The effect of menstrual cycling on genomic predictive biomarkers in premenopausal breast cancer

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February 2020

A thesis submitted to The University of Adelaide in fulfilment of the requirements for the admission to the degree Doctor of Philosophy

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Abstract

Emerging in the clinic are new assays that use gene expression profiling to predict breast cancer response to treatment. A leading genomic assay is Oncotype DX, which quantifies a panel of 21 genes to calculate a Recurrence Score. The Oncotype DX 21-gene Recurrence Score is predictive of adjuvant chemotherapy benefit for women with early-stage, hormone receptor (HR)-positive, HER2-negative breast cancer and is recommended in clinical guidelines for guiding adjuvant chemotherapy treatment decisions. However, genomic assays such as Oncotype DX were largely developed in postmenopausal women and it remains unclear whether they are suitable for use in premenopausal women, where fluctuations in estrogen and progesterone during the menstrual cycle could affect genomic biomarker expression. The studies described in this thesis aimed to determine how menstrual cycling affects the Oncotype DX 21-gene signature using paired breast cancer samples and mouse models of breast cancer.

To investigate how Oncotype DX Recurrence Scores fluctuate in HR-positive breast cancer, paired formalin-fixed paraffin-embedded invasive breast cancer samples were collected approximately 2 weeks apart from women <50 years old, and compared to women >50 years old and women with HR-negative disease. The Oncotype DX 21-gene signature was assessed through quantitative RT-PCR and 21-gene Recurrence Scores were calculated using the Oncotype DX Recurrence Score algorithm. Increased discordance in Recurrence Scores was observed between paired breast cancer samples collected from younger women (3.2±2.5; mean±stdev) compared to older women (2.0±1.7; p=0.04) and women with HR-negative disease (2.3±1.0; p=0.02). Linear regression analysis revealed that for every one-year decrease in age, discordance in Recurrence Scores increased by 0.07 units (p=0.0035). In young women, discordances were driven by variable expression of Proliferation- and HER2-group genes.

To determine whether the ovarian cycle contributes to the increased variability in Recurrence Scores observed in younger women, HR-positive mammary tumours were dissected from naturally cycling MMTV-PyMT mice at either the estrus or diestrus phase of the ovarian cycle. Tumours collected at diestrus show significant differences in expression of 6 Oncotype DX signature genes (*Ki67*, *Ccnb1*, *Esr1*, *Erbb2*, *Grb7*, *Bag1*; p≤0.05) and a significant increase in Recurrence Score (21.8±2.4; mean±SEM) compared to tumours dissected at estrus (15.5±1.9; p=0.03). Clustering analysis revealed a subgroup of tumours collected at diestrus characterised by increased expression of Proliferation- (p<0.001) and Invasion-group (p=0.01) genes, and increased Recurrence Score (p=0.01). These tumours also exhibited higher expression of

Estrogen-group genes (p=0.005) suggesting increased sensitivity to hormonal fluctuations during the ovarian cycle.

Variable concentrations of progesterone at the time of tissue collection may influence biomarker expression and affect Recurrence Score. We defined the effects of progesterone on biomarker expression in HR-positive breast cancer cells (T-47D, ZR-75-1) using mammary fat pad-xenograft BALB/c nude mice treated with exogenous estrogen with or without co-treatment with progesterone. In T-47D xenograft tumours, progesterone co-treatment reduced *PGR* (p=0.03) and *KI67* (p=0.03) gene expression; an effect mirrored in their protein expression (p=0.03, p=0.02 respectively). In ZR-75-1 cell lines, progesterone co-treatment reduced *PGR* (p=0.05) gene expression, while no differences were observed in protein expression (p>0.05).

Our results suggest that menstrual cycling affects the Oncotype DX 21-gene signature and influences Recurrence Scores. Oncotype DX may be less effective for guiding chemotherapy treatment decisions for cycling premenopausal women, as Recurrence Scores might partially depend on the menstrual cycle stage at the time of tissue collection. There is a pressing need for large-scale, long-term prospective studies to investigate the effects of menstrual cycling on genomic predictive biomarkers, which is required for assays such as Oncotype DX to be tailored for use in premenopausal women.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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February 2020

Statement of author contribution

I conducted 99% of experiments reported in the thesis with supervisory input from Associate Professor Wendy Ingman, Professor Timothy Price, and Dr Amanda Townsend. Input provided by others is reported below for each chapter.

Chapter 3

I completed a majority of the work reported in the chapter, including all patient identification and recruitment, RNA extraction, real-time PCR and gene expression analysis, and immunohistochemical and histological experiments. Dr Pallave Dasari assisted me with obtaining ethical and governance approval for both retrospective and prospective projects. Formalin-fixed paraffin-embedded tissue sections were cut by The Queen Elizabeth Hospital Pathology Department for the retrospective study. I performed hematoxylin and eosin stains on all these samples, which were then assessed by Associate Professor Wendy Raymond to confirm presence of invasive disease. Dr David Walsh provided access to patient samples during the prospective project. I completed all data analysis, with statistical support provided by Ms Suzanne Edwards.

Chapter 4

I completed the majority of work reported in this chapter, including general animal procedures (mouse breeding, genotyping, estrous cycling tracking), tissue collection and processing. I also completed the protein analysis (tissue sectioning, hematoxylin and eosin staining, and staining and analysing expression of biomarkers through immunohistochemistry) and gene expression analysis (RNA extraction, real-time PCR and analysis). Hematoxylin and eosin stained tumours were assessed by Dr Lucy Woolford assessed to define tumour grade. Dr Lachlan Moldenhauer assisted with the t-sne analysis of gene expression data.

Chapter 5

Dr Danielle Glynn provided support with animal ethical applications and training in animal procedures. I performed all remaining experiments, including; *in vitro* hormone treatment (cell culture, hormone treatments) and *in vivo* mammary fat pad xenografts (including estradiol pellet implantation, mammary fat pad inoculations, tumour monitoring, estrus cycle tracking, and tissue collection). I also performed all gene and protein analysis, as detailed above for chapter 4.

Acknowledgements

First and foremost, I would like to thank my principal supervisor, Associate Professor Wendy Ingman, for the extensive support, encouragement, and guidance offered over the years. I am immensely grateful for the wonderful opportunities and advice you have provided me. I have enjoyed our interactions at both the academic and personal level, and appreciate your genuine joy and enthusiasm when you see your students succeed. I must also acknowledge my co-supervisors, Professor Tim Price and Dr Amanda Townsend, for their invaluable advice, input, and support throughout my project.

I would also like to thank the other members of the Breast Biology and Cancer Unit past and present that I have had the pleasure of working alongside. Thank you for your constant support and friendship over the years, and for making the lab a warm, positive and enjoyable environment. A special acknowledgement goes to Dr Pallave Dasari, for not only her immense support throughout this project, but also for her sincere advice and guidance, and helping me make the most of my potential. I must also thank Ms Leigh Hodson and Dr Danielle Glynn for their technical expertise, scientific input, and assistance with experiments. Many thanks also go to everyone else at the Basil Hetzel Institute, as well as The Hospital Research Foundation and The University of Adelaide, for their support during my candidature.

This project involved a large group of people, of whose support and guidance I am immensely grateful for. I am truly grateful to the surgeons at The Queen Elizabeth Hospital for not only their assistance in sample collection, but also for their intellectual expertise and input into this project. I must also thank the oncologists, pathologists, radiologists, and nursing staff at The Queen Elizabeth Hospital for their help, which without this project would not have been possible. I am also truly thankful to the patients at The Queen Elizabeth Hospital who kindly provided tissue samples for my research.

Last but not least, I am extremely grateful to my friends and family for their constant support throughout the years. To my parents, thank you for your never-ending support and encouragement; and for all the pick-ups and drop-offs at odd hours. I am extremely grateful for your patience and reassurance through the rather challenging days, but also for you enthusiasm and pride at my successes. To my fellow PhD friends, Amy and Lisa, thank you for your friendship and compassion—and the copious amounts of coffee and cake—throughout the years. I am glad to have shared this journey with you; celebrating our successes together and supporting each other through the more difficult times. I am very lucky to have such supportive family and friends, which without I would not have been able to complete my studies.

Publications arising from this thesis

Journal articles

Bernhardt SM, Dasari P, Walsh D, Townsend AR, Price TJ and Ingman WV. Breast cancer surgery – is there an optimal time of the month? Oncology Letters. doi: 10.3892/ol.2020.11771 (Appendix A)

Bernhardt SM, Dasari P, Walsh D, Townsend AR, Price TJ and Ingman WV (2016). Hormonal modulation of breast cancer gene expression: implications for intrinsic subtyping in pre-menopausal women. Front. Oncol. 6:241. doi: 10.3389/fonc.2016.00241
(Appendix B)

Manuscripts in preparation

Bernhardt SM, Dasari P, Glynn DJ, Woolford L, Raymond W, Moldenhauer LM, Walsh D, Townsend AR, Price TJ and Ingman WV. Menstrual cycling critically affects the Oncotype DX 21-gene signature: implications for predictive biomarker assays in premenopausal women.

Abstracts arising from this thesis

2019

Bernhardt SM, Dasari P, Glynn DJ, Woolford L, Raymond W, Moldenhauer LM, Walsh D, Townsend AR, Price TJ and Ingman WV. "Menstrual cycling critically affects the Oncotype DX 21-gene signature: implications for predictive biomarker assays in premenopausal women" San Antonio Breast Cancer Symposium. San Antonio, Texas, USA. Poster presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "The impact of menstrual cycling on Oncotype DX Recurrence Scores in premenopausal breast cancer patients". The Joint Annual Scientific Meetings of the Endocrine Society of Australia and the Society for Reproductive Biology. Sydney, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Woolford L, Raymond W, Moldenhauer LM, Walsh D, Townsend AR, Price TJ, Ingman WV. "Menstrual cycling critically affects the Oncotype DX 21-gene signature: implications for predictive biomarker assays in premenopausal women". The Robinson Research Institute Symposium. Adelaide, Australia. Poster presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "The impact of menstrual cycling on Oncotype DX Recurrence Scores in premenopausal breast cancer patients". The Queen Elizabeth Hospital Research Expo. Adelaide, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "The impact of menstrual cycling on Oncotype DX Recurrence Scores in premenopausal breast cancer patients". Florey Postgraduate Research Conference. Adelaide, Australia. Poster presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "The clinical utility of breast cancer genomic tests for premenopausal women". Australian Society for Medical Research. Adelaide, Australia. Oral presentation.

2018

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Ovarian hormones modulate the expression of genes involved in breast cancer diagnosis". Australian Society for Medical Research. Adelaide, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Ovarian hormones modulate the expression of genes involved in breast cancer diagnosis". The Joint Annual

Scientific Meetings of the Endocrine Society of Australia and the Society for Reproductive Biology. Adelaide, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Ovarian cycle stage affects intrinsic subtyping of mouse mammary tumours". Breast Screen Australia. Adelaide, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Ovarian hormones alter breast cancer gene expression and diagnosis in mouse models". South Australian Breast Cancer Study Group meeting. Adelaide, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Ovarian hormones modulate the expression of genes involved in breast cancer diagnosis". The Queen Elizabeth Hospital Research Expo. Adelaide, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Ovarian hormones alter gene expression and diagnosis in mouse models of breast cancer". Florey Postgraduate Research Conference. Adelaide, Australia. Poster presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Ovarian hormones modulate the expression of genes involved in breast cancer diagnosis". Australian Society for Medical Research. Adelaide, Australia. Oral presentation.

2017

Bernhardt SM, Townsend AR, Price TJ, Ingman WV. "Hormonal modulation of breast cancer gene expression: Implications for premenopausal women". The Joint Annual Scientific Meetings of the Endocrine Society of Australia and the Society for Reproductive Biology. Perth, Australia. Oral presentation.

Bernhardt SM, Dasari P, Glynn DJ, Townsend AR, Price TJ, Ingman WV. "Expression of key genes used for subtyping are altered by ovarian cycle stage". The Robinson Research Institute Symposium. Adelaide, Australia. Poster presentation.

Bernhardt SM, Townsend AR, Price TJ, Ingman WV. "Hormonal modulation of breast cancer gene expression: Implications for pre-menopausal women". The Queen Elizabeth Hospital Research Day. Adelaide, Australia. Oral presentation.

Bernhardt SM, Townsend AR, Price TJ, Ingman WV. "Hormonal modulation of breast cancer gene expression: Implications for pre-menopausal women". Florey Postgraduate Research Conference. Adelaide, Australia. Poster presentation.

Bernhardt SM, Townsend AR, Price TJ, Ingman WV. "Hormonal modulation of breast cancer gene expression: Implications for pre-menopausal women". Australian Society for Medical Research. Adelaide, Australia. Poster presentation.

2016

Bernhardt SM, Townsend AR, Price TJ, Ingman WV. "Ovarian hormones regulate the expression of key genes involved in breast cancer diagnosis". The Robinson Research Institute Symposium. Adelaide, Australia. Poster presentation.

Bernhardt SM, Townsend AR, Price TJ, Ingman WV. "Estrogen and progesterone modulate gene expression in breast cancer cell lines". The Queen Elizabeth Hospital Research Day. Adelaide, Australia. Poster presentation.

Awards and honours

2019

- Robinson Research Institute Best Student Poster Award, Robinson Research Institute
- John Barker Poster Prize for Cancer Research, The University of Adelaide
- Florey Medical Research Foundation Prize, Florey Medical Research Foundation
- Adelaide Medical School Poster Prize, The University of Adelaide
- Endocrine Society of Australia Travel Award, The Endocrine Society of Australia
- Novartis Junior Scientist Award Finalist, The Endocrine Society of Australia
- Ross Wishart Memorial Award Finalist, Australian Society for Medical Research
- Ross Wishart People's Choice Award, Australian Society for Medical Research

2018

- Adelaide Medical School Poster Prize, The University of Adelaide
- Adelaide Medical School Research Travel Award, The University of Adelaide
- Novartis Junior Scientist Award Finalist, The Endocrine Society of Australia
- Science in Public Media & Communications Workshop Award, The Hospital Research Foundation and The Basil Hetzel Institute
- Three Minute Thesis (3MT) University Finalist, The University of Adelaide

2017

- Adelaide Medical School Research Travel Award, The University of Adelaide

Abbreviations

AR	Androgen receptor
Areg	Amphiregulin
ATCC	American type culture collection
bp	Base pair
CDK	Cyclin dependant kinases
cDNA	Complementary DNA
CT	Cycle threshold
DAB	3, 3′-diaminobenzidine
DCC	Dextran-coated charcoal
DCIS	Ductal carcinoma *in situ*
DFS	Disease free survival
E2	Estrogen
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial–mesenchymal transition
ER	Estrogen receptor
EtOH	Ethanol
FBS	Foetal bovine serum
FDA	U.S. Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
FSH	Follicle-stimulating hormone
HER2	Human epidermal growth factor receptor-2
HR	Hormone receptor
HRP	Horseradish peroxidase
IDC	Invasive ductal carcinoma
IGF-IR	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
Ki67	Marker of proliferation Ki67
LCIS	Lobular carcinoma *in situ*

LH	Luteinising hormone
MMTV	Mouse mammary tumour virus
OS	Overall survival
P4	Progesterone
p53	Tumour protein p53
PBS	Phosphate buffered saline
pCR	Pathological complete response
PDX	Patient derived xenograft
PFA	Paraformaldehyde
PR	Progesterone receptor
PyMT	Polyomavirus middle T antigen
RANK	Receptor activator of nuclear factor κb
Rb	Retinoblastoma protein
ROR	Risk of recurrence
RS	Recurrence score
Rsu	Unscaled recurrence score
RT-PCR	Real time-PCR
SD	Standard deviation
SEM	Standard error of mean
TAE	Tris-acetate-EDTA
TGFβ	Transforming growth factor β
t-SNE	T-Distributed Stochastic Neighbour Embedding
VEGF	Vascular endothelial growth factor
VC	Vehicle Control

Gene abbreviations

Oncotype DX 21-gene signature

ACTB	Actin Beta
BAG1	BCL2-Associated Athanogene
BCL2	B-Cell CLL/Lymphoma 2
BIRC5	Baculoviral IAP Repeat-Containing 5 (Survivin)
CCNB1	Cyclin B1
CD68	CD68 Molecule
CSTL2	Cathepsin L2

ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ESR1	Estrogen Receptor
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
GRB7	Growth Factor Receptor-Bound Protein 7
GSTM1	Glutathione *S*-Transferase Mu 1
GUSB	Glucuronidase Beta
MKI67	Marker Of Proliferation Ki-67
MMP11	Matrix Metallopeptidase 11 (Stromelysin 3)
MYBL2	Myb Proto-Oncogene Like 2
PGR	Progesterone Receptor
RPLPO	Ribosomal Protein Lateral Stalk Subunit P0
SCUBE2	Signal Peptide, Cub Domain And Egf Like Domain Containing 2
STK15	Aurora Kinase A
TFRC	Transferrin Receptor

Other Genes

BAX	Bcl2-Like Protein 4
CCND1	Cyclin D
CCNE1	Cyclin E
CDC20	Cell Division Cycle 20
CDC6	Cell Division Cycle 6
CDH1/3	Cadherin 1/3
CLDN3/4/7	Claudin 3/4/7
FASL	Fas Ligand
FOXA1	Forkhead Box Protein A1
GATA3	Gata-Binding Factor 3
HOXB13:IL17BR	Homeobox B13:Interleukin 17 Receptor B Two-Gene Ratio
MYC	Myc Proto-Oncogene, BHLH Transcription Factor
SNAI1/2	Snail Family Transcriptional Repressor 1/2
TFF1	Trefoil Factor 1
TWIST1/2	Twist Family BHLH Transcription Factor 1/2
WNT-4	Wnt Family Member 4
ZEB2	Zinc Finger E-Box Binding Homeobox 2

Chapter One

Literature Review and Aims

1.1. Introduction

Approximately 25% of breast cancers are diagnosed in women under the age of 50 (1). When breast cancer is diagnosed in young women it carries a high burden, with reduced 5 year survival rates compared to breast cancer in older women (2, 3) and a devastating impact on young families. Breast cancer is considered a chronic disease, with increased mortality extending over the next 40 years, even if the breast cancer is diagnosed at an early stage (3).

Breast cancer is not a single disease; there are many mutated genes that drive tumour development. Biomarkers are essential to classify breast cancer into different subtypes, each of which responds best to different therapies. Currently, immunohistochemical assays are used to identify expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and determine the rate of tumour proliferation (Ki67). Together with clinicopathological variables, these biomarkers are used to determine the best treatment option for an individual patient.

Now, clinics are increasingly adopting gene expression profiling to help guide treatment decisions for breast cancer patients; promising improved decision-making capabilities compared to traditional protein-based methods. Currently, five genomic tests (Prosigna, Oncotype DX, MammaPrint, EndoPredict, Breast Cancer Index) are recommended in clinical guidelines for guiding adjuvant chemotherapy and extended endocrine therapy decisions. In the clinic, their use has a significant impact on treatment decision-making (4).

However, despite their use in guiding treatment decisions for premenopausal women, tests that use gene expression profiling were largely developed using breast cancer samples from postmenopausal women. In premenopausal women, fluctuations in ovarian hormones during the menstrual cycle impact breast cancer gene expression (5, 6). However, the extent to which menstrual cycling impacts genomic testing and treatment decision-making is not well defined.

In this literature review, we outline the role of ovarian hormones in the regulation of gene expression in the breast and highlight the deficiencies in knowledge around gene expression profiling in premenopausal breast cancer. We conclude that while genomic testing offers significant advantages over traditional subtyping methods, currently available genomic tests have not been sufficiently validated in the context of premenopausal breast cancers, where there are significant fluctuations in estrogen and progesterone. There is a pressing need for more research into hormonal modulation of breast cancer gene expression in order to provide the optimal treatment for premenopausal women.

1.2. The menstrual cycle

1.2.1. Hormonal fluctuations during the menstrual cycle

During the follicular phase of the menstrual cycle, increasing concentrations of FSH produced by the pituitary stimulate maturation of estrogen-secreting ovarian follicles. Estrogen acts on the pituitary to further increase the production of FSH and LH. Eventually, the concentration of estrogen peaks, stimulating a peak in LH secretion that triggers ovulation. Ovulation signals transition from the follicular phase of the menstrual cycle to the luteal phase. During the luteal phase, LH promotes differentiation of the ovarian follicle into the progesterone producing corpus luteum. The luteal phase is characterised by a high concentration of progesterone, and is accompanied by a smaller second rise of estrogen. Progesterone supresses FSH and LH production, resulting in a decrease in estrogen concentration. Concentrations of progesterone begin to decrease as the corpus luteum ceases to produce progesterone and collapses. Consequently, the end of the luteal phase is characterised by low circulating hormones, which in turn relieve the negative inhibition of FSH and LH allowing progression into the next follicular phase (7, 8)(Figure 1).

