. For most women, having breast cancer had not changed their diet. Health concerns unrelated to cancer, the needs of other family members, cooking on a budget and physical appearance all seemed more important than the impact of the cancer diagnosis.

**Discussion** Variation in phytoestrogen intakes and metabolite excretion reflect food preferences, dietary analysis database limitations and likely variations in existing knowledge combined with a lack of routine access to dietary information. In the absence of definitive advice, more immediate health and social concerns influence food choice rather than past breast cancer diagnosis.

**Conclusion** No data previously existed on intake in this potentially vulnerable group and these data will help evaluate the health implications related to such phytoestrogen consumption patterns.

**Acknowledgement** Funded by the Food Standards Agency, UK.

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## P93

### Homeopathy service in an NHS hospital breast cancer clinic: outcome study

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*Breast Cancer Res* 2008, **10(Suppl 2):**P93 (doi: 10.1186/bcr 1977)

**Background** Since 2004, Burton-upon-Trent's NHS Hospital Breast Care Unit has run a homeopathy service providing an alternative therapy for symptoms affecting women during and after treatment of their primary disease. The majority suffer from menopause-type symptoms arising from breast cancer treatment. Such symptoms can be bad enough to affect long-term compliance with drug regimes. Patients receive a course of treatment from a qualified homeopath, consisting of a series of patient-centred consultations plus individualised homeopathic medicines. The present study aimed to evaluate the benefit gained by women attending the homeopathic service between April 2005 and March 2007.

**Methods** Routine data gathered at each homeopathic consultation included a validated patient-generated and assessed outcome measure (MYMOP2), in which patients choose their worst symptoms, and score them and their general wellbeing on a seven-point Lickert scale from 0 (very good) to 6 (very bad). A change >0.8 is considered clinically significant improvement.

**Results** Initial and final MYMOP2 data were collected from 104 women, mean age 51.2 years, range 19–74 years. The most frequently chosen worst symptoms were hot flushes (46%), breast pain (19%), depression/anxiety (10%) and aches/pains (9%). The mean worst symptom score at presentation was 4.1 ± 0.126, and at the end of the course was 2.34 ± 0.16 ($P < 0.001$), with 73% reporting an improvement ≥1. General wellbeing at presentation scored 3.2 ± 0.13 and at the end 2.3 ± 0.15 ($P < 0.001$).

**Conclusion** These results indicate that homeopathy can offer a valuable addition to mainstream conventional therapy for breast cancer patients, possibly helping to improve compliance and therefore long-term survival.

## P94

### Primary ductal carcinoma *in situ* mammosphere formation: importance of the epidermal growth factor and Notch receptor signalling pathways

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The cancer stem cell hypothesis suggests that targeting stem-like cells in cancer will improve current therapeutic strategies. *In vitro* culture of mammospheres (MS), colonies that are analogous to neurospheres, has been used to study factors affecting the self-renewal and growth of ductal carcinoma *in situ* (DCIS) in 29 cases. The MS culture system demonstrates a small subset of DCIS cells with self-renewal clonogenic capacity showing 1.5 ± 0.1% MS forming efficiency (MFE), which is greater than normal breast MFE, 0.5 ± 0.1% ($P < 0.0001$). DCIS MS demonstrated an increased growth rate compared with normal, yielding MS >60 μm within 3 days rather than 7 days. The MFE was greater in high (1.6 ± 0.1%) compared with low (1.1 ± 0.1%, $P = 0.012$) histological grade DCIS, suggesting a link between the number of MS-initiating cells and recurrence rates.

Since normal breast MS formation was known to depend on epidermal growth factor (EGF) and Notch receptor signalling, we investigated these pathways in DCIS MS. Only high-grade DCIS MFE was decreased in the presence of the EGF receptor inhibitor, Gefitinib, when no EGF was present in the media (1.36 ± 0.16% to 0.56 ± 0.2183, $P = 0.0017$). This suggests high-grade DCIS secrete growth factors that signal via the EGF receptor. Notch was aberrantly activated in DCIS compared with normal breast, demonstrated by increased levels of activated Notch intracellular domain (NICD) and downstream targets Notch 4 and Hes-1. A γ-secretase inhibitor, DAPT, which inhibits the activating cleavage of Notch receptors, reduced DCIS MFE from 0.88 ± 0.07% to 0.51 ± 0.08% ($P = 0.0005$). A Notch 4 receptor neutralising antibody reduced DCIS MFE, 0.97 ± 0.1% to 0.2 ± 0.05%, resulting in no MS formation in two out of six cases ($P < 0.0001$). Furthermore, presence of NICD by immunohistochemistry predicted recurrence

in patients with 5 years' follow up after surgery ($n = 50$, $P = 0.012$).

These data indicate that Notch and EGF receptor signalling pathways are important in DCIS MS formation, and therapeutic inhibition of the Notch signalling may increase recurrence-free survival after surgery.

**Acknowledgement** Funded by Breast Cancer Campaign (grant# MAY2005:21).

**P95**

## PARP-1 inhibitor monotherapy and combination therapy in a preclinical mouse model of Brca2 mutant breast cancer

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**Background** Women who inherit a germline mutation in the *BRCA2* gene are predisposed to breast cancer [1]. Therapies targeted specifically at these cancers have so far remained elusive. Recently, inhibition of poly-ADP-(ribose)-polymerase-1 (PARP-1) was shown to cause high levels of death in cells and xenografts deficient in BRCA2 [2-4].

**Methods** We have conditionally deleted *Brca2* and *p53* within murine mammary epithelium, resulting in the development of naturally arising tumours from 6 months of age. These tumours have been treated *in situ* with a highly potent inhibitor of PARP-1, either alone or in combination with carboplatin. The tumour size was followed by regular measurement with calipers.

**Results** Daily exposure to 50 mg/kg PARP-1 inhibitor caused significant regression or growth inhibition in the majority of *Brca2*/*p53*-deficient tumours, in comparison with *p53*-deficient or *pten*-deficient control tumours. Combination treatment with carboplatin did not enhance initial tumour regression compared with carboplatin treatment alone. However, prolonged treatment with PARP-1 inhibitor, after an initial 28-day combination therapy, increased the time to tumour relapse compared with 28 days of carboplatin monotherapy or combination therapy.

**Conclusion** This is the first preclinical study to show *in vivo* hypersensitivity of naturally arising *Brca2*-deficient mammary tumours to PARP-1 inhibition monotherapy and combination therapy.

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