1.2.2. Hormone-driven changes in the breast during the menstrual cycle

Fluctuations in estrogen and progesterone across the menstrual cycle direct the mammary gland epithelium to undergo sequential waves of proliferation, differentiation and apoptosis (9-12). The highest proliferative activity of mammary epithelium is observed during the luteal phase, with rising concentrations of progesterone. During the luteal phase there is an increase in secondary branching, alveoli budding and stromal development, accompanied by changes to the extracellular matrix (7, 13, 14). Epithelial apoptosis increases at the end of the menstrual cycle, with decreasing concentrations of estrogen and progesterone (15), and is associated with an atrophy of the epithelium, closing of the alveolar lumen, condensation of intralobular stroma, and a variable inflammatory infiltrate (13) (Figure 1).

Hormonally-driven morphological changes are associated with altered gene and protein expression. Estrogen is a potent proliferative agent, and regulates many genes involved in cell cycle progression, such as *CCND1* (16) and *c-MYC* (17, 18). Estrogen is also involved in the activation of Cyclin E complexes (19) and induces cyclin dependant kinases (Cdk) (19, 20) to promote cell cycle progression. In parallel, estrogen inhibits the expression of genes responsible for the suppression of cell growth, such as *p21* and tumour suppressor *p53* (21), and induces retinoblastoma protein phosphorylation (19, 20) to promote cell cycle progression.

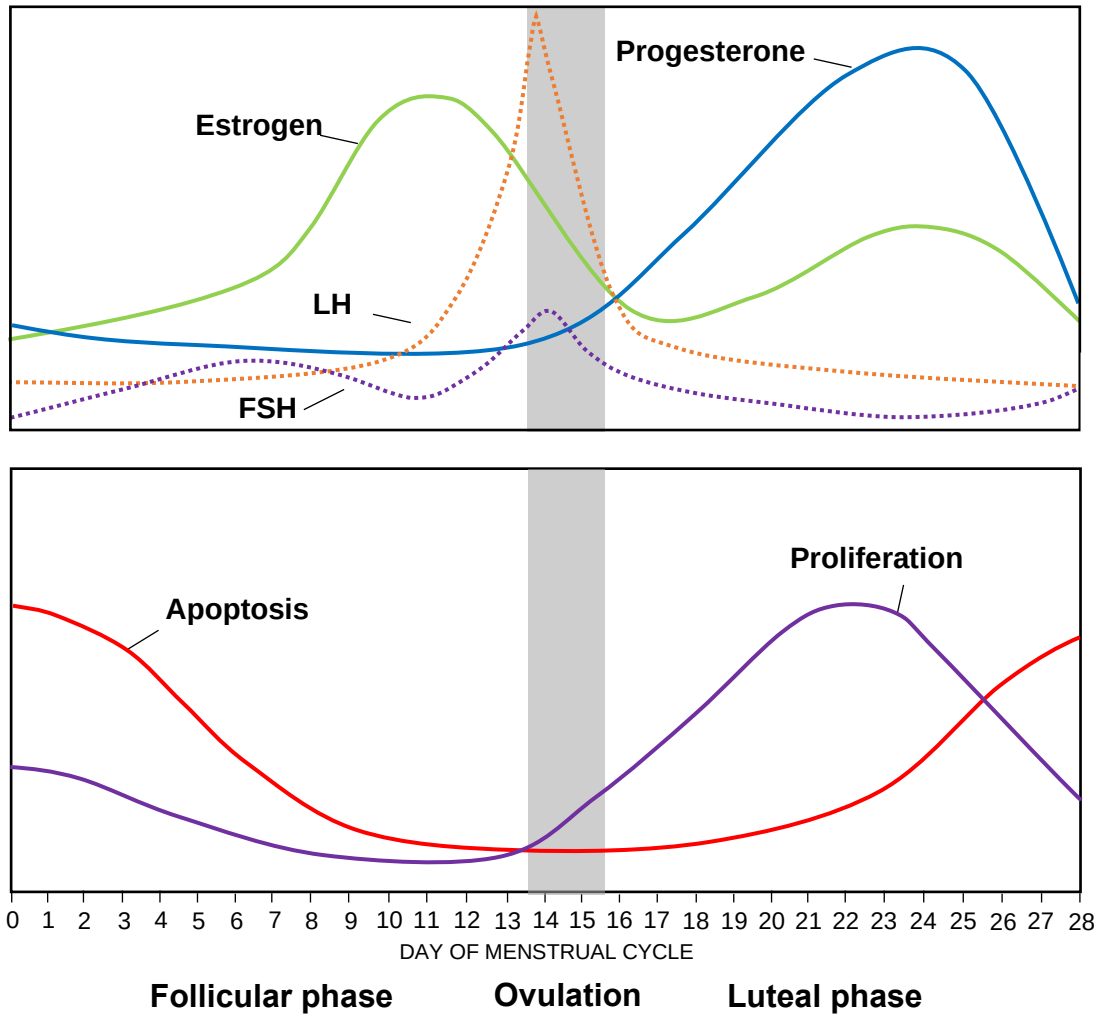


Figure 1 Changes in hormone concentrations in accordance with the menstrual cycle. (A) The fluctuations of estrogen (green), progesterone (blue), follicle-stimulating hormone (FSH; purple), and luteinizing hormone (LH; orange) during the human menstrual cycle. (B) Net apoptosis (red) and proliferation (purple) in the mammary gland in accordance with the menstrual phase.

Progesterone also plays an important role in cell proliferation in the breast, specifically acting during the luteal phase of the menstrual cycle. The proliferative role of progesterone is likely mediated by the regulation of cell cycle progression genes, including *CCND1* (22, 23), *c-MYC* (22-24), and Cdk activity (25). In addition to stimulating genes associated with cell cycle progression, progesterone inhibits the expression of genes responsible for the suppression of cell growth, such as *p53* (26), and induces retinoblastoma protein phosphorylation (27).

Progesterone elicits its proliferative function mainly through a paracrine mechanism. Recently, RANKL has been identified as an important paracrine mediator of progesterone-induced proliferation in the mammary gland (28, 29). Expression of RANKL is increased during the luteal phase of the menstrual cycle (30, 31), and is implicated upstream of Cyclin D (28). Furthermore, overexpression of its receptor, RANK, in mice increases proliferation of mammary epithelial cells (32, 33). Similarly, Wnt-4 has been identified as a paracrine mediator of progesterone signalling important for mammary gland epithelium proliferation (28, 34).

To promote optimal proliferation of mammary epithelial cells, estrogen induces expression of the progesterone receptor. This leads to proliferation of mammary epithelial cells through elevated expression of cell cycle genes when both estrogen and progesterone are present (35). Conversely, progesterone down-regulates its receptor and inhibits synthesis of ER (36), in turn reducing HR-mediated cell proliferation.

Estrogen and progesterone also play roles in epithelial apoptosis. Estrogen is an inhibitor of apoptosis, and increases the expression of anti-apoptotic proteins, such as Bcl2 and Bcl-xL (37). Consistent with this, Bcl2 is expressed almost exclusively in ER positive breast cancers and is associated with a good prognosis (38). Similarly, the fall in estrogen and progesterone at the end of the luteal phase is associated with an increase in apoptotic proteins, such as BAX (7) and FasL (39), and a decrease in antiapoptotic proteins, such as Bcl2 (7).

1.2.3. Crosstalk between hormone receptor and growth factor receptor signalling pathways

In the breast, estrogen and progesterone play key roles in regulating crosstalk between hormone receptor and growth factor receptor signalling pathways. Estrogen and progesterone modulate the expression of epidermal growth factor receptor family members, including the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER2) (40, 41). Similarly, many key genes associated with growth factor receptor signalling are implicated downstream of estrogen- (42) and progesterone-signalling (40, 43) in the mammary gland.

Estrogen and progesterone can induce phosphorylation of growth factor receptors, and directly interact with signal transduction pathways to activate MAPK, JAK/STAT, Src, and PI3K pathways. This in turn promotes mammary epithelial cell proliferation. In parallel, EGFR and HER2 phosphorylate and activate ER and PR (44). It has also been shown that expression of growth factor ligands are induced by estrogen and progesterone, including EGF, TGFα and amphiregulin (Areg) (40, 43, 45-48), and act as an important mediators of paracrine-induced proliferation. The interplay between hormone receptor and growth factor receptor signalling is illustrated in Figure 2.

Recent studies have also indicated that expression of vascular endothelial growth factor receptor (VEGF), a receptor involved in tumour growth, is induced by estrogen treatment *in vitro* (49) and *in vivo* (50); an effect attenuated by treatment with estrogen antagonists (51, 52). Furthermore, VEGF expression is reduced with increasing concentrations of progesterone (53). Similarly, a relationship has been identified between ER and the insulin-like growth factor I receptor (IGF-IR), a receptor involved in cell survival and proliferation. Crosstalk between ER and IGF-IR promotes cell proliferation when estrogen and/or IGF-I are present (54, 55).

In breast cancer, increased growth factor signalling is associated with a more aggressive phenotype. Overexpression of growth factor receptors has been associated with increased metastasis and poor survival (56-58), together with a lack of response to endocrine therapy (59). Preclinical studies have shown that overexpression of growth factor receptors in HR-positive tumours is associated with resistance to tamoxifen; conversely, targeting or blocking growth factor signalling in these tumours can restore tamoxifen sensitivity (60). Similarly, breast cancers that are resistant to growth factor receptor inhibitors such as gefitinib and lapatinib are characterised by an increase in ER-driven tumour growth (61).

The cross-talk between hormone receptor and growth factor receptor signalling is thought to be mediated by progesterone (43). Therefore, it is possible that progesterone may play a role in driving the development of endocrine therapy resistance. For premenopausal women, increasing concentrations of progesterone during the luteal phase of the menstrual cycle may promote growth factor-driven signalling, and reduce responsiveness of the cancer to endocrine therapies. Consistent with this, breast tumours in young women often have higher EGFR or HER2 expression and a worse prognosis, compared to breast cancer in older women (62, 63).

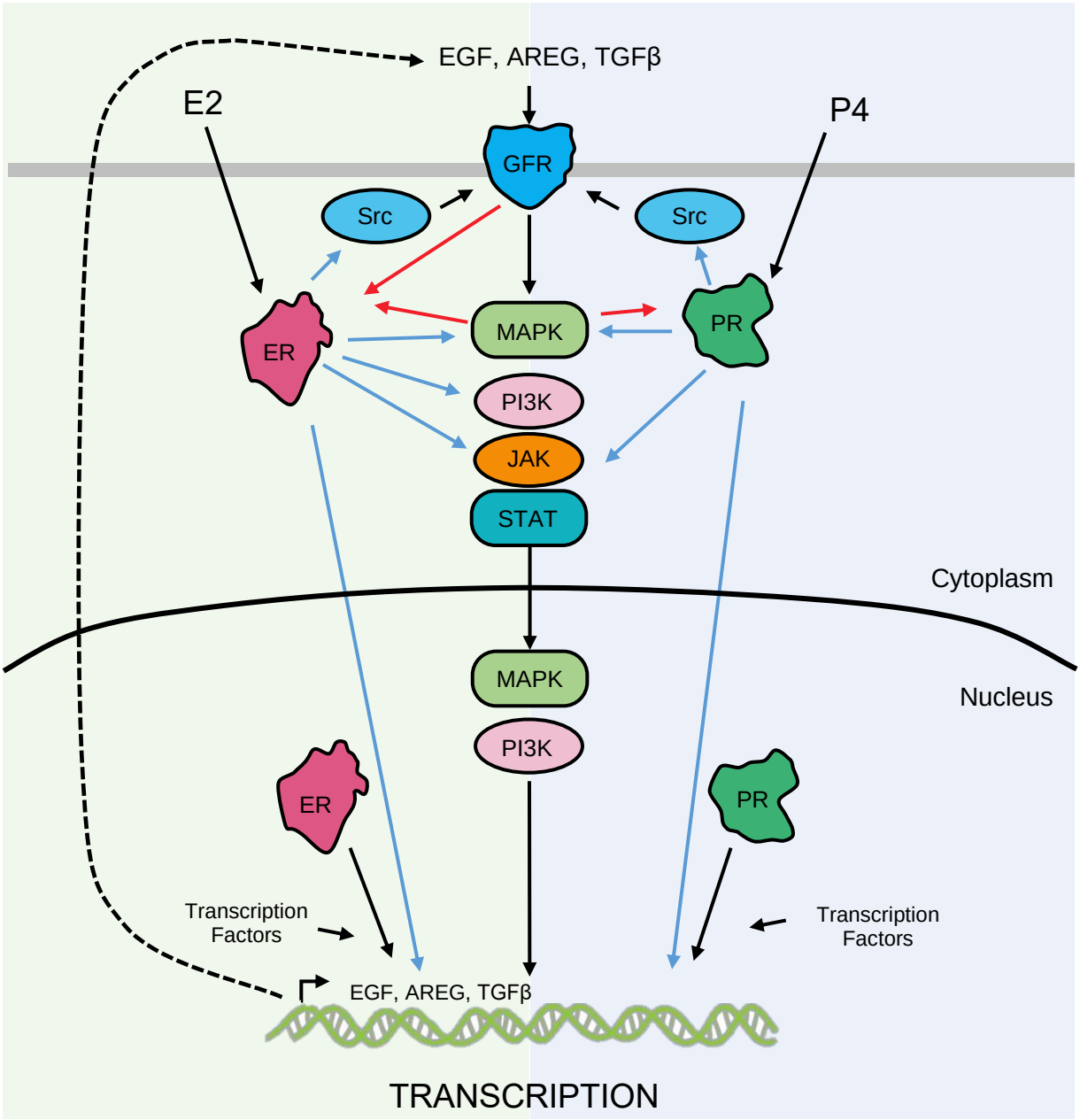


Figure 2 The interplay between hormone receptor and growth factor receptor signalling. Hormone receptors regulate gene transcription either by binding directly to DNA response elements or by recruiting transcription factors and co-regulators. In addition, crosstalk occurs between the estrogen receptor (ER), progesterone receptor (PR), and growth factor receptors to regulate gene expression. ER and PR regulate growth factor receptor activity by either: (i) directly interfering with transduction pathways, to activate MAPK, JAK/STAT, SRC, or PI3K signalling downstream of growth factor receptors, or (ii) inducing expression and secretion of paracrine growth factors, such as AREG, TGFβ or EGF which act on growth factor receptors to activate pathways involved in cell proliferation, survival and metastasis. In parallel, growth factor receptors can in turn phosphorylate and activate ER and PR. Adapted from (44).

1.3. Molecular subtypes of breast cancer

1.3.1. Classification of breast cancer subtypes through immunohistochemistry

Breast cancer is a heterogeneous disease, due to its diverse molecular and cellular features, with different therapeutic strategies required depending on the tumour type and stage. Traditionally, evaluation of ER, PR, HER2 and Ki67 immunoreactivity, together with clinicopathological variables including tumour size, type and grade, are used to classify breast tumours and guide treatment decisions. Breast cancer can be classified into five major subtypes, i.e Luminal A, Luminal B, HER2-enriched, Basal-like and Normal breast-like. These subtypes exhibit significant differences in incidence, survival and clinical outcomes (64-67).

Luminal A tumours are the most common, representing 50-60% of all breast cancers (68). Patients with Luminal A breast cancer have a good prognosis; displaying increased overall and disease free survival compared to other breast cancer subtypes (64, 66, 67). Treatment of early stage Luminal A breast cancer is based mainly on hormonal therapies, with the addition of adjuvant chemotherapy dependant on the clinical stage. The immunohistochemical profile of Luminal A tumours is characterised by high expression of ER, PR, an absence of HER2 overexpression, and a low rate of proliferation (68, 69).

Luminal B tumours account for 15-20% of all breast cancers (68). Patients with Luminal B breast cancer have poorer outcomes from endocrine therapy, however have a better response to chemotherapy, achieving a pathological complete response (pCR) to neoadjuvant chemotherapy in 16% of tumours compared to 6% in Luminal A tumours (70). From the immunohistochemical point of view, Luminal B tumours are characterised by a lower expression of ER and PR, and a higher Ki67 index, compared to Luminal A tumours (69).

HER2-enriched tumours represent 15-20% of breast cancer subtypes (68). Patients with HER2-enriched tumours show poor prognosis and overall survival (64, 66). The immunohistochemical profile of HER2-enriched tumours is characterised by variable ER or PR expression, and HER2 overexpression (69). Consequently, treatment of these tumours includes monoclonal antibodies that directly target the HER2 receptor, given in conjunction with chemotherapy (71).

Basal-like tumours comprise 15-20% of all breast cancers (69), and are associated with an aggressive clinical behaviour and a high rate of metastasis (72). Patients with Basal-like tumours have a poor prognosis; displaying lower overall and disease free survival compared to other subtypes (64, 66, 67). Basal-like tumours are characterised by a triple-negative immunohistochemical phenotype; ER, PR and HER2 negative. Consequently, these tumours

cannot be treated with endocrine therapy nor anti-HER2 antibodies, and treatment strategies are dependent on systemic chemotherapy.

Normal breast-like tumours account for 5-10% of all breast cancers (68). They lack the expression of ER, PR and HER2, however are not considered Basal-like tumours as expression of basal cytokeratin 5 and EGFR is absent (69). However, Normal breast-like tumours are poorly defined, and it is argued that they are an artefact of having a high percentage of normal breast epithelium cells in the tumour specimen (73, 74). It has been suggested that these tumours could be grouped into the recently discovered Claudin-low subtype, which also displays Basal-like characteristics, while also sharing biomarkers in common with normal breast epithelial cells. Similar to the Basal-like subtype, Claudin-low tumours have been associated with therapeutic resistance and poor survival outcomes (75), due to their highly migratory nature. The treatment for both Normal breast-like and Claudin-low subtypes is systemic chemotherapy.

In clinical practice identifying triple negative and HER2-positive breast cancers can be achieved with standard pathological testing and recommendations for appropriate adjuvant therapy in early stage disease are well defined. However, for patients with ER-positive and HER2-negative disease, distinguishing between those with Luminal A disease and those with Luminal B disease is more challenging and has implications for treatment recommendations (76). Emerging research suggests that a majority of women with Luminal breast cancer who are receiving chemotherapy do not derive a significant benefit from it, and they could be adequately treated with endocrine therapy alone (77-79).

Ideally, the decision to use adjuvant chemotherapy should be based on the prediction of the degree of benefit, to minimise the number of patients receiving unnecessary treatment. Identifying patients with good prognosis Luminal A disease who will have a minimal absolute benefit from adjuvant chemotherapy, can allow these women to avoid unnecessary chemotherapy and its associated side effects. Conversely, identifying women with Luminal B disease and a higher risk of relapse can prevent under treatment in this group (76, 80). However, this is not easily achieved with standard pathological testing alone.

1.3.2. Classification of intrinsic breast cancer subtypes through gene expression profiling

In 2000, Perou *et al.* (65) proposed a new classification system for breast cancer subtypes, separating them into distinct subgroups based on gene expression profiles. This new approach was a significant departure from traditional subtyping methods which relied on protein expression and immunohistochemistry to classify subtypes. Intrinsic subtyping by gene expression profiling is prognostic of overall and relapse free survival (64, 66, 67, 81, 82), and

can predict the relative risk of disease recurrence, and the patient's benefit from hormonal- and chemotherapies (74, 83). Therefore, gene expression profiling can be used to inform risk prediction and better guide treatment decisions; decreasing the number of patients receiving a suboptimal therapy. The main genes associated with each intrinsic subtype, together with pathological characteristics and prognosis are summarised in Table 1.

Luminal A tumours are characterised by a high ER protein expression, and as such display an increased expression of genes associated with ER function such as *FOXA1*, *PGR*, *BCL2*, *GATA3*, and *TFF1* (65, 66). Similar to Luminal A tumours, Luminal B tumours also express genes associated with ER activation; however increased expression of proliferative genes such as *CCNB1*, *CCND1*, *CCNE1*, *MYBL2*, and *MKI67* are the hallmark of Luminal B tumours (66, 67, 84). Consequently, Luminal B tumours possess a more aggressive phenotype, higher proliferative index, and worse prognosis, compared to Luminal A tumours (64, 66, 67, 70, 84).

HER2-enriched tumours are characterized by a high expression of human epidermal growth factor receptor-2 gene, *ERBB2*, and other genes associated with the HER2 pathway (68, 85). These tumours also overexpress *GRB7*, an adaptor protein involved in receptor tyrosine kinase signalling. Overexpression of *ERBB2* and *GRB7* promote activation of PI3K/AKT, JAK/STAT, Ras/Raf, and Src signalling pathways to allow for sustained proliferative signalling (69). Consequently, HER2-enriched tumours are characterised by an increased expression of proliferative genes such as *CCNE1*, *CCND1*, *MYBL2*, and *MKI67* (69), and exhibit a more aggressive and highly proliferative phenotype, compared to other breast cancer subtypes (85).

Basal-like tumours do not express ER or PR, and therefore exhibit low expression of estrogen-regulated genes. Conversely, *EGFR* is often overexpressed in these tumours, where increased EGFR expression correlates with poor patient survival. Basal-like tumours therefore display a dysregulation in PI3K/AKT, JAK/STAT, Ras/Raf, and Src signalling pathways, and exhibit an increased expression of genes implicated downstream of EGFR signalling (86). Consequently, Basal-like tumours show high expression of proliferative genes such as *MYC*, *CCNE1*, *CCND1*, *CDC20*, *CDC6*, and *BIRC5* (43, 69), which together contribute to their highly proliferative phenotype.

Claudin-low and Normal breast-like tumours possess a similar phenotype to Basal-like tumours, however, are enriched for epithelial-to-mesenchymal transition (EMT) markers such as *SNAI1/2*, *TWIST1/2*, and *ZEB2* (75), and exhibit low expression of genes encoding the tight junction proteins; *CLDN3/4/7*, and *CDH1/3* (75). Consequently, these subtypes are highly migratory and exhibit poor prognosis (87).

Table 1 Summary of clinical and pathological characteristics, prognosis and gene expression changes of breast cancer subtypes.

		Biomarker profile				Prognosis				
Subtype	**Incidence (%)**	**ER**	**PR**	**HER2**	**Ki67**	**OS (%)**	**5 year DFS (%)**	**10 year DFS (%)**	**Gene expression profile**	**Treatment**
Luminal A	50-60	+	+	-	Low	89-95	79-85	70-78	Increased expression in genes associated with ER function: *FOXA1*, *PGR*, *BCL2*, *ESR1*, *GATA3*, *TFF1*	Hormonal therapies +/- chemotherapy
Luminal B	15-20	+	+	-	Mod	71-85	60-75	50-60	Increased expression in genes associated with ER function: *FOXA1*, *PGR*, *BCL2*, *ESR1*, *GATA3*. Increased expression of proliferative genes *CCNB1*, *CCND1*, *CCNE1*, *MYBL2*, *MKI67*.	Hormonal therapies +/- chemotherapy
HER2-enriched	15-20	+/-	+/-	+	High	43-78	41-65	45-51	Amplification of *ERBB2* & *GRB7* and PI3K pathway activation. Increased expression of proliferative genes *BIRC5*, *CCNE1*, *CCND1*, *MYBL2*, *MKI67*.	HER2 targeted therapy + chemotherapy
Basal-like	15-20	-	-	-	High	53-73	48-72	48-65	Increased expression of *EGFR* and downstream signalling. Increased expression of proliferative genes *MYC*, *CCNE1*, *CCND1*, *CDC20*, *CDC6*, *BIRC5*	Chemotherapy
Claudin-low	12-14	-	-	-	Low	x	67	x	Loss of tight junction proteins: *CLDN3/4/7*, *CDH1*. Enrichment for EMT transition markers: *SNAI1/2*, *TWIST1/2*, *ZEB2*	Chemotherapy
Normal breast-like	5-10	-	-	-	High	93	79-87	85	Loss of tight junction proteins: *CLDN3/5/7*, *CDH1*	Chemotherapy
Refs	(2, 3)		(2)		(2)		(4-8)		(2, 3, 7, 9, 10)	(2)

1.4. Comparison between protein-based versus gene-based subtyping

In 2009, Parker *et al.* developed a 50-gene subtyping tool, designated PAM50, which can be used to classify tumours into intrinsic subtypes based on the expression of 50 cancer-associated genes. These 50 genes were identified from a list of 1,906 genes that had been identified in four previous microarray studies. The list was minimised to genes that had passed previously established formalin-fixed paraffin-embedded (FFPE) performance criteria, and were further refined through statistical analyses, allowing for identification of genes that showed the highest correlation to each intrinsic subtype. Differential gene expression between subtypes is shown by microarray in Figure 3.

Classification of intrinsic subtypes through PAM50 retains the prognostic and predictive significance that is characteristic to intrinsic breast cancer subtypes (64, 73, 80, 88, 89). Furthermore, several studies have suggested that the PAM50 classification method provides better information on prognosis compared to immunohistochemistry-based surrogates (64, 67, 81). Therefore, subtyping by immunohistochemistry may be inferior to genomic profiling and the use of gene expression profiling in a clinical setting could improve patient outcomes.

Accurate testing of biomarkers is important, as discrepancies between immunohistochemical and intrinsic subtyping of breast tumours may lead to differences in treatment decisions. Several studies have addressed this, comparing agreement in subtyping between immunohistochemical and gene profiling methods. A study by Cheang *et al.* reported that of HR-positive Luminal tumours as defined by PAM50, 8% did not stain positive for ER through immunohistochemistry (81). Similarly, Chia *et al.* identified 8% of HR-positive Luminal tumours instead being classified as either HER2-positive, or triple negative through immunohistochemistry (64).

Discordances in HER2 expression have also been investigated. While several studies have reported high overall concordance in HER2 expression between gene expression profiling and immunohistochemistry (90-93), others report low concordance (73, 88). A study by Chia *et al.* found that only 66% of HER2-enriched tumours identified by PAM50 exhibited HER2 overexpression through immunohistochemistry. Instead, 31% of HER2-enriched tumours were classified as Luminal A or B tumours, and 4% classified as Basal-like. Similarly, Cheang *et al.* also identified a low concordance in HER2 status between PAM50 and immunohistochemistry, with 6% of tumours instead classified as Luminal A tumours and 16% as triple negative (81). As patients with ER-negative or HER2-positive tumours will receive chemotherapy, such discordances in classification could have critical implications on treatment decisions, where women may receive unnecessary chemotherapy with no benefit.

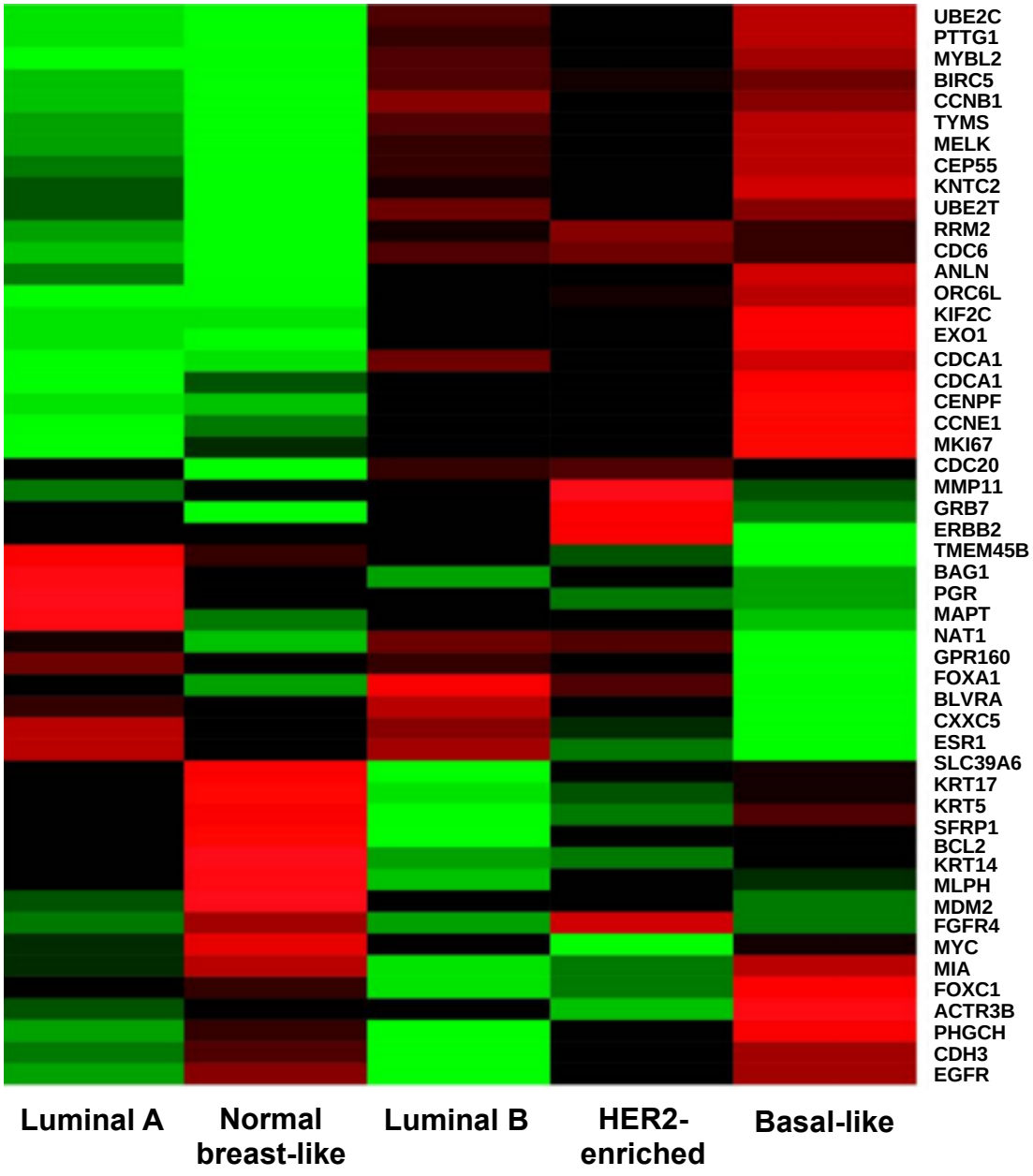


Figure 3 Microarray heatmap of PAM50 gene expression in 'intrinsic' breast cancer subtypes. Molecular profiles have distinct gene expression profiles. Expression values of genes included in the PAM50 signature are shown as red/green according to their relative expression level for each subtype. Highest gene expression (red), lowest (green) & average (black) (74).

As different subtypes each respond best to different treatments, discrepancies between subtyping methods pose a significant question that is yet to be answered: in discordant cases, which result should be used to guide treatment decisions, and is it appropriate to deny a patient treatment that would otherwise be indicated by a different subtyping method? Studies by Chia *et al.* (64) and Cheang *et al.* (81) included only premenopausal women and described low concordance between immunohistochemistry and gene expression profiling. This low concordance may be due to the fluctuations in circulating hormones during the menstrual cycle, and the relative effect of hormonal stimulation on gene versus protein expression. While it is believed that gene expression profiling is more reflective of true tumour biology compared to protein-based immunohistochemistry, the paucity of data on premenopausal women makes it difficult to determine the efficacy of gene expression profiling compared to the traditional gold standard for tumour subtyping.

1.5. The use of genomic tests for guiding adjuvant therapy decisions

Genomic tests are now being integrated into clinical practice to help guide treatment decisions for women with breast cancer, promising improved treatment decision-making capabilities compared to traditional protein-based methods. Five tests (Prosigna, Oncotype DX, MammaPrint, EndoPredict, and the Breast Cancer Index) are recommended in international guidelines for guiding adjuvant chemotherapy or extended endocrine therapy treatment decisions (76, 94-96), and have been shown to impact treatment decision-making in the clinic (97). However, despite their use in the clinic, these tests have been developed predominantly in postmenopausal women, and there is a scarcity of literature on whether they are appropriate for use in premenopausal women.

1.5.1. Prosigna

Prosigna is an *in vitro* diagnostic assay based on the PAM50 gene signature assay. The Prosigna test is performed on FFPE tissue, identifies a tumour's intrinsic subtype and the patient's risk of distant disease recurrence, and aims to help guide clinicians and patients in treatment decision-making. The clinical studies involved in the development of Prosigna from PAM50 are summarised in Table 2. Of note, the validation of the Prosigna test—necessary for FDA approval—was based on two clinical trials (TransATAC and ABCSG-8 clinical trials) incorporating data from over 2,400 postmenopausal women enrolled in adjuvant aromatase inhibitor trials.

Table 2 Development of Prosigna, a PAM50-based subtype classifier. Following the development of a 50-gene subtype classifier by Parker *et al.* in 2009, subsequent studies by the same group clinically and analytically validated the prognostic value of the 50-gene signature. TransATAC and ABCSG-8 trials provided evidence of the clinical validity of Prosigna. Currently in recruitment is a study evaluating the treatment impact of Prosigna. The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

			Menopausal Status			ER Status		
Authors	**Year**	**Total**	**Pre-menopausal**	**Post-menopausal**	**Unkn**	**ER+**	**ER-**	**Ref**
Parker *et al.*	2009	761	-	-	761	544	195	(74)
Neilsen *et al.*	2010	786	20	752	14	768	9	(88)
Bastien *et al.*	2012	154	49	101	4	100	49	(73)
Chia *et al.*	2012	398	398	0		291	107	(64)
Cheung et al.	2012	476	476	0		300	168	(81)
Martin *et al.*	2013	820	443	377		645	172	(98)
Dowsett *et al.*	2013	1007	0	1007		1007	0	(99)
Liu *et al.*	2014	1094	757	337		638	456	(100)
Nielsen *et al.*	2014	43	-	-	43	43	0	(101)
Sestak *et al.*	2014	2137	0	2137		213	0	(102)
Gnant *et al.*	2014	1478	0	1478		1464	17	(80)
Wallden *et al.*	2015	746	91	433	222	547	177	(89)
Laenkholm *et al.*	2018	2558	0	2558		2558	0	(79)
Jensen *et al.*	2018	460	460	0		329	114	(103)

Several studies have examined the use of PAM50 in managing adjuvant therapy explicitly in premenopausal breast cancer patients (64, 81, 103). Most recently, a retrospective analysis of 460 premenopausal women evaluated the prognostic and predictive value of Prosigna for use in these women (103). The authors identified an association between Prosigna intrinsic subtypes and benefit of chemotherapy, where women with Basal-like and Luminal B breast cancer showed improved disease free and overall survival with chemotherapy treatment. Interestingly, this chemotherapy benefit was not observed for women with HER2-enriched tumours. Critically, the authors noted no significant association between continuous Prosigna risk scores and disease free or overall survival in premenopausal women. These findings raise concerns for the use of Prosigna in premenopausal women, and whether Prosigna is appropriate for guiding chemotherapy treatment decisions for these women is unclear.

1.5.2. Oncotype DX

The Oncotype DX 21-gene Recurrence Score assay guides adjuvant chemotherapy treatment decisions for women with early-stage, HR-positive, HER2-negative breast cancer. Oncotype DX evaluates the expression of 21 genes (16 cancer-associated genes and 5 reference genes; Table 3) in FFPE breast cancer samples to calculate a Recurrence Score, which significantly correlates with the probability of distant breast cancer recurrence, and can predict the likely benefit from the addition of chemotherapy to endocrine treatment (104-108). Similar to Prosigna, the Oncotype DX assay relies heavily on genes associated with proliferation, and estrogen- and growth factor-signalling, which are differentially expressed by normal breast epithelial across the menstrual cycle, as discussed earlier (section 1.2.2).

To calculate Recurrence Scores, the Oncotype DX algorithm weights genes included in the 21-gene signature differently, depending on the strength of their association with distant disease recurrence. Proliferation-, Invasion-, and HER2-group genes, along with *CD68* expression, are all weighted positively in the Oncotype DX algorithm, as their increased expression is associated with an increased risk of disease recurrence. Conversely, expression of Estrogen-group genes, along with *GSTM1* and *BAG1* expression, are weighted negatively, as their increased expression is associated with lower rates of disease recurrence. The genes included in the 21-gene signature, along with their weighting, are highlighted in Table 3.

The development of Oncotype DX is summarised in Table 4. To note, the Oncotype DX assay was largely developed using breast cancer samples collected from postmenopausal women. In 2018, results from a prospective clinical study were published that support the possibility that the Oncotype DX assay may be less precise in younger women. The TAILORx study (77)

Table 3 Panel of 21 genes used in the Oncotype DX assay to determine the risk of distant recurrence. In 2004, Paik *et al.* identified 250 candidate genes from published literature and genomic databases that have been shown to be correlated with disease outcome. The list of genes was reduced to 16 cancer-associated genes, which showed the highest correlation to distant recurrence after 10 years, and 5 reference genes. Genes are grouped on the basis of function, correlated expression, or both. Each group is weighted differently depending on its correlation with distant recurrence. The weighting of groups are shown in the table.

Proliferation	**Invasion**	**HER2**	**Estrogen**	**Other**	**Reference**
KI67	*MMP11*	*GRB7*	*ESR1*	*GSTM1*	*ACTB*
STK15	*CTSL2*	*ERBB2*	*PGR*	*CD68*	*GADPH*
BIRC5			*BCL2*	*BAG1*	*RPLPO*
CCNB1			*SCUBE2*		*GUS*
MYBL2					*TRFC*
Weighting:					
+1.04	+0.10	+0.47	-0.34	-0.08 +0.05 -0.07	x

Table 4 The development of Oncotype DX, a 21-gene assay which identifies patient benefit from adjuvant chemotherapy. Following the identification of the 21-gene signature, which showed high correlation to distant recurrence of breast cancer at 10 years, subsequent studies verified its predictive and prognostic value. The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

			Menopausal Status		ER Status		
Authors	**Year**	**Total**	**Pre-menopausal**	**Post-menopausal**	**ER+**	**ER-**	**Ref**
Paik *et al.*	2004	668	194	474	668	0	(104)
Esteva *et al.*	2005	149	122	27	103	46	(109)
Gianni *et al.*	2005	89	-	-	52	31	(110)
Habel *et al.*	2006	790	209	581	682	108	(111)
Paik *et al.*	2006	651	289	362	651	0	(106)
Goldstein *et al.*	2008	465	193	272	465	0	(112)
Albain *et al.*	2010	367	0	367	367	0	(107)
Mamounas *et al.*	2010	1023	298	725	1023	0	(113)
Dowsett *et al.*	2013	1231	0	1231	1231	0	(99)
Sparano *et al.*	2015	1623	480	1143	1621	5	(105)
Sparano *et al.*	2018	9719	3300	6419	9665	54	(77)
Sparano *et al.*	2019	9427	2958	6469	-	-	(114)
Sparano *et al.*	2019	1389	407	982	1349	40	(115)

incorporated data from 9,719 women with breast cancer (n=3,054 women aged ≤50 years; n=6,665 women aged >50 years). The authors reported that for women over the age of 50 years with Oncotype DX Recurrence Scores ≤26, endocrine therapy alone was not inferior to chemoendocrine therapy in terms of disease free and overall survival. However, women under the age of 50 with Recurrence Scores between 16 to 26 still exhibit some benefit from chemotherapy. When clinical information was integrated, this chemotherapy benefit was instead observed for young women with Recurrence Scores of 20-25 (114). The biological basis of this age-related difference in chemotherapy benefit is not well defined; however, it is possible that menstrual cycling in premenopausal women contributes to this difference.

1.5.3. MammaPrint

MammaPrint is a microarray-based assay that evaluates the expression of 70 genes associated with metastasis, proliferation, invasion, survival and angiogenesis (116), using frozen tumour tissue samples. The list of 70 genes was identified from whole genome expression arrays, and selected for on the basis of those that significantly correlated with disease outcome (116). Interestingly, MammaPrint does not measure expression of commonly used diagnostic markers ER, PR or HER2.

Through the relative expression of these 70 genes, MammaPrint classifies tumours into high or low risk groups, which corresponds with patient's clinical outcome. Studies have shown that risk groups identified by MammaPrint correspond with patients overall survival (117), disease free metastasis (82, 118), and the benefit from adjuvant chemotherapy (119, 120). Most studies validating the diagnostic capabilities of MammaPrint were small scale retrospective studies which included both pre- and postmenopausal women, as summarised in Table 5. MammaPrint had initially been developed and validated in patients under the age of 55, suggesting that MammaPrint is targeted towards the younger population.

In 2016, results from a prospective study were published, comparing MammaPrint to clinicopathological tools for selecting patients for adjuvant chemotherapy (78). The median age of the patients was 55 years. The study found that approximately 46% of patients that were classified as high risk by clinicopathological features, were classified as a low risk of metastasis by MammaPrint. Although these tumours presented with a high clinical risk, the results from the study suggested that these patients received no significant benefit from adjuvant chemotherapy. Therefore, the authors concluded that using MammaPrint to guide treatment decisions, compared to traditional clinicopathological features, can reduce the number of patients receiving unnecessary chemotherapy.

Table 5 The development and clinical validation of the MammaPrint assay. The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

			Menopausal Status			
Authors	**Year**	**Total**	**Pre-menopausal**	**Post-menopausal**	**Unkn**	**Ref.**
Van't Veer *et al.*	2002	97	66	31		(116)
Van't de Vijver *et al.*	2002	295	246	49		(82)
Buyse *et al.*	2006	302	203	99		(118)
Bueno-de-Mesquita *et al.*	2007	427	292	135		(121)
Wittner *et al.*	2008	100	24	76		(122)
Bueno-de-Mesquita *et al.*	2009	123	83	40		(117)
Mook *et al.*	2009	241	125	116		(123)
Mook *et al.*	2010	148	0	148		(124)
Knauer *et al.*	2010	541	231	310		(120)
Straver *et al.*	2010	167	119	39	9	(119)
Drukker *et al.*	2013	427	292	135		(125)
Drukker *et al.*	2014	295	246	49		(126)
Cardoso *et al.*	2016	6693	2226	4467		(78)

1.5.4. EndoPredict

EndoPredict is an RT-PCR based diagnostic test that evaluates the expression of 8 proliferative and HR-associated genes using FFPE tissue samples. In combination with clinicopathological features of the tumour, it identifies the risk of distant metastasis within 10 years (127). The EndoPredict assay is used to guide adjuvant chemotherapy and extended endocrine treatment decisions for women with HR-positive, HER2-negative breast cancer.

The EndoPredict gene signature was identified from gene expression profiles of breast cancer samples taken predominantly from postmenopausal women (127). Initial clinical validation of EndoPredict was based on clinical trials (ABCSG-6, ABCSG-8, TransATAC) that incorporated data exclusively from postmenopausal women who were enrolled in aromatase inhibitor trials. Similarly, subsequent studies confirming the validity of EndoPredict were performed using breast cancer samples from these same retrospective cohorts of postmenopausal women. The development of EndoPredict is summarised in Table 6.

Recently, a study by Sestak *et al.* (128) incorporated data from 5 retrospective datasets (GEICAM/9906, GEICAM 2003/02, ABCSG-6, ABCSG-8, TransATAC) to validate the EndoPredict assay for use in both pre- and postmenopausal women. However, despite being the largest study to date, this study was again performed largely using postmenopausal breast cancer samples. Currently, there are a lack of studies investigating the efficacy of EndoPredict for premenopausal women, and whether EndoPredict is an appropriate tool for guiding treatment decisions in younger women has not been sufficiently investigated.

1.5.5. Breast Cancer Index

The Breast Cancer Index is an RT-PCR based assay that classifies patients into risk groups to predict the risk of early or late disease recurrence and the likelihood of benefit from extended endocrine therapy (129-132). The Breast Cancer Index evaluates two independent biomarkers; the HOXB13:IL17BR gene ratio, which is associated with endocrine therapy response (133), and the molecular grade index, which is determined by the expression of 5 proliferation-related genes (134). Classification of breast cancer through the expression of these 7 genes aims to identify patients who are most likely to benefit from extended endocrine therapy. The development of the Breast Cancer Index is summarised in Table 7. Similar to Prosigna, the clinical validation of the Breast Cancer Index was based on retrospective studies that used samples exclusively from postmenopausal women, and therefore results cannot be generalised to premenopausal women.

Table 6 The development and clinical validation of the EndoPredict assay. The development and clinical validation of the EndoPredict gene signature was predominantly performed using the same retrospective datasets, of which many incorporated data exclusively from postmenopausal women enrolled in aromatase inhibitor trials. The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

			Menopausal Status				
Authors	**Year**	**Total**	**Pre-menopausal**	**Post-menopausal**	**Unkn**	**Study Cohort**	**Ref.**
Filipits *et al.*	2011	1702	0	1702		ABCSG-6, ABCSG-8	(127)
Dubsky *et al.*	2013	1702	0	1702		ABCSG-6, ABCSG-8	(135)
Dubsky *et al.*	2013	1702	0	1702		ABCSG-6, ABCSG-8	(136)
Muller *et al.*	2013	167	-	-	167	N/A	(137)
Martin *et al.*	2014	555	300	255		GEICAM-9906	(138)
Buus *et al.*	2016	928	0	928		TransATAC	(139)
Martin *et al.*	2016	536	-	-	536	GEICAM-9906	(140)
Sestak *et al.*	2018	774	0	774		TransATAC	(141)
Filipits *et al.*	2019	1702	0	1702		ABCSG-6, ABCSG-8	(142)
Sestak *et al.*	2019	3746	572	3174		GEICAM-9906, GEICAM -2003/02, ABCSG-6, ABCSG-8, TransATAC	(128)

Table 7 The development and clinical validation of the Breast Cancer Index assay. The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

			Menopausal Status			
Authors	**Year**	**Total**	**Premenopausal**	**Postmenopausal**	**Unkn**	**Ref.**
Ma *et al.*	2004	80	2	78		(143)
Ma *et al.*	2008	836	81	327	428	(134)
Jankowitz *et al.*	2011	265	80	185		(144)
Jerevall *et al.*	2011	588	0	588		(132)
Mathieu *et al.*	2012	150	66	84		(145)
Sgroi *et al.*	2013	665	0	665		(130)
Zhang *et al.*	2013	958	0	958		(129)
Habel *et al.*	2013	608	162	446		(146)
Sanft *et al.*	2015	96	13	76		(147)
Sgroi *et al.*	2016	292	0	292		(131)

1.6. Hormonal modulation of breast cancer biomarkers

1.6.1. Impact of menstrual cycle stage on the expression of traditional protein biomarkers

Hormonal fluctuations during the menstrual cycle can influence the expression of breast cancer biomarkers. In premenopausal women, hormone receptor protein expression fluctuates across the menstrual cycle in normal breast epithelium (148, 149) and breast cancer samples (6, 150, 151), in accordance with concentrations of estrogen and progesterone. Highest expression of ER is observed during the follicular phase of the menstrual cycle, compared to ovulatory and luteal phases. Conversely, PR expression is highest during the ovulatory phase, compared to follicular and luteal phases (6, 150, 151). Tumour proliferation, as assessed through Ki67 protein expression, also fluctuates across the menstrual cycle stage, with highest proliferative activity observed during the luteal phase (152).

HER2 expression has also been reported to fluctuate with menstrual cycle stage; however, there is controversy in the literature as to when HER2 expression peaks. Several studies have suggested that expression of HER2 is increased during the follicular phase of the menstrual cycle (153, 154). Conversely, other studies instead suggest that HER2 expression is highest during the luteal phase (155), and that its expression is inversely related to ER expression which peaks during the follicular phase (156).

Given that the assessment of ER, PR, HER2 and Ki67 protein expression is the current gold standard for breast cancer management, changes in their expression with menstrual cycle stage could potentially influence the choice of adjuvant therapy in some cases. Furthermore, as expression of these biomarkers reflects response to therapy (157, 158), changes in biomarker expression might also influence the extent to which the tumour responds to treatment.

1.6.2. Impact of menstrual cycle stage on breast cancer gene expression

Changes in hormone receptor protein expression are associated with downstream changes in gene expression. Several studies have reported that the expression of estrogen-regulated genes fluctuates across the menstrual cycle, with highest expression observed during the follicular phase (6, 159, 160), when ER expression peaks. Similarly, studies have shown that expression of estrogen-related genes significantly correlates with serum concentrations of estrogen (161); and that their expression is significantly higher in premenopausal women compared to postmenopausal women (5).

Proliferative gene expression also fluctuates across the menstrual cycle. A study by Haynes *et al.* (159) measured proliferative gene expression in HR-positive breast cancer samples collected

from women at different stages of the menstrual cycle. The authors reported that four of the five proliferative genes included in Oncotype DX gene signature (*MKI67*, *BIRC5*, *CCNB1*, *MYBL2*) showed differential expression with menstrual cycle stage. Furthermore, the authors demonstrated that expression of RANKL, an important paracrine mediator of progesterone-induced proliferation, was increased during the luteal phase of the menstrual cycle with increasing concentrations of progesterone.

Clearly, fluctuating concentrations of estrogen and progesterone during the menstrual cycle can affect breast cancer gene expression. As genomic tests rely heavily on the expression of estrogen and proliferation genes for risk prediction, changes in their expression with menstrual cycle stage could significantly impact genomic testing. In support of this possibility, a recent *in vitro* study has shown that the combination of estrogen and progesterone significantly impacts gene expression in HR-positive breast cancer cell lines, and this results in the switching from a Luminal A to Basal-like PAM50 subtype and increases Oncotype DX Recurrence Scores (43). However, current studies have only measured gene expression at a single time-point in each patient (i.e. at the time of surgical excision) and were therefore limited to looking at differences between groups of patients who were at different menstrual cycle stages. Consequently, the extent to which menstrual cycling affects gene expression within the same tumour is not well defined.

If menstrual cycle stage affects breast cancer gene expression it is expected that there would be discrepancies between paired diagnostic and surgical breast cancer samples, which would be taken from the woman at different times and therefore different stages of the menstrual cycle. However, studies on concordance between biopsy and surgical samples have not specifically investigated premenopausal women. In 2012, Riis *et al.* compared gene expression profiles of 13 women (1/13 premenopausal; 12/13 postmenopausal) between paired core needle biopsies and surgical excisions, identifying 228 genes differentially expressed between samples. A majority of genes were immunoregulatory or stress-related (162). One gene from the Prosigna and Oncotype DX gene signatures showed differential expression between samples; *GRB7*. Similarly, Jeselsohn *et al.* compared gene expression profiles between core biopsies and surgical excisions in postmenopausal women with HR-positive breast cancer (163). The authors identified significant changes in the expression of 14 genes, a majority of which were immunoregulatory. Two genes included in the Prosigna and Oncotype DX gene signatures, *MYC* and *CCNB1*, showed differential expression between core biopsies and surgical excisions.

Most recently, a study used a genome wide approach to identify concordance in intrinsic subtyping between core biopsies and surgical excisions. The authors collected 56 paired core-

cuts from postmenopausal breast cancer patients, and classified tumours into one of the 5 intrinsic subtypes based on the PAM50 gene signature (164). No systematic differences in categorisation of the tumours into intrinsic subtypes were identified, however discordances were identified between the Luminal A versus Luminal B subtype. While these studies have generally found good concordance between diagnostic and surgical samples, concordance in premenopausal women has not been specifically investigated.

A change in biomarker status can have important clinical consequences for adjuvant treatment. Studies evaluating the concordance in gene expression profiles between core biopsies and surgical excisions suggest that biopsies taken at diagnosis are representative of the whole tumour. However, these studies were performed predominantly in postmenopausal women, and it is possible that hormonal fluctuations in premenopausal women may alter the expression of these biomarkers. As such, the biopsy taken at diagnosis or during surgery may be influenced by fluctuating concentrations of estrogen and progesterone, and therefore not represent the true tumour gene profile. This could have critical implications for genomic profiling tests; leading to suboptimal treatment recommendations and reduced survival outcomes for premenopausal women.

1.7. Conclusion

Breast cancer clinics are increasingly adopting gene expression profiling to help guide adjuvant chemotherapy treatment decisions. However, despite their availability to young women, such tests were largely developed and validated in postmenopausal women – patients in whom fluctuations in estrogen and progesterone associated with the menstrual cycle are absent. Yet these hormones are highly likely to affect breast cancer gene expression in premenopausal women, and the diagnosis and treatment trajectories that stem from its measurement could fundamentally depend on a patient's menstrual cycle stage at the time of tissue sampling. It is important to understand how hormonal fluctuations might affect genomic tests in order to provide premenopausal women with the optimal treatment for their individual cancer.

1.8. Hypothesis and aims

The Oncotype DX 21-gene assay is the leading gene expression profiling test in Australia, with the strongest evidence supporting its predictive and prognostic capabilities (77). The Oncotype DX assay has been included in guidelines for guiding adjuvant chemotherapy treatment decisions (76, 94-96), and its use results in a 12% net reduction in chemotherapy use in Australian clinics (165). Currently, the Oncotype DX 21-gene assay is being considered by the Medical Services Advisory Committee to be included on the Medicare Benefits Schedule.

However, despite its use in the clinic for guiding adjuvant chemotherapy treatment decisions in premenopausal breast cancer patients, there is a scarcity of literature on whether Oncotype DX is suitable for use in these women, where ovarian hormones estrogen and progesterone fluctuate dramatically across the course of the menstrual cycle. The extent to which menstrual cycling impacts the Oncotype DX 21-gene signature and subsequent treatment decision-making is not well understood.

The experiments described in this thesis aim to address the following hypothesis:

Fluctuations in estrogen and progesterone during the menstrual cycle of premenopausal women affect the Oncotype DX 21-gene signature.

The validity of this hypothesis was investigated by addressing the following aims:

(i) To identify how the Oncotype DX 21-gene signature varies between paired HR-positive breast cancer samples collected from premenopausal women compared to postmenopausal women.

(ii) To define how natural fluctuations in estrogen and progesterone during the ovarian cycle affect the Oncotype DX 21-gene signature in HR-positive mouse mammary tumours.

(iii) To investigate the effects of estrogen and progesterone on the Oncotype DX 21-gene signature in HR-positive breast cancer cell lines *in vitro* and in mice *in vivo*.

Chapter Two

Materials and Methods

2.1. Human breast cancer biopsy collection

2.1.1. Study 1 – Retrospective study of breast cancer biomarkers between paired samples

Human ethics committee approval was obtained from The Queen Elizabeth Hospital (Approval number Q20170106) and informed consent was obtained from the patients. Paired formalin-fixed paraffin-embedded (FFPE) breast cancer samples were collected from women who underwent breast cancer diagnosis and treatment at The Queen Elizabeth Hospital between 2000 – 2015. The inclusion and exclusion criteria of the study are as follows:

Inclusion criteria:

- Female aged 18 years or over
- Treated for breast cancer at TQEH between years 2000 – 2015
- Two FFPE tumour blocks available on site at The Queen Elizabeth Hospital
- Must give written informed consent

Exclusion criteria:

- Patients with benign breast disease
- Patients who received any neoadjuvant therapy
- Patients unable to provide informed consent

Paired samples were collected from the same tumour at different times; 30 women had paired core needle biopsies and corresponding surgical excisions, while 5 women had paired surgical excisions and corresponding re-excisions with residual disease. Sample 1 was defined as the earlier collected sample, with sample 2 as the corresponding later collected pair. Figure 4 shows a timeline that describes when tissue samples were collected.

Archived FFPE tissue blocks were retrieved and sectioned by The Queen Elizabeth Hospital pathology department. For each FFPE tissue block, 4 x 5µM sections were cut and mounted on coated glass slides (Trajan), and histopathology was performed on these sections as detailed below. An additional 6 x 10µM tissue sections were cut and mounted on glass slides (HD Scientific Supplies) to be used for RNA extraction and gene expression analysis.

2.1.2. Study 2 – Prospective study on the effect of the menstrual cycle on breast cancer biomarkers in premenopausal women

Human ethics committee approval was obtained from The Queen Elizabeth Hospital (Approval numbers Q20160309 and Q20160806) and informed consent was obtained from the patients prior to tissue collection. The inclusion and exclusion criteria of the study are as follows:

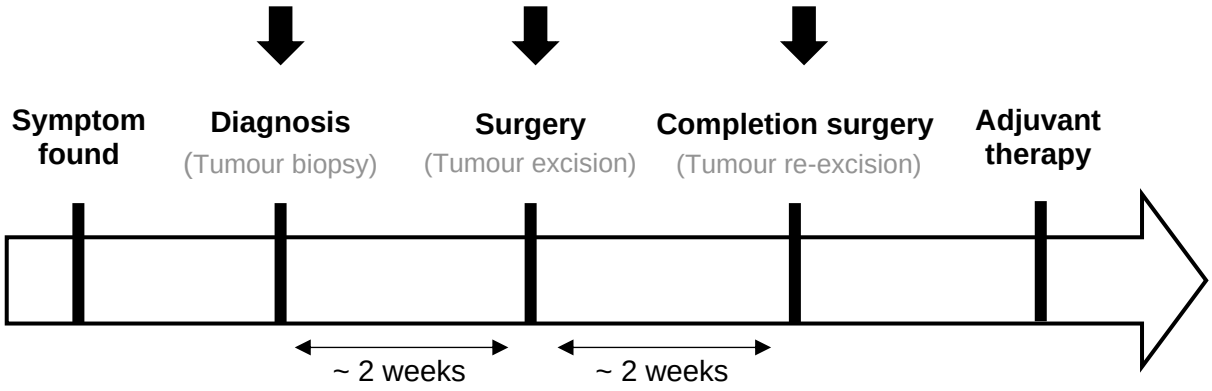


Figure 4 The timeline for the diagnosis and treatment of breast cancer. After finding a breast cancer symptom, a woman will present to the clinic for diagnosis. At the clinic, imaging is performed and a biopsy is collected for diagnostic purposes. Following a breast cancer diagnosis, the woman is scheduled to have the tumour surgically removed. At The Queen Elizabeth Hospital, the time between diagnosis and surgery is approximately 2 weeks. In cases where there is residual disease the patient is scheduled for a second completion surgery, approximately 2 weeks after the primary surgery to excise any residual cancer. Following the completion surgery the patient will receive further treatment, such as radiotherapy, endocrine therapy, and/or chemotherapy. For our retrospective study paired samples were collected from the same patient at different times during the management of the breast cancer, including the time of diagnostic biopsy, the time of surgical excision, or the time of surgical re-excision. For our prospective study paired biopsies were collected at the time of diagnostic biopsy and the time of surgery only.

Inclusion criteria:

- Premenopausal women over the age of 18
- No evidence of metastatic breast cancer
- Breast lesion to be at least 15 mm in size
- Must give written informed

Exclusion Criteria:

- Presence of metastatic breast cancer
- Pregnant women or breast feeding mothers
- Women who received neoadjuvant therapy
- Participants with any other uncontrolled medical or psychiatric conditions

Paired breast cancer biopsies were collected from premenopausal women undergoing diagnosis and surgery for breast cancer at The Queen Elizabeth Hospital. Paired samples were collected at two different time points: at the time of diagnostic biopsy and during the surgical excision of the breast cancer. Figure 4 shows a timeline describing when tissue samples were collected. Blood samples were collected at the same time as tissue collection.

Sample 1 (diagnostic biopsy)

Premenopausal women presenting to The Queen Elizabeth Hospital with breast lesions were recruited into the study. During the diagnostic investigation of the lesion, an additional core biopsy from the lesion was collected, placed in 10mL RNA later (Invitrogen), and stored at -80°C until use. A core biopsy of surrounding normal breast tissue was also collected at the time of diagnostic biopsy, and fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) overnight at 4°C. The normal breast tissue biopsy was washed with 1×PBS and stored in 70% ethanol, prior to processing and embedding in paraffin wax. An 8 mL blood sample was also collected in serum tubes at the time of diagnostic biopsy. Blood was centrifuged at 2,000g for 10 minutes, to separate serum. Serum was aliquoted into cryovials in a 1mL volume, and stored at -80°C.

Sample 2 (surgical biopsy)

Prior to surgery, patients completed a questionnaire detailing dates of their last menstrual period, menstrual cycle length, and pregnancy and oral contraceptive history. Following surgery, a core biopsy of normal breast tissue and of the breast tumour was collected from the excised surgical sample by TQEH pathological department. Normal tissue was fixed in 4% PFA and embedded in paraffin wax as previously described. Biopsies of the breast tumour were placed in RNA later, and stored at -80°C until use. An 8mL blood sample was also collected, and processed as previously described to collect serum.

2.2. Cell lines and culture

2.2.1. Cell culture

Hormone receptor (HR)-positive human breast cancer cell lines (T-47D, ZR-75-1) were obtained from American Type Culture Collection (ATCC). Both cell lines express ER and PR, and do not overexpress HER2. The T-47D cell line is characteristic of a Luminal A subtype, and exhibits a lower proliferative index compared to the Luminal B ZR-75-1 cell line (Table 8). As the Oncotype DX 21-gene assay was developed for use in HR-positive, HER2-negative breast cancers, these cell lines were ideal for studying the effects of ovarian hormones on the Oncotype DX 21-gene signature.

Table 8 Characteristics of the breast cancer cell lines.

	ER	PR	HER2	Ki67	Subtype
T-47D	+	+	-	Low	Luminal A
ZR-75-1	+	+	-	High	Luminal B

Breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% foetal bovine serum (FBS; Serana), 1% sodium pyruvate, (Life Technologies) and 1% penicillin/streptomycin (Life Technologies), and maintained in at 37°C in 5% CO_2 and 95% humidity. Our lab has previously established that these culture conditions do not exhibit high background hormone concentrations, and therefore will not obscure any effects of estrogen and progesterone on breast cancer gene expression.

2.2.2. *In vitro* hormone treatments

Breast cancer cell lines (T-47D, ZR-75-1) were seeded in triplicate in 6-well plates (5×10^5 cells/well, 2mL) for 72 hours. Cells were treated with 10nM 17β-estradiol (Sigma-Aldrich) for 72 hours, prior to treatment for 16 hours with either a vehicle control (ethanol; Sigma-Aldrich) or with 10nM progesterone (Sigma-Aldrich). Hormones were diluted in culture medium, and treatments were performed by overlaying the hormone treatment on the existing media, so that cells were treated with either 10nM estrogen alone (E), or 10nM estrogen + 10nM progesterone (E + P). In parallel, cells treated with the vehicle control alone (EtOH) were included as vehicle-treated controls. Following hormonal treatment, cells were trypsinised and used for RNA extraction, as described in 2.4.3.

To trypsinise, cells were washed with 1× phosphate buffered saline (PBS; Life Technologies) prior to treating with trypsin (Life Technologies) for 2 minutes at 37°C. Warmed complete medium was added to neutralise trypsin, and the total volume was centrifuged at 1200g for 5 minutes. Cells were resuspended in 1mL of complete medium. A cell count was performed by mixing 10µL cell suspension with 10µL 0.5M trypan blue, and counting viable cells using a Neubauer Haemocytometer.

2.3. Animal and general procedures

2.3.1. Mice

All animal experiments were approved by The University of Adelaide Animal Ethics Committee (Approval number: M2016-124), and performed in accordance with the Australian Code of Practice for the Care and use of Animals for Scientific Purposes (166). All mice were maintained in specific pathogen-free conditions with 12:12 light-dark cycles, and controlled temperature at the Laboratory Animal Services Medical School facility.

2.3.1.1. MMTV-PyMT transgenic mice

Transgenic mice harbouring the polyomavirus middle T-antigen oncogene (PyMT), driven by the mammary gland specific mouse mammary tumour virus promoter (MMTV) were used (167). Female MMTV-PyMT$^{+/-}$ mice on a FVB background spontaneously develop epithelial tumours in the mammary gland with 100% penetrance and an average latency of 6 weeks, and were used to model the effect of ovarian cycle stage on breast cancer biomarker expression.

2.3.1.2. BALB/c nude mice

Immunocompromised BALB/c nude mice were used to model the effects of ovarian hormones on human breast cancer cell line xenografts. BALB/c nude mice harbour a genetic mutation in the *Foxn1* gene, which results in a deteriorated or absent thymus and reduced T cell immune response. The immunocompromised nature of BALB/c nude mice allow human breast cancer cell lines to be grown in a mouse model, where we can manipulate the hormonal environment and timing of tissue collection. Female BALB/c nude mice of 6 weeks old were purchased from the Animal Resource Centre (Australia), and allowed to acclimatize for one week prior to experimentation. Mice were provided with antibiotic water (Baytril (enrofloxacin); Bayer) for the duration of the study.

2.3.2. Animal Procedures

2.3.2.1. Genotyping mice

Tail tips were collected from 3 week old mice in sterile 0.6mL Eppendorf tubes. Tissue was digested at 55°C overnight in 350µL digestion buffer (Table 9), with 0.1mg proteinase K (Sigma-Aldrich) added. Digested tails were diluted 1 in 20 with H_2O, and heated to 95°C to inactivate proteinase K. Extracted genomic DNA was stored at 4°C prior to PCR analysis.

Genotyping was determined by PCR amplification using allele-specific primers. Identification of the MMTV-PyMT allele was performed using MMTV-PyMT primers: MMTV 490 (F) 5'-CGTCCAGAAAACCACAGTCA-3' and MMTV 685 (R) 5'-CCGCTCGTCACTTAT CCTTC-3' (band size, 195 base pairs). The PCR reaction contained 1×DNA polymerase reaction buffer (Fisher Biotec, WA, Australia), 2.5mM $MgCl_2$ (Fisher Biotec), 200µM dNTPs (Roche), 5µM for each reverse and forward primer (Integrated DNA Technologies), 0.55U Taq polymerase (Fisher Biotec), and 2µL extracted DNA in a final reaction volume of 25µL. PCR reaction conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; and 72°C for 7 minutes.

PCR products were separated by size by gel electrophoresis. PCR products containing 1×loading buffer were run on a 2% agarose gel containing GelRed (Biotium) in TAE buffer for 40 minutes. Buffer compositions are described in Table 9. pUC19 (Geneworks) was used as a size marker. The gel was visualised under UV light to detect bands of PCR products. The presence of a 195bp product confirmed that mice carried the MMTV-PyMT gene (Figure 5).

2.3.2.2. Tumour monitoring

Mice were monitored weekly for tumour development by palpation. Briefly, mice were restrained by grasping the scruff of the neck between the thumb and fore-finger, and the base of the tail with the little finger. Mice were palpated from the first mammary gland to the tenth mammary gland to identify presence of any tumour. Following identification of the first mammary tumour, tumour growth was monitored weekly by using a set of digital callipers to measure the length and the width of the tumour. Tumour volumes were calculated as:

$$\text{Tumour volume} = \frac{length \times width \times width \times \pi}{6}\ \text{mm}^3$$

2.3.2.3. Estrous cycle tracking

The estrous cycle stage was tracked by vaginal smearing, as previously described (168). Briefly, the vagina was flushed with 20µL of sterile PBS, and the contents were pipetted on to a glass

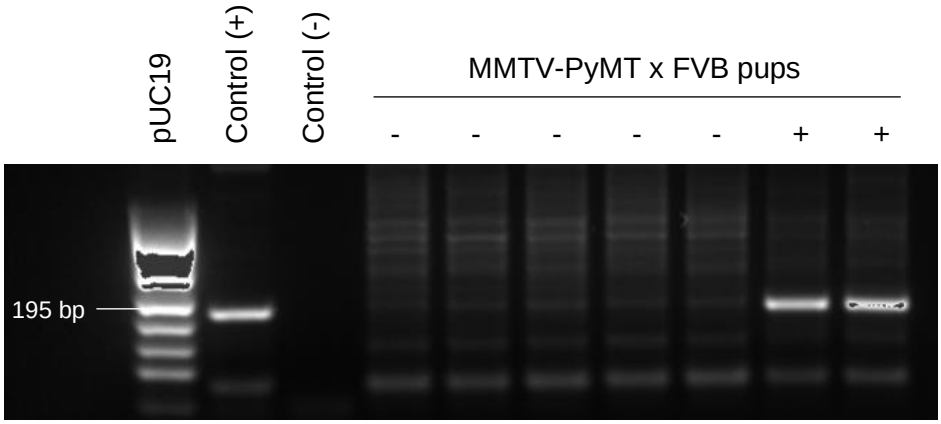


Figure 5 Genotyping MMTV transgene by PCR. Female FVB were mated with MMTV-PyMT+ males. Pups were weaned at 21 days, and genotyped. DNA from mice was amplified by PCR using MMTV primers, and the presence of a 195bp product identified that mice were carrying the transgene. Female pups carrying the MMTV-PyMT transgene were included in the study.

slide with a coverslip. The cellular contents were examined under a phase contrast microscope, and estrous cycle stage was determined based on the relative proportion of cell types (Figure 6). Estrus cycles were tracked for at least two full cycles, beginning when tumours were first palpable. MMTV-PyMT transgenic mice were euthanized at either the estrus or diestrus stage of the estrous cycle, and mammary tumours were dissected.

2.3.2.4. Blood collection

Cardiac puncture was used to collect blood from MMTV-PyMT mice under deep anaesthesia. Mice were injected intraperitoneally with 500µl 2% Avertin (Sigma-Aldrich). Following anaesthesia, up to 1mL of blood was collected directly from the heart using a 23G needle (BD). Blood was centrifuged for 10 minutes at 10,000g. Serum was collected and stored at -20 °C.

2.3.2.5. Estradiol pellet implantation

At 7 weeks of age, BALB/c nude mice were subcutaneously implanted with slow-release 17β-estradiol pellets under anaesthesia. Prior to surgery, surgical tools were sterilised through autoclaving, and washed in 70% ethanol. Mice were anaesthetised through administration of 2% isoflurane (Veterinary Companies of Australia Pty Ltd., Australia) as an inhalant, delivered with oxygen using a precision inhaler. Slow-release 17β-estradiol pellets (Innovative Research of America, 0.36mg/pellet, 60 day release) were subcutaneously implanted in the dorsal neck region of mice. The surgical incisions were closed using wound clips (Kent Scientific). Once regaining consciousness, mice were injected subcutaneously with buprenorphine analgesia (Temgesic; Intervet, Schering-Plough, Victoria, Australia) at 0.8 µg/10g of body weight. Following surgery, mice were maintained on a 37°C heating pad for 1 hour.

2.3.2.6. Inoculation of breast cancer cell lines

One week following 17β-estradiol pellet implantation, breast cancer cell lines were inoculated into the mammary fat pad of immunocompromised BALB/c nude mice under anaesthesia. Breast cancer cell lines T-47D or ZR-75-1 were cultured in T125 flasks under conditions previously described. Cells were washed with PBS and trypsinised. Cell counts were performed, and cells were resuspended in 1.1mL PBS at a final concentration of 2×10^7 cells/mL (T-47D cells) or 1×10^8 cells/mL (ZR-75-1 cells). Cell resuspensions were mixed with an equal volume of growth factor-reduced Matrigel (Life Sciences), and kept on ice. Mice were anaesthetised as previously described, and 100µL of cells were injected into the mammary fat pad using a 23G syringe, delivering a total of 1×10^6 (T-47D) or 5×10^6 (ZR-75-1) cells. Following surgery, mice were maintained on a 37°C heating pad for 1 hour.

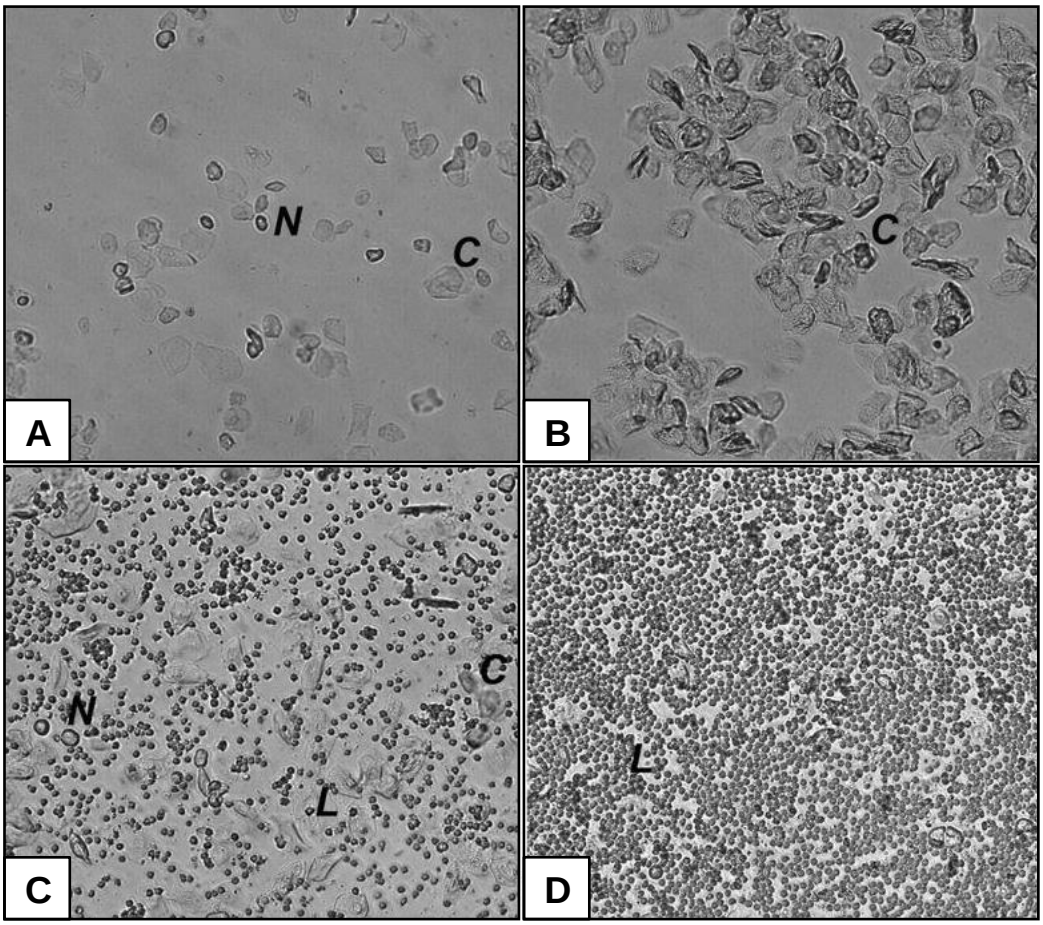


Figure 6 Estrous cycle stage determination by vaginal smears. Representative images of vaginal cytology at the (A) proestrus; (B) estrus; (C) metestrus; and (D) diestrus phase of the mouse estrous cycle. Abbreviations: N = nucleated epithelial cell, C = cornified epithelial cell, L = leukocyte. Image derived from (169).

2.3.2.7. Progesterone treatment

Progesterone (Sigma-Aldrich) was dissolved in absolute ethanol (Sigma-Aldrich) and diluted in sesame oil (Sigma-Aldrich) to a final concentration of 10mg/mL. For the vehicle control, absolute ethanol alone was diluted in sesame oil. Tumours were palpated weekly to monitor growth, as previously described. Once xenografted tumours reached 100-150mm^2, BALB/c nude mice were randomised into treatment groups and treated with either 100µL progesterone (to deliver 1mg progesterone), or 100µL of the vehicle control. Mice were treated daily for 3 days. Mice were culled by cervical dislocation 24 hours following the final treatment, and tissue was collected.

2.3.2.8. Tissue collection from mice

Primary tumours dissected from MMTV-PyMT and BALB/c nude mice were cut in half. Half the tumour was fixed in in 4% paraformaldehyde (PFA; Sigma-Aldrich) overnight at 4°C, washed thrice with 1×PBS and stored in 70% ethanol, prior to processing and embedding in paraffin wax. The other half of the tumour was immediately snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction was performed.

Table 9 Composition of buffers and solutions used in the animal experiments. All reagents were supplied by Sigma-Aldrich.

10 × PBS	2g potassium dihydrogen phosphate, 17.8g disodium hydrogen phosphate, 80g sodium chloride, 2g potassium chloride. Make up to 2L with H_2O. Adjust pH to 7.2 - 7.4. Store at room temperature. Dilute 1:10 with H_2O for 1×stock.
Tail digestion buffer	2.5 mL 1M Tris, 3.75 mL 5M NaCl, 12.5 mL 0.5M EDTA, 25 mL 10% SDS. Make up to 250 mL with H_2O. Adjust pH to 7.8. Store at room temperature.
10 × TAE buffer	48.4g Tris base, 20mL 0.5M EDTA, 11.42mL glacial acetic acid. Make up to 1L with H_2O. Store at room temperature. Dilute 1:10 with H_2O for 1×stock.
Agarose gel	2g agarose in 100mL in 1×TAE buffer. Add 10µL GelRed.
6 × Loading buffer	3ml 100% Glycerol, 25mg Bromophenol blue. Make up to 10ml with H_2O. Store at room temperature.
Avertin	1g 2,2,2-triromomethanol dissolved in 1mL 2methyl-2-butanol. Make up to 50mL with H_2O. Aliquot and store at -20°C.

2.4. RNA extraction

2.4.1. Formalin-fixed paraffin-embedded human breast cancer samples

Six 10μm thick sections were cut from FFPE tissue blocks by The Queen Elizabeth Hospital pathology department, and mounted on glass slides. Tissue sections were scraped off the glass slides into an Eppendorf tube, and RNA was extracted using the PureLink FFPE RNA Isolation kit (Invitrogen) as per the manufacturer's protocol. To elute RNA, columns were washed once with 20μL RNase-free H_2O (to be used for downstream cDNA synthesis), and again with 50μL RNase-free H_2O (to retrieve remaining RNA). RNA was quantified using the Nanodrop Spectrometer 2000. Absorbance ratios at 260/280 were assessed to confirm RNA purity. All samples exhibited absorbance ratios of ~2.0 and were considered pure, and were included in downstream analyses.

2.4.2. Human breast cancer biopsies

Human breast cancer biopsies were stored in RNA later at -80°C until RNA extraction was performed. Breast cancer biopsies were homogenised using TRIzol as described above, prior to RNA being extracted using the PureLink RNA Mini Kit (Invitrogen) as per the manufacturer's instructions. To elute RNA, columns were washed once with 20μL RNase-free H_2O (to be used for downstream cDNA synthesis), and again with 50μL RNase-free H_2O (to retrieve remaining RNA). RNA was quantified using the Nanodrop Spectrometer 2000. Absorbance ratios at 260/280 were assessed to confirm RNA purity, as described above.

2.4.3. Breast cancer cell lines

Total RNA was extracted from *in vitro* cell lines using an RNeasy kit (Qiagen) as per the manufacturer's protocol. Total RNA was eluted in 35μl of RNase-free H_2O (Invitrogen), and treated with DNase (DNase-free, Invitrogen). RNA was quantified using Nanodrop Spectrometer 2000 (ThermoScientific). Absorbance ratios at 260/280 were assessed to confirm RNA purity, as described above.

2.4.4. Breast cancer cell line xenografts and MMTV-PyMT mammary tumours

For mammary tumours dissected from mice, total RNA was extracted from snap frozen tissue using TRIzol (Life Technologies). Tumours were transferred into Eppendorf tubes containing 0.6g of 1.4mm ceramic beads (Qiagen) and 1mL TRIzol (Invitrogen) and were homogenised using the Powerlyzer 24 homogenizer (MoBio, USA)(30Hz, 5 minutes). Samples were incubated on ice for 10 minutes before 200μL of chloroform (Sigma-Aldrich) was added.

Samples were mixed vigorously by manual shaking, and incubated on ice for 15 minutes. Samples were then centrifuged for 15 minutes at 11,000g at 4°C, and the top aqueous layer (RNA containing) was transferred into a new Eppendorf tube. Equal volumes of isopropanol (approximately 500µL; Sigma-Aldrich) were added to the tubes, and samples were incubated overnight at -20°C. Samples were centrifuged for 30 minutes at 11,000g at 4°C, and supernatant discarded. The RNA pellet was washed twice with 500µL ice cold 70% ethanol, and centrifuged at 11,000g for 10 minutes at 4°C between washes. The RNA pellet was air dried on the bench for 30 minutes, before being resuspended in 50µl of RNase-free H_2O.

Samples were treated with DNase to remove genomic DNA contamination, using a TURBO DNA-free kit (Life Technologies) according to the manufacturer's instructions. Briefly, 5µL of 10×TURBO DNase Buffer and 1µL TURBO DNase to the RNA was added, and RNA samples were incubated at 37°C for 30 minutes. To inactivate the DNase enzyme, 5µL of DNase Inactivation Reagent (Life Technologies) was added to each sample, and incubated for 5 minutes at room temperature, with occasional mixing to redisperse the DNase Inactivation Reagent. Samples were then centrifuged at 10,000g for 1.5 minutes at 4°C, and RNA was transferred to a fresh Eppendorf. RNA was quantified using Nanodrop Spectrometer 2000. Absorbance ratios were assessed to confirm RNA purity, as described above.

2.4.5. RNA storage by precipitation

For long term storage, RNA was precipitated in 2.5× sample volume of 100% ethanol (Sigma-Aldrich) and 0.1× sample volume of 3M sodium acetate (Life Technologies). The tube was mixed by inverting, and stored at -20°C. To resuspend RNA for use, samples were centrifuged for 20 minutes at 11,000g at 4°C, and supernatant discarded. The RNA pellet was washed twice with 500µL ice cold 70% ethanol, and centrifuged at 11,000g for 10 minutes at 4°C between washes. The RNA pellet was air dried on the bench for 30 minutes, before being resuspended in 50µl of RNase-free H_2O.

2.5. Complementary DNA (cDNA) synthesis

For FFPE tissues, cDNA was reverse transcribed from between 100 to 500ng of RNA, depending on the quantity of RNA retrieved during RNA extraction. cDNA was synthesised using random hexamer primers and SuperScript IV VILO with ezDNase Enzyme (Invitrogen) as per the manufacturer's instructions. Briefly, 1µL of 10×ezDNase Buffer and 1µL ezDNase enzyme were added to an appropriate volume of RNA, and samples were incubated at 37°C for 2 minutes. Samples were centrifuged briefly, and 4µL SuperScript IV VILO Master Mix and

6µL RNase-free H_2O was added. Reactions were incubated at 25°C for 10 minutes, 50°C for 10 minutes, and 85°C for 5 minutes. Synthesised cDNA was diluted 1:5 in RNase-free H_2O, and stored at -80°C until use.

For human breast cancer biopsies, cDNA was reverse transcribed from 500ng of RNA using iScript cDNA synthesis kit (Bio-Rad). Reactions were incubated at 25°C for 5 minutes, 42°C for 25 minutes, and 85°C for 5 minutes. Synthesised cDNA was diluted 1:10 in RNase-free H_2O, and stored at -20°C until use.

For breast cancer cell lines and mouse mammary tumours, cDNA was reverse transcribed from 500ng of RNA using iScript cDNA synthesis kit (Bio-Rad). Reactions contained an appropriate volume of RNA (for 500ng), 1×iScript reaction mix (continuing dNTPs, MgCl2 and buffer), and 1µL iScript reverse transcriptase in a 20µL total volume. Reactions were incubated at 25°C for 5 minutes, 42°C for 25 minutes, and 85°C for 5 minutes. Synthesised cDNA was diluted 1:10 in RNase-free H_2O, and stored at -20°C until use.

2.6. TaqMan arrays

Gene expression was measured in FFPE breast cancer samples and prospectively collected breast cancer samples using custom designed 384-well TaqMan array cards (ThermoFisher). Each array card comprised of 8 channels, each which measured expression for 24 individual genes, in duplicate. Primers for each gene were designed by ThermoFisher.

Cards were loaded with 100µL of 1:1 mix of cDNA and TaqMan Fast Advanced Master Mix (Applied Biosystems). Array cards were centrifuged twice at 1200g for 1 minute, sealed, and inlet ports removed. Array cards were run using a QuantStudio12K Real-Time PCR system (Applied Biosystems). PCR conditions were 2 minutes at 50°C, 10 minutes at 92°C followed by 50 two-step cycles of 1 second at 95°C and 20 seconds at 60°C.

2.7. Quantitative real-time PCR

For *in vitro* cell lines and cell line xenografts, real-time PCR (RT-PCR) amplification was performed on Bio-Rad CFX96 using SYBR Green PCR Master Mix (Bio-Rad). Primer pairs specific to human genes were obtained from Integrated DNA Technologies and are detailed in Table 10. Reactions were performed in a total volume of 10ul, containing 1×SYBR green master mix (Bio-Rad), 4µM forward and reverse primers, and cDNA template (2µL). PCR conditions were 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 30 seconds.

Table 10 PCR primers used to quantify gene expression in T-47D and ZR-75-1 breast cancer cell lines *in vitro* and *in vivo*. Primers recognise human genes. Housekeeping genes are highlighted in grey.

Gene	Primer sequence	Product length (bp)
MKI67	5' - TCCAGACGCCAAAATAAGACTG 3' - TCCATCTCTGGGGAGGTCTTC	119
STK15	5' - GAGGTCCAAAACGTGTTCTCG 3' - ACAGGATGAGGTACACTGGTTG	205
BIRC5A	5' - AGGACCACCGCATCTCTACAT 3' - AAGTCTGGCTCGTTCTCAGTG	118
CCNB1	5' - AATAAGGCGAAGATCAACATGGC 3' - TTTGTTACCAATGTCCCCAAGAG	111
MYBL2	5' - GGTGGCTGAGAGTTTTGAATCC 3' - CCTCCTCGGTCCAGCAAGA	210
ESR1	5' - TGATTGGTCTCGTCTGGCGCT 3' - GCACACAAACTCCTCTCCCTGC	179
PGR	5' - CGCGCTCTACCCTGCACTC 3' - TGAATCCGGCCTCAGGTAGTT	121
BCL 2	5' - CTGCACCTGACGCCCTTCACC 3' - CACATGACCCCACCGAACTCAAAGA	119
SCUBE2	5' - CCAGGCAATTATCGTTGCACT 3' - TGACGTTGACACAGGTATGCT	124
ERBB2	5' - CTGAACTGGTGTATGCAGATTGC 3' - TTCCGAGCGGCCAAGTC	83
GRB7	5' - CCATCTGCATCCATCTTGTT 3' - GGCCACCAGGGTATTATCTG	67
MMP11	5' - AAGGTATGGAGCGATGTGACG 3' - GTCCAGGTCTCATCATAGTCGAA	197
CTSL	5' - CACCGGCTTTGTGGACATC 3' - ATGACCTGCATCAATAGCAACA	94
GSTM1	5' - GGCCCAGCTTGAATTTTTCA 3' - AAGCTATGAGGAAAAGAAGTACACGAT	86
CD68	5' - ATGATGAGAGGCAGCAAGATGG 3' - GCTACATGGCGGTGGAGTACAA	262
BAG1	5' - TCATAGGGGTTCCACAGTCTTTTC 3' - AACATGACCCGGCAACCAT	112
EGFR	5' - CGAGTCGGGCTCTGGAGGAAAAGA 3' - ATTCCCAAGGACCACCTCACAGTT	131
FOXA1	5' - GGGGGTTTGTCTGGCATAGC 3' - GCACTGGGGGAAAGGTTGTG	147
MRPL19	5' - TGCCAGTGGAAAAATCAGCCA 3' - CAAAGCAAATCTCGACACCTTG	119
ACTB	5' - CATCCGCAAAGACCTGTACG 3' - AGTACTTGCGCTCAGGAGG	149
GAPDH	5' - GTCATGGGTGTGAACCATGAGA 3' - GGTCATGAGTCCTTCCACGATAC	135
RPLPO	5' - TGCAGCCCAGAACACTGGTC 3' - ACTCAGGATTTCAATGGTGCC	100
GUSB	5' - GTCTGCGGCATTTTGTCGG 3' - CACACGATGGCATAGGAATGG	127
TFRC	5' - GGCTACTTGGGCTATTGTAAAGG 3' - CAGTTTCTCCGACAACTTTCTCT	156

For mammary tumours dissected from MMTV-PyMT transgenic mice, RT-PCR amplification was performed on a Bio-Rad CFX384 Real Time Detection System using SYBR Green PCR Master Mix (Bio-Rad). Reactions were loaded into 384-well plates in triplicate, using a QIAgility robot (Qiagen). Reactions were performed in a total volume of 10µl, containing 1×SYBR green master mix, 4µM forward and reverse primers, and cDNA template (2µL). No template controls were included with each run, and consisted of nuclease-free H_2O in place of cDNA. Primer pairs specific to mouse genes were obtained from Integrated DNA Technologies and are detailed in Table 11. PCR conditions were 95°C for 10 minutes, then 50 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 30 seconds.

Gene expression was measured in triplicate, and defined as the average number of cycles required to reach threshold (CT). Normalised gene expression was then calculated using the ΔCt method, relative to the expression of appropriate housekeeping gene(s), where:

$$\text{Normalised gene expression} = 2^{-((\text{gene of interest})-(\text{average of housekeeping gene(s)}))}$$

2.8. Calculation of Oncotype DX Recurrence Scores

Oncotype DX Recurrence Scores were calculated from reference-normalised gene expression, following the algorithm described in (104). Normalised gene expression measurements were calculated as Δ CT = CT (gene of interest) – CT (mean of five reference genes) as per (170). A 1-unit increase in reference-normalised expression measurements reflects a doubling of RNA.

To calculate Oncotype DX Recurrence Scores, group scores were first calculated using normalised gene expression measurements of individual genes:

$$\text{GRB7 group score} = 0.9\times\mathit{GRB7} + 0.1\times\mathit{ERBB2}$$

$$\text{ER group score} = (0.8\times\mathit{ESR1} + 1.2\times\mathit{PGR} + \mathit{BCL2} + \mathit{SCUBE2}) \div 4$$

$$\text{Proliferation group score} = (\mathit{BIRC5} + \mathit{KI67} + \mathit{MYBL2} + \mathit{CCNB1} + \mathit{STK15}) \div 5$$

$$\text{Invasion group score} = (\mathit{CTSL2} + \mathit{MMP11}) \div 2$$

The unscaled recurrence score (RSu) was calculated from the above group scores:

$$\text{RSu} = +0.47\times\text{GRB7 group score} - 0.34\times\text{ER group score} + 1.04\times\text{proliferation group score} + 0.10\times\text{invasion group score} + 0.05\mathit{CD68} - 0.08\times\mathit{GSTM1} - 0.07\times\mathit{BAG1}$$

The scaled recurrence score (RS) was then calculated from the unscaled recurrence score:

Table 11 PCR primers used to quantify gene expression in MMTV-PyMT mammary tumours. Primers recognise mouse genes. Housekeeping genes are highlighted in grey.

Gene	Primer sequence	Product length (bp)
Mki67	5' - AATCCAACTCAAGTAAACGGGG 3' - TTGGCTTGCTTCCATCCTCA	127
Stk15	5' - CTGGATGCTGCAAACGGATAG 3' - CGAAGGGAACAGTGGTCTTAACA	105
Birc5a	5' - CTACCGAGAACGAGCCTGATT 3' - AGCCTTCCAATTCCTTAAAGCAG	60
Ccnb1	5' - AGAGCTATCCTCATTGACTGGC 3' - AACATGGCCGTTACACCGAC	155
Mybl2	5' - GTGAGGCAGTTTGGACAGCAA 3' - GGATTCAAAACCCTCAGCCA	101
Esr1	5' - TGATTGGTCTCGTCTGGCGCT 3' - GCACACAAACTCTTCTCCCTGC	179
Pgr	5' - CGCCATCTACCAGCCGCTC 3' - TGAATCTGGCCTCAGGTAGTT	121
Bcl 2	5' - ATGCCTTTGTGGAACTATATGGC 3' - GGTATGCACCCAGAGTGATGC	120
Scube2	5' - GGCTGTGTCCACGACTGTTTA 3' - GTTCTCCAAGCATTCGTCCAT	117
Erbb2	5' - TGCTCAACTGGTGTGTTCAGATT 3' - TTCGGGCAGCTAGGTCC	84
Grb7	5' - ACAAACAGGCATATCCCATGAAG 3' - TAGAGGCCAGATCGACGCA	159
Mmp11	5' - GCCTGATGTACTGAATGCCC 3' - GCTCCCTTACAAGCTGCCA	119
Ctsv	5' - ATCAAACCTTTAGTGCAGAGTGG 3' - CTGTATTCCCCGTTGTGTAGC	136
Gstm1	5' - ATACTGGGATACTGGAACGTCC 3' - AGTCAGGGTTGTAACAGAGCAT	349
Cd68	5' - TGTCTGATCTTGCTAGGACCG 3' - GAGAGTAACGGCCTTTTTGTGA	75
Bag1	5' - GCAGCAGGGAGTTGACTAGAA 3' - TTACTTCCTCGGTTTGGGTCG	111
Actb	5' - GTGTGACGTTGACATCCGTAAAG 3' - CTCAGGAGGAGCAATGATCTTGAT	151
Gapdh	5' - ACACATTGGGGGTAGGAACA 3' - AACTTTGGCATTGTGGAAGG	223
Rplpo	5' - AGATTCGGGATATGCTGTTGGC 3' - TCGGGTCCTAGACCAGTGTTC	109
Gusb	5' - CGGGCTGGTGACCTACTGGATT 3' - TGGCACTGGGAACCTGAAGT	134
Tfrc	5' - GTTTCTGCCAGCCCCTTATTAT 3' - GCAAGGAAAGGATATGCAGCA	152

RS = 0 if RSu < 0

RS = 20×(RSu – 6.7) if 0 ≤RSu≤100

RS = 100 if RSu > 100

Tumours were classified into 3 categories based on the Recurrence Scores; low risk (RS < 18), intermediate risk (RS 18-30), high risk (RS > 30).

2.9. Histology and Immunohistochemistry

2.9.1. Haematoxylin Eosin staining

Haematoxylin and eosin staining was performed on 5µM paraffin-embedded sections cut using a Microtome (Leica Biosystems, Australia). Sections were dewaxed in xylene, and subsequently passed through 100%, 90%, 70% and 50% ethanol for rehydration. Slides were stained with haematoxylin (Sigma-Aldrich) and counterstained with eosin (Sigma-Aldrich), prior to dehydrating and mounting with Entellan mounting medium (Merck).

2.9.2. Immunohistochemistry for ER, PR, and Ki67

For cell line xenografts and tumours dissected from MMTV-PyMT mice, staining of ER, PR, and Ki67 protein were assessed on 5µM sections mounted on coated glass slides. Sections were dewaxed in xylene, and subsequently passed through 100%, 90%, 70% and 50% ethanol for rehydration. Antigen retrieval was performed at 95°C for 20 minutes, using either Dako EnVision low pH or high pH antigen retrieval solution (Dako), depending on the primary antibody used. Sections were blocked for endogenous peroxidases (peroxidase blocking solution, Dako REAL), prior to incubation with appropriate primary and secondary antibodies. Antibody binding was detected using DAB (Dako), and sections were counterstained with haematoxylin. Sections were dehydrated by passing through increasing ethanol concentrations, then xylene, before mounting with Entellan mounting medium. Serial sections stained with secondary antibodies only were included as negative controls. Stained tissue sections were captured as a digital image using a NanoZoomer 1.0 (Hamamatsu, Shizouka, Japan) at a zoom equivalent to a 40× objective lens.

The antibodies and conditions used for staining breast cancer cell line xenografts (Table 12) and MMTV-PyMT mammary tumours (Table 13) are described below.

Table 12 Antibodies and conditions used for formalin-fixed paraffin-embedded breast cancer cell line T-47D and ZR-75-1 xenografts

Antigen	Clone	Antigen retrieval	1° antibody incubation	Secondary antibody
Estrogen Receptor (Dako, M704729-2)	1D5	High	1:100 at 4°C overnight	antibody:DaKO HRP (RTU); 1 hour room temperature
Progesterone Receptor (Dako, M356929-2)	PgR636	High	1:200 at 4°C overnight	antibody:DaKO HRP (RTU); 1 hour room temperature
Ki67 (Abcam, ab16667)	SP6	Low	1:100 at 4°C overnight	Goat α-Rabbit (Dako, 1:500); 1 hour room temperature

Table 13 Antibodies and conditions used for formalin-fixed paraffin-embedded MMTV-PyMT mammary tumours

Antigen	Clone	Antigen retrieval	1° antibody incubation	Secondary antibody
Estrogen Receptor (Santa Cruz, sc8005)	D12	High	1:50 at 4°C overnight	antibody:DaKO HRP (RTU); 1 hour room temperature
Ki67 (Abcam, ab16667)	SP6	Low	1:100 at 4°C overnight	Goat α-Rabbit (Dako, 1:500); 1 hour room temperature

2.9.3. Digital quantification of staining

To determine the number of positive stained cells in tumours, staining was quantified using the ImmunoRatio plugin within ImageJ software, which identified percent positive staining by calculating (DAB stain)/(Hematoxylin stain). Protein expression was assessed across an entire section of the tumour. Corresponding serial sections stained with the secondary antibody only were used to subtract background, as described in Figure 7.

2.9.4. Pathological assessment of tumours

Haematoxylin and eosin staining of MMTV-PyMT mammary tumours were assessed by a veterinary pathologist. Tumours were graded and cytological atypia defined. Staining of ER and KI67 was assessed to define percent positivity.

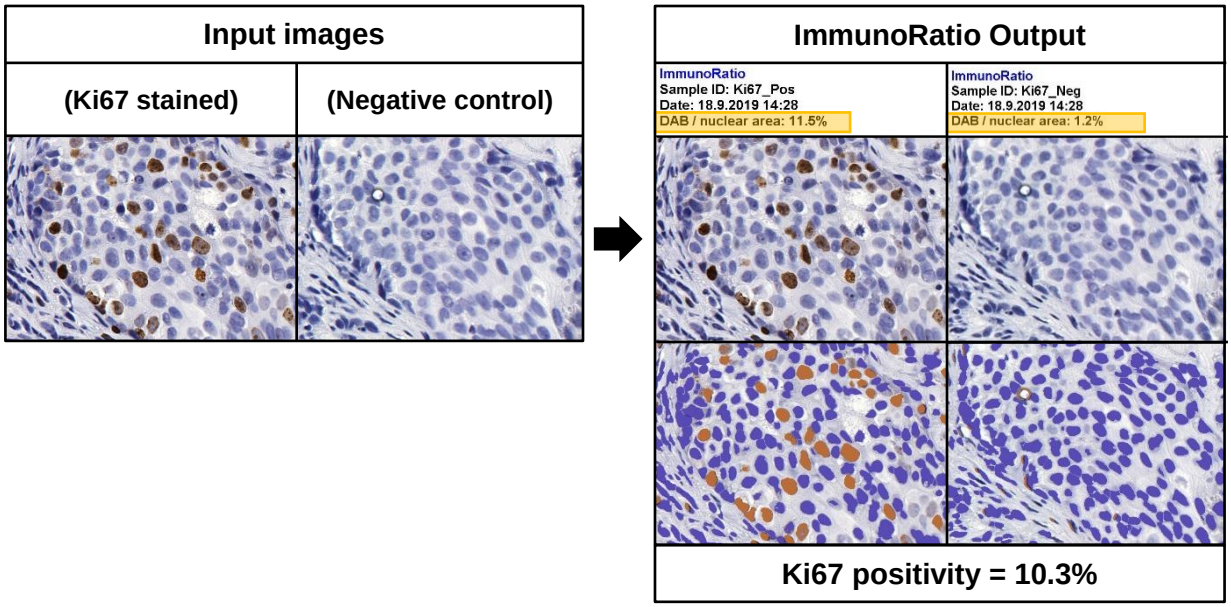


Figure 7 Quantification of staining using the ImmunoRatio plugin within the ImageJ software. Stained tissue sections were captured as a digital image using a NanoZoomer. (A) Images at a 60× magnification were input into the ImmunoRatio plugin within Image J software. (B) The ImmunoRatio algorithm identified the percent positive staining by calculating (DAB stain)/(Hematoxylin stain). Corresponding serial sections stained with the secondary antibody only were used to subtract background. The plugin only identifies cells, ignoring the surrounding matrix. The above example above identified an 11.5% positivity in the Ki67 stained section, and a 1.2% positivity in the negative control. Therefore, the overall Ki67 positivity for this individual image was calculated as 10.3%. This process was performed multiple times for each tumour for protein expression to be assessed across the whole tumour section.

Haematoxylin and eosin staining of human breast cancer samples were assessed by a pathologist to confirm presence of invasive disease. The hormone receptor status and proliferative index of breast cancer samples was obtained from reports completed by a pathologist at the time of breast cancer diagnosis.

2.10. Statistical analyses

All data were assessed using SPSS Statistics Version 24 (IBM Corporation, Armonk, NY, USA) or SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Linear mixed-effects models were performed to investigate concordance in the expression of Oncotype DX signature genes between paired diagnostic and surgical breast cancer samples, for women aged <50 years and women aged >50 years. Unstructured covariance structures were used to adjust for repeated measurements over time. The magnitude of change in gene expression and Oncotype DX Recurrence Scores between paired samples were assessed (i) with age as a continuous variable; and (ii) with age as a categorical variable (i.e, women aged <50 years compared to women aged >50 years old) using linear regression analysis. Variation between age groups was assessed using Levene's test for equality of variances. In mouse studies, average expression of Oncotype DX signature genes and Oncotype DX Recurrence Scores were compared between estrous cycle stages and treatment groups using T-tests for normally distributed data. Differences in gene expression and Oncotype DX Recurrence Scores between clusters were assessed using one way analysis of variance with post hoc comparisons performed. Data were considered significant when $p<0.05$.

Chapter Three

Variability in the Oncotype DX 21-gene signature between paired breast cancer samples

3.1. Introduction

Hormone receptor (HR)-positive breast cancers account for approximately 70-80% of all breast cancer cases. Within this breast cancer subtype there are highly variable responses to chemotherapy and heterogeneous clinical outcomes. Traditionally, the decision to treat HR-positive breast cancers with adjuvant chemotherapy is based on the clinical and pathological features of the tumour, with many women recommended chemotherapy based on these criteria (171). However, this results in significant over-treatment with chemotherapy, as many women would have been adequately treated with endocrine therapy alone (77-79)

The Oncotype DX 21-gene Recurrence Score assay is recommended in international guidelines to assist adjuvant chemotherapy treatment decisions for women with early stage, HR-positive, HER2-negative breast cancer (76, 94, 172, 173); promising improved decision making capabilities compared to traditional protein-based methods. The Oncotype DX assay evaluates the expression of 21 genes to calculate a Recurrence Score, which reflects the patient's underlying risk of disease recurrence and predicts the likely benefit from the addition of chemotherapy to endocrine treatment (77, 104-108). This assay has been shown to influence treatment decision-making in the clinic, with a 12% reduction in chemotherapy use (4). However, while Oncotype DX is available to both premenopausal and postmenopausal women to assist treatment decision-making, it was developed predominantly in postmenopausal women, and there is a scarcity of literature on whether it is suitable for use in premenopausal women (174).

In premenopausal women, ovarian hormones estrogen and progesterone fluctuate during the menstrual cycle. Studies have shown that HR protein expression fluctuates across the menstrual cycle, in response to fluctuating concentrations of estrogen and progesterone (6, 151, 175), and is associated with downstream gene expression changes (5, 6). Changes in gene expression with menstrual cycle stage could impact Oncotype DX Recurrence Scores. Indeed, previous studies suggest that co-treatment of breast cancer cell lines with estrogen and progesterone *in vitro* increases Oncotype DX Recurrence Scores, compared to estrogen treatment alone (43).

Results from a prospective analysis further support the possibility that the Oncotype DX assay may be inherently different in prediction of risk of recurrence between premenopausal and postmenopausal women. The TAILORx study (77) incorporated data from 9,719 women with breast cancer (n=3,054 women aged ≤50 years; n=6,665 women aged >50 years). The authors reported that for women over the age of 50 years with Oncotype DX Recurrence Scores ≤26, endocrine therapy alone was not inferior to chemoendocrine therapy in terms of disease free

and overall survival. However, women under the age of 50 with Oncotype DX Recurrence Scores between 16 and 26 still exhibit some benefit from chemotherapy treatment. When clinical information was integrated with Oncotype DX Recurrence Score, the prediction of which premenopausal patients would have a substantial benefit from chemotherapy was not improved (114). The biological basis of this age-related difference in chemotherapy benefit is not well defined; however, it is possible that hormonal fluctuations during the menstrual cycle of premenopausal women contribute to this difference.

Currently, it remains unclear whether Oncotype DX Recurrence Scores are affected by fluctuating concentrations of estrogen and progesterone during the menstrual cycle. To begin to address this, a retrospective study of paired breast cancer samples collected from younger and older women was conducted. As this is a retrospective study, there were no details of patient menopausal status or menstrual cycle stage at the time of tissue collection. However, if menstrual cycling affects Oncotype DX Recurrence Scores, it is expected that there would be high variability in Recurrence Scores between paired breast cancer samples in younger women with hormone-responsive breast cancer, as the samples could have been collected at different stages of their menstrual cycle. Therefore, it is hypothesised that there will be increased discordance in Recurrence Scores between paired samples collected from younger women with HR-positive disease, compared to non-cycling older women or women with HR-negative disease.

Paired FFPE invasive breast cancer samples were collected approximately 2 weeks apart from women aged under 50 years old and compared to women aged over 50 years old. HR-negative tumours were included as a hormone-unresponsive control. The Oncotype DX 21-gene signature was assessed through quantitative RT-PCR and Oncotype DX Recurrence Scores were calculated using the Oncotype DX Recurrence Score algorithm. Results from this chapter show that discordances in Recurrence Scores are more variable between paired samples collected from younger women, compared to older women and women with HR-negative tumours, and are driven by a variable expression of Proliferation- and HER2-group genes.

3.2. Results

3.2.1. Patient identification and recruitment

Potential patients to be included in the study were identified from a list of 878 breast cancer patients who had been referred to The Queen Elizabeth Hospital Oncology Unit between 2000 and 2015. For each patient, medical records were individually reviewed to determine if they met the eligibility criteria for the study. Of all patients, a total of 794 patients did not meet this criteria as they either; (i) did not have paired samples available; (ii) had received neoadjuvant therapy which would have introduced confounding bias into our results; (iii) were deceased, or (iv) were male. These patients were excluded from the study.

Patient information sheets and consent forms were sent to the 84 women who met the inclusion criteria, and consent was sought via telephone call. A total of 53 women agreed to participate. The Queen Elizabeth Hospital Pathology Department retrieved and sectioned FFPE tissue blocks for 46 of these women, as tissue blocks for 7 women could not be sectioned as there was insufficient sample available remaining in the block or the block was stored off site.

Of the blocks sectioned, hematoxylin and eosin stains were performed to confirm presence of malignant breast disease prior to RNA extraction. As Oncotype DX testing is recommended for use in women with invasive breast cancer, only patients with invasive disease present in the sample were included in the analysis. Four patients had either benign disease or insufficient invasive disease present in their FFPE samples, and were therefore excluded from analysis. Examples of hematoxylin and eosin stained tissue sections are presented in Figure 8.

RNA was extracted from 42 paired invasive breast cancer samples; however, a further 7 paired samples were excluded from analysis due to insufficient RNA yield, as discussed in section 3.2.2. A total of 35 patients were included in the study; 29 patients with HR-positive breast cancer (18 women <50 years old; 11 women >50 years old); and 6 women with HR-negative breast cancer. The process for identifying and recruiting patients is highlighted in Figure 9.

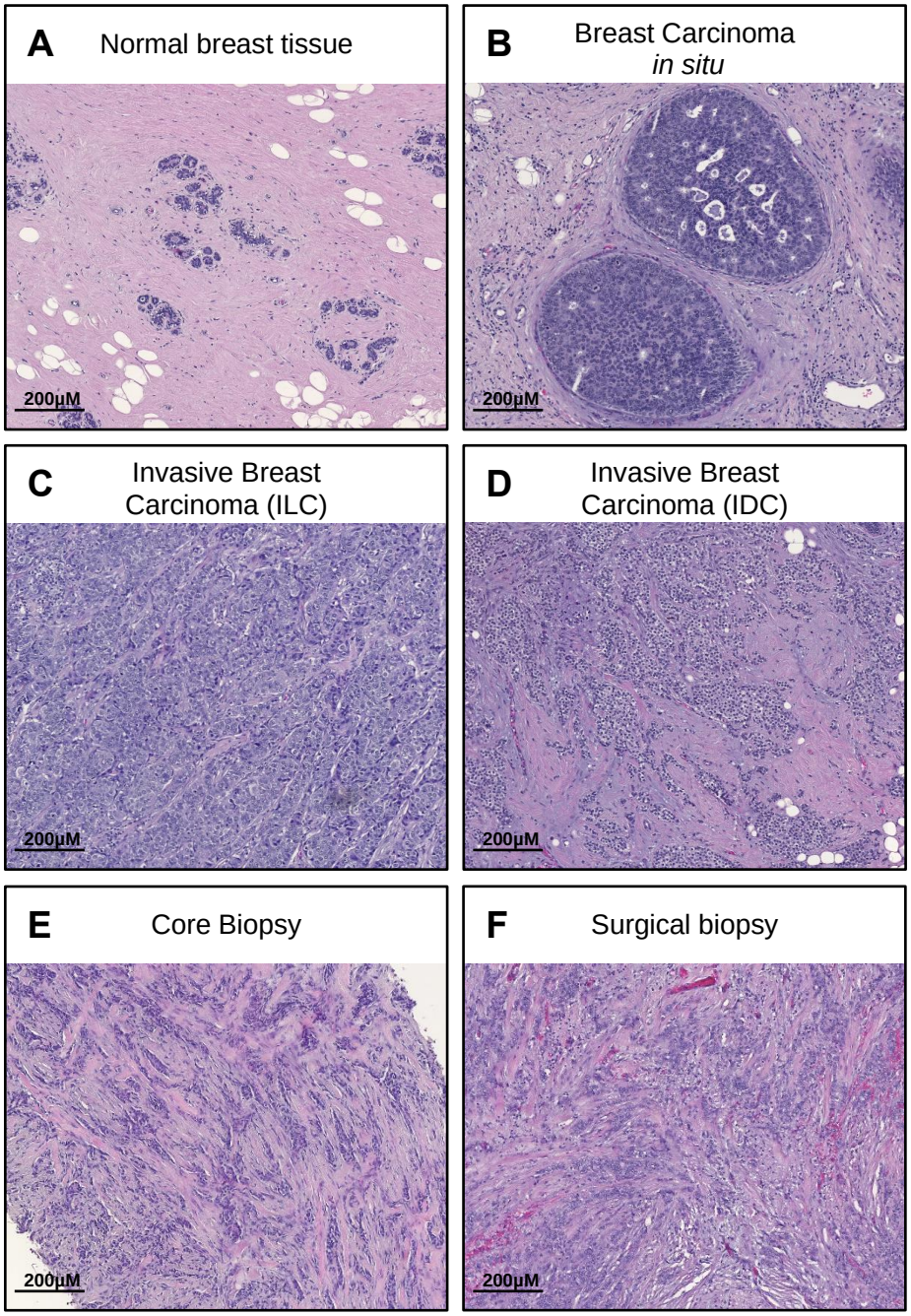


Figure 8 Haematoxylin and eosin stains of formalin-fixed paraffin-embedded tissue sections collected from breast cancer patients. FFPE tissue blocks were retrieved from 46 patients, and hematoxylin and eosin stains were performed to confirm presence of malignant breast disease. Examples of (A) normal breast tissue; (B) carcinoma *in situ;* (C) invasive lobular carcinoma (ILC); and (D) invasive ductal carcinoma (IDC). Samples that did not contain invasive disease were excluded from analysis. (E-F) Hematoxylin and eosin stain of a core biopsy and corresponding surgically excised tumour from a patient with a grade 3 invasive ductal carcinoma. Bars represent 200µM.

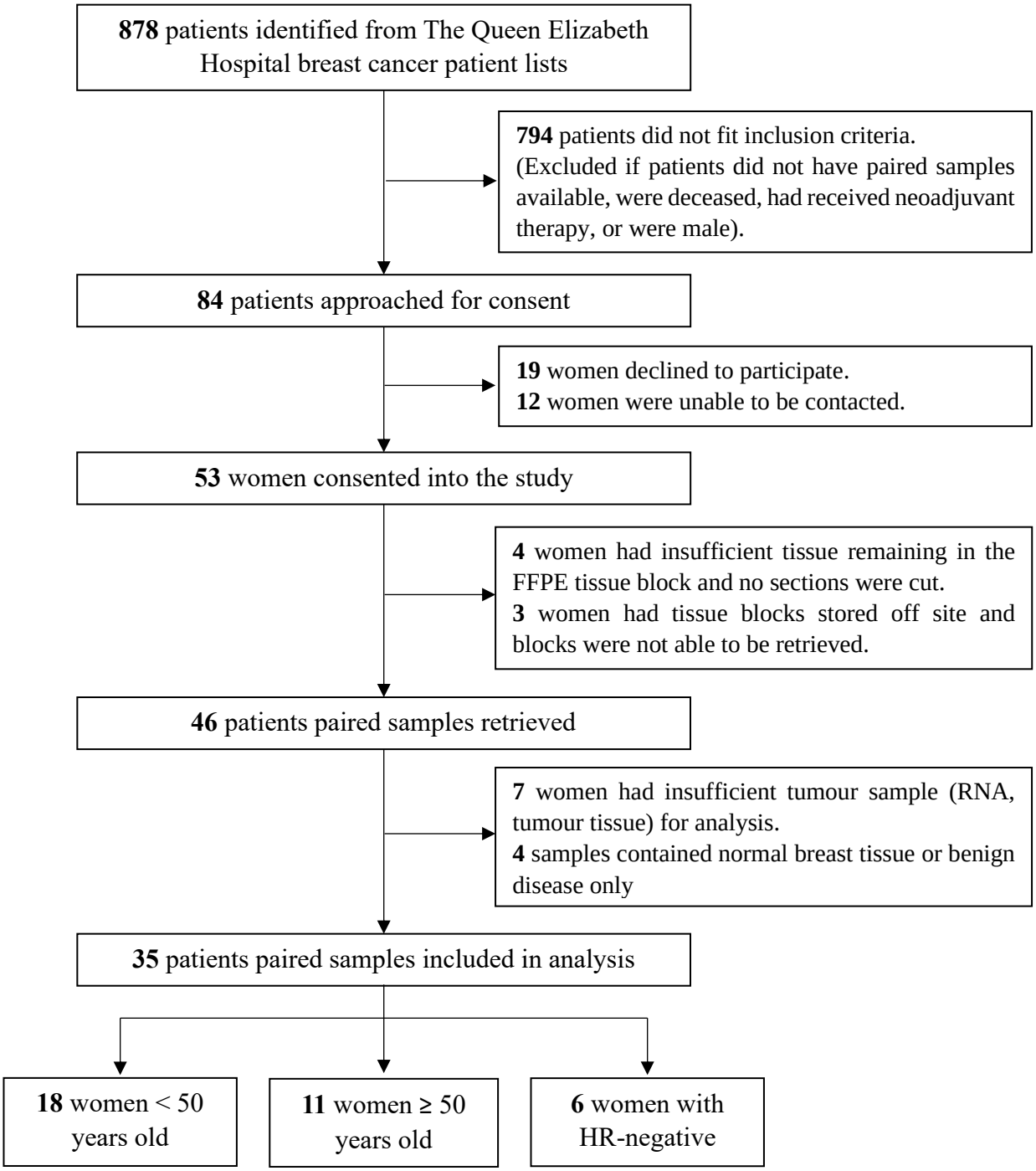


Figure 9 Flow chart showing patient recruitment. Potential patients for inclusion in the study were identified from The Queen Elizabeth Hospital breast cancer patient lists. Of the 878 patients initially identified, 35 women were included in the study; 18 women aged under 50, 11 women aged over 50; and 6 women with HR-negative disease.

3.2.2. Assessing the purity and quality of RNA in archival formalin-fixed paraffin-embedded breast cancer samples

Challenges arise when using FFPE tissue samples for gene expression analysis, as RNA extracted from archival samples can be of a low quality and partially degraded. As this has the potential to introduce bias into results (176), it is important to confirm that the quality of RNA is acceptable prior to use in downstream applications.

Previous studies suggest that the quality and quantity of RNA can be influenced by the extraction method used (177). Therefore, to overcome limitations in using FFPE tissue samples, we extracted RNA from FFPE breast cancer samples using an RNA isolation kit specifically designed for use with archival tissue samples. RNA was extracted from 42 paired breast cancer samples, for a total of 84 samples, and the quantity and quality of extracted RNA was assessed using the Nanodrop spectrophotometer. The amount of RNA isolated from FFPE tissue samples ranged from 22ng – 2464.6ng (mean 363.8ng), which was of sufficient yield for downstream quantitative RT-PCR analysis. The amount of RNA varied significantly between samples depending on the tissue collection method (i.e, biopsy versus surgery), where an increased amount of RNA was extracted from surgical samples.

To confirm the purity of RNA, absorbance ratios were also measured using the Nanodrop spectrophotometer. For all RNA samples, the 260:280 absorbance ratios fell within the desired range, ranging from 1.83 – 2.02. This suggests that the samples are free from DNA, phenol, or other contaminants, and that the RNA is of suitable purity for gene expression analysis.

To further confirm the quality of RNA, cDNA was prepared from the RNA and quantitative RT-PCR was performed to detect expression of housekeeping gene *ACTB* and breast cancer biomarker *ESR1*, using well-established primers. The CT values of *ACTB* (mean CT = 33.5) and *ESR1* (mean CT = 39.3) were detected at appropriate levels for 35 of the 41 samples. The remaining 7 samples showed CT values of >45 and were therefore excluded from further analysis. Of the 35 samples detected through RT-PCR, no non-specific amplification was present as indicated by the single peak in the melt curve analysis and the absence of amplification in the negative controls (Figure 10). Together, these experiments confirm that the RNA extracted from FFPE tissue samples is of appropriate quality for use in downstream gene expression analysis.

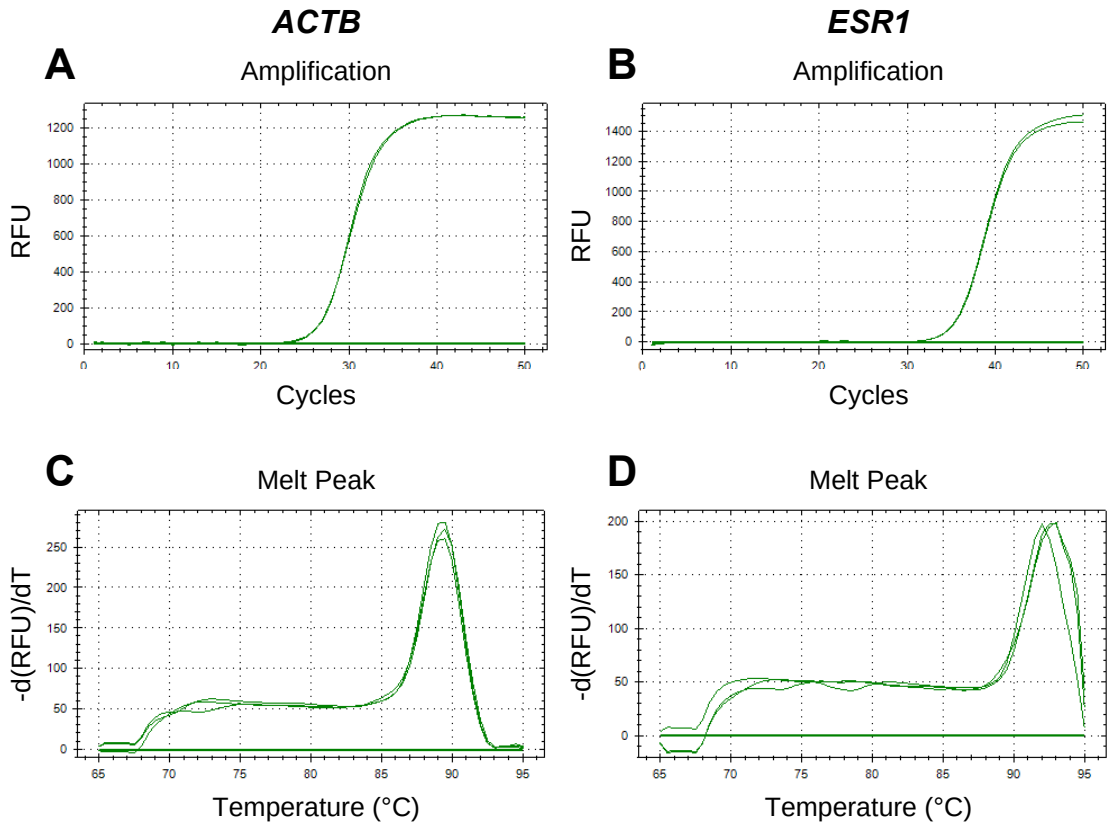


Figure 10 Assessing the quality and purity of RNA extracted from archival formalin-fixed paraffin-embedded breast cancer samples. RNA was extracted from 46 paired FFPE breast cancer samples using the PureLink FFPE RNA isolation kit. Following assessment of RNA quantity and purity using the Nanodrop spectrometer, the quality of RNA was further confirmed through quantitative real-time PCR analysis. cDNA was synthesised from extracted RNA, and real-time PCR was performed using primers recognising housekeeping gene *ACTB* and breast cancer biomarker *ESR1*. Examples of amplification curves for (A) *ACTB* and (B) *ESR1*, and melting peaks for (C) *ACTB* and (D) *ESR1* for one sample run in triplicate. No amplification was detected in no template controls.

3.2.3. Patient characteristics

Twenty-nine patients with HR-positive, invasive breast cancer were included in the analysis (18 women <50 years old; 11 women >50 years old). Six women with HR-negative invasive breast cancer were also studied, and acted as a hormone-unresponsive control. Hormone receptor status was obtained from pathology reports completed during the routine breast cancer diagnosis. Patient and tumour characteristics are detailed in Table 14.

Paired samples were collected from the same tumour at different times; 30 women had paired core needle biopsies and corresponding surgical excisions, while 5 women had paired surgical excisions and corresponding re-excisions with residual disease. Sample 1 was defined as the earlier collected sample, with sample 2 as the corresponding later collected pair. The dates of sample collection were obtained from electronic medical records. Paired samples were collected an average of 17 days apart in women under the age of 50, an average of 18 days apart in women over the age of 50, and an average of 17 days apart in HR-negative tumours.

Table 14 Patient and tumour characteristics. Tumour characteristics including HR-status, and tumour type, grade and size were obtained from reports completed by a pathologist during the routine breast cancer diagnosis. Patient details including date of birth and the dates of biopsy and surgery were recorded from medical records. Women were separated into groups based on their age at diagnosis (i.e, <50 years old versus >50 years old). The age of 50 was selected as it is the best age for distinguishing premenopausal women from postmenopausal women when menopausal status is unknown (178). Abbreviations: IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma.

Characteristics	Women <50yrs n (%)	Women >50yrs n (%)	HR-negative n (%)
Total Number			
	18	11	6
Median age at diagnosis (years; range)			
	45 (33 – 49)	67 (52 – 77)	51 (33 – 78)
Median days between samples (days; range)			
	18 (2 – 42)	17 (6 – 49)	17 (13 – 24)
Tumour Type			
IDC	14 (78)	7 (64)	6 (100)
ILC	3 (17)	4 (36)	0 (0)
Other	1 (6)	0 (0)	0 (0)
Tumour Grade			
1	5 (28)	1 (9)	0 (0)
2	7 (39)	9 (82)	0 (0)
3	5 (28)	1 (9)	6 (100)
Unknown	1 (6)	0 (0)	0 (0)
Tumour size			
≤5mm	1 (6)	3 (27)	0 (0)
6-10mm	1 (6)	0 (0)	1 (17)
11-20mm	8 (44)	2 (18)	3 (50)
21-50mm	6 (33)	3 (27)	2 (33)
>50mm	1 (6)	3 (27)	0 (0)
Unknown	1 (6)	0 (0)	0 (0)
Lymph node status			
Negative	12 (67)	3 (27)	5 (83)
Positive	5 (28)	8 (73)	0 (0)
Unknown	1 (6)	0 (0)	1 (17)
Lymphovascular invasion			
Present	5 (28)	4 (36)	5 (83)
Absent	13 (72)	6 (55)	0 (0)
Unknown	0 (0)	1 (9)	1 (17)

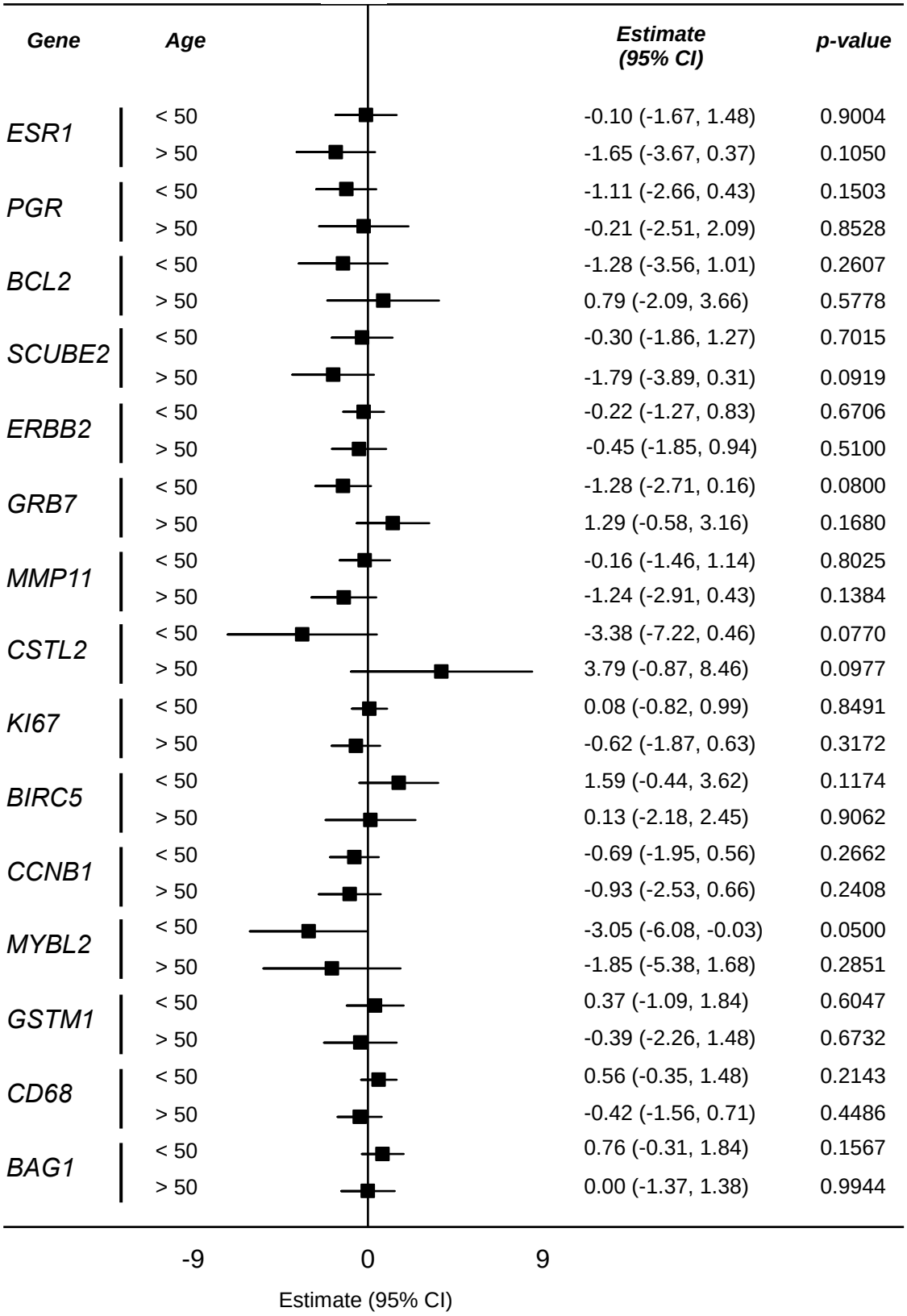
3.2.4. Expression of the Oncotype DX 21-gene signature between paired breast cancer samples collected from younger women, compared to older women.

Samples collected from the same breast cancer on different days are anticipated to show a degree of variability in gene expression due to extrinsic factors such as the precise part of the tumour biopsied, and differences in tumour fixation and processing (179, 180). This variability is expected to be similar between premenopausal and postmenopausal women.

Indeed, we found no significant differences in expression of any Oncotype DX signature genes between sample 1 and sample 2 in both younger women (<50 years old) and older women (>50 years old) when assessed using linear mixed effect models adjusted for repeated measurements ($p>0.05$). Additionally, the magnitude of change in gene expression between paired samples collected from younger women did not differ statistically compared to older women, when assessed through linear regression analysis ($p>0.05$; Figure 11).

Together, these findings confirm that extrinsic factors—including tumour heterogeneity and tissue collection and processing methods—affect gene expression to a similar extent in younger women versus older women, and that the two samples that comprise each pair are related in their gene expression signature.

Figure 11 Concordance in Oncotype signature gene expression between paired breast cancer samples. Paired breast cancer samples were collected from women under the age of 50 (n=18) and compared to women over the age of 50 (n=11). Expression of the 21 genes included in the Oncotype DX gene signature were measured by quantitative real-time PCR, and gene expression was normalised to the average of 5 reference genes (*ACTB*, *GAPDH*, *RPLPO*, *GUS*, *TFRC*). Results are presented as a forest plot showing concordance in gene expression between sample 1 and sample 2, for younger women (<50) and older women (>50). Statistical significance was assessed with age as a continuous variable using linear mixed-effect models adjusted for multiple comparisons. No data were statistically significant ($p>0.05$).


Gene
Age
Estimate (95% CI)
p-value
ESR1
< 50
-0.10 (-1.67, 1.48)
0.9004
> 50
-1.65 (-3.67, 0.37)
0.1050
PGR
< 50
-1.11 (-2.66, 0.43)
0.1503
> 50
-0.21 (-2.51, 2.09)
0.8528
BCL2
< 50
-1.28 (-3.56, 1.01)
0.2607
> 50
0.79 (-2.09, 3.66)
0.5778
SCUBE2
< 50
-0.30 (-1.86, 1.27)
0.7015
> 50
-1.79 (-3.89, 0.31)
0.0919
ERBB2
< 50
-0.22 (-1.27, 0.83)
0.6706
> 50
-0.45 (-1.85, 0.94)
0.5100
GRB7
< 50
-1.28 (-2.71, 0.16)
0.0800
> 50
1.29 (-0.58, 3.16)
0.1680
MMP11
< 50
-0.16 (-1.46, 1.14)
0.8025
> 50
-1.24 (-2.91, 0.43)
0.1384
CSTL2
< 50
-3.38 (-7.22, 0.46)
0.0770
> 50
3.79 (-0.87, 8.46)
0.0977
KI67
< 50
0.08 (-0.82, 0.99)
0.8491
> 50
-0.62 (-1.87, 0.63)
0.3172
BIRC5
< 50
1.59 (-0.44, 3.62)
0.1174
> 50
0.13 (-2.18, 2.45)
0.9062
CCNB1
< 50
-0.69 (-1.95, 0.56)
0.2662
> 50
-0.93 (-2.53, 0.66)
0.2408
MYBL2
< 50
-3.05 (-6.08, -0.03)
0.0500
> 50
-1.85 (-5.38, 1.68)
0.2851
GSTM1
< 50
0.37 (-1.09, 1.84)
0.6047
> 50
-0.39 (-2.26, 1.48)
0.6732
CD68
< 50
0.56 (-0.35, 1.48)
0.2143
> 50
-0.42 (-1.56, 0.71)
0.4486
BAG1
< 50
0.76 (-0.31, 1.84)
0.1567
> 50
0.00 (-1.37, 1.38)
0.9944
-9
0
9
Estimate (95% CI)

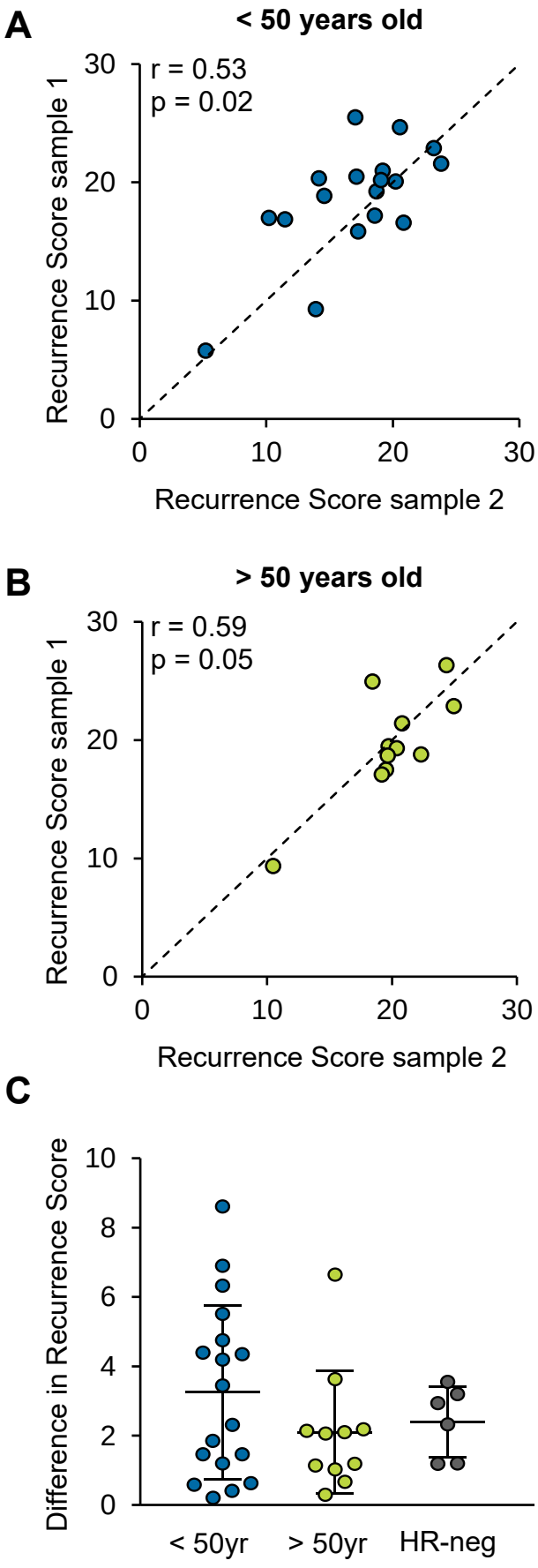
3.2.5. Comparing variability in Oncotype DX Recurrence Scores between paired breast cancer samples

Oncotype DX calculates Recurrence Scores on the basis of gene expression. Therefore, we sought to determine how variation in the expression of Oncotype DX signature genes between paired samples might impact Oncotype DX Recurrence Scores. Using reference normalised gene expression, Recurrence Scores were calculated using the Oncotype DX Recurrence Score algorithm. Recurrence Scores of sample 1 significantly correlated with Recurrence Scores of sample 2, in both younger women (Spearman's correlation coefficient r=0.53; p=0.02; Figure 12A) and older women (r=0.59; p=0.05; p=0.0003; Figure 12B).

To quantify discordance in Recurrence Scores between paired breast cancer samples, the absolute difference in Recurrence Scores between sample 1 and sample 2 was calculated. The average difference in Recurrence Scores was not statistically different between younger women (3.2±2.5; mean±stdev), as compared to older women (2.0±1.7; p=0.08). However, younger women had a more variable discordance in Oncotype DX Recurrence Scores between paired samples, as reflected by the significantly larger standard deviation when assessed using the Levene's test for equality of variances (p=0.04; Figure 12C).

Paired breast cancer samples from a cohort of women with HR-negative disease were also investigated. These tumours do not express estrogen or progesterone receptors and are expected to be less responsive to fluctuations in ovarian hormone during the menstrual cycle. Differences in Recurrence Scores between paired HR-negative samples (2.3±1.0) did not differ significantly, compared to paired samples collected from women aged under 50 (p=0.43) or women aged over 50 (p=0.68). However, younger women exhibited a more variable discordance in Recurrence Scores, compared to women with HR-negative disease (p=0.02). No differences in variation were observed between HR-negative tumours and women aged over 50 (p=0.64; Figure 12C)

Figure 12 Concordance in Oncotype DX Recurrence Scores between paired breast cancer samples. Correlation in Oncotype DX Recurrence Scores for (A) women aged <50 years (n=18) and (B) women aged >50 years (n=11). Spearman's correlation coefficients and p-values are presented. (C) Discordance in Recurrence Scores between paired breast cancer samples collected from women under the age of 50, women over the age of 50, or HR-negative tumours (n=6). Oncotype DX Recurrence Scores were calculated from reference-normalised gene expression, and discordances were quantified by calculating the absolute difference in Recurrence Scores between sample 1 and sample 2. Mean discordances were compared using linear regression analysis. The variation from mean was assessed using Levene's test for equality of variances. Data are presented as individual values. Bars represent mean±stdev.


A
< 50 years old
r = 0.53
p = 0.02
Recurrence Score sample 1
Recurrence Score sample 2
0
10
20
30
B
> 50 years old
r = 0.59
p = 0.05
Recurrence Score sample 1
Recurrence Score sample 2
C
Difference in Recurrence Score
0
2
4
6
8
10
< 50yr
> 50yr
HR-neg

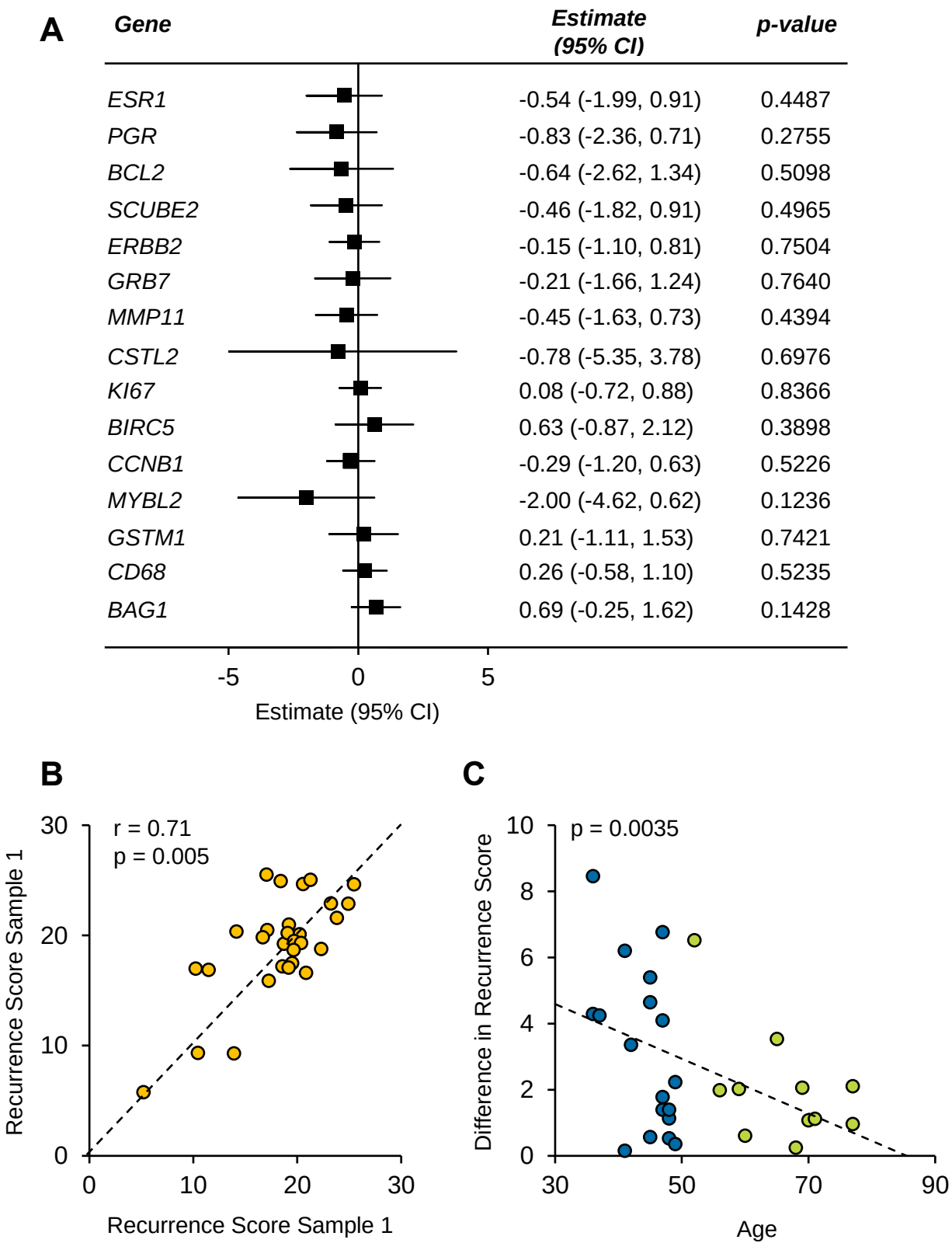
3.2.6. Comparing variability in Oncotype DX Recurrence Scores between paired breast cancer samples as a factor of age

As the menopausal status of women was unknown, women were defined as "premenopausal" or "postmenopausal" using the age of 50 years as a cut-off. Although this age is suggested to be the optimal age-based proxy to define menopausal status (178), it may not accurately distinguish between premenopausal and postmenopausal women, and does not take into account perimenopausal women. Critically, an incorrect classification of menopausal status can introduce bias into our results.

To address this issue, we also assessed variability in the Oncotype DX 21-gene signature using age as a continuous variable. In agreement with findings from our categorical analysis of women aged <50 years compared to women aged >50 years, we found that patient age did not influence the magnitude of change in gene expression between paired samples when assessed using linear mixed-effect models adjusted for repeated measurements ($p>0.05$; Figure 13A). Similarly, there was a significant correlation in Oncotype DX Recurrence Scores between sample 1 and sample 2 for all women of all ages ($r=0.71$; $p=0.005$; Figure 13B); further suggesting that extrinsic factors similarly affected gene expression in both younger and older women.

We next analysed discordances in Recurrence Scores with age as a continuous variable. Consistent with findings from our categorical analysis, we found a significant inverse association between patient age and discordance in Recurrence Scores between paired samples. For every one year decrease in age, the difference in Recurrence Scores between paired samples increased by 0.07 units (95% confidence interval: -0.12, -0.02; $p=0.0035$; Figure 13C).

Figure 13 Concordance in Oncotype DX Recurrence Scores between paired breast cancer samples with age as a continuous variable. Paired breast cancer samples were collected from women with invasive, HR-positive breast cancer (n=29), and the Oncotype DX 21-gene signature was assessed through real time-PCR. Forest plot showing concordance in gene expression between sample 1 and sample 2 for the genes which comprise the Oncotype DX 21-gene signature. Statistical significance was assessed with age as a continuous variable using linear mixed-effect models adjusted for multiple comparisons. No data were statistically significant ($p>0.05$). (B) Correlation in Oncotype DX Recurrence Scores for all women. Spearman's correlations and p-values are presented. (C) Difference in Oncotype DX Recurrence Scores between paired breast cancer samples by age. Linear regressions were performed to investigate the association between the difference in RS and age as a continuous variable. Data are presented as individual values.


A
Gene
Estimate (95% CI)
p-value
ESR1 -0.54 (-1.99, 0.91) 0.4487
PGR -0.83 (-2.36, 0.71) 0.2755
BCL2 -0.64 (-2.62, 1.34) 0.5098
SCUBE2 -0.46 (-1.82, 0.91) 0.4965
ERBB2 -0.15 (-1.10, 0.81) 0.7504
GRB7 -0.21 (-1.66, 1.24) 0.7640
MMP11 -0.45 (-1.63, 0.73) 0.4394
CSTL2 -0.78 (-5.35, 3.78) 0.6976
KI67 0.08 (-0.72, 0.88) 0.8366
BIRC5 0.63 (-0.87, 2.12) 0.3898
CCNB1 -0.29 (-1.20, 0.63) 0.5226
MYBL2 -2.00 (-4.62, 0.62) 0.1236
GSTM1 0.21 (-1.11, 1.53) 0.7421
CD68 0.26 (-0.58, 1.10) 0.5235
BAG1 0.69 (-0.25, 1.62) 0.1428
-5
0
5
Estimate (95% CI)
B
r = 0.71
p = 0.005
Recurrence Score Sample 1
Recurrence Score Sample 1
C
p = 0.0035
Difference in Recurrence Score
Age

3.2.7. Identifying the underlying gene expression changes that drive increased variability in Oncotype DX Recurrence Scores in younger women

Having found that discordances in Recurrence Scores were more variable in younger women compared to older women, the underlying gene expression changes that contributed to the increased discordance was investigated. Paired breast cancer samples collected from women <50 years with minimal discordance were separated from paired samples with larger discordance by setting an arbitrary discordance threshold of 3 units (Figure 14A).

Paired breast cancer samples with discordance >3 units showed greater differences in the expression of Proliferation- ($p=0.05$; Figure 14B) and HER2-group scores ($p=0.02$; Figure 14D) between paired samples, compared to paired samples with discordance ≤3 units. Conversely, expression of Estrogen- ($p=0.99$; Figure 14C) or Invasion- ($p=0.97$; Figure 14E) group scores did not differ significantly between groups. Patient and tumour characteristics were similar between groups, and not likely to contribute to discordances (Table 15).

For older women and women with HR-negative tumours, there was an insufficient number of samples showing changes in Recurrence Scores greater than 3 units, and therefore these groups could not be statistically analysed.

Figure 14 Discordances in Oncotype DX group scores between paired breast cancer samples collected from younger women. (A) For women aged <50 years old, an arbitrary threshold of 3 units was set to distinguish between paired samples showing small differences in Recurrence Scores of ≤3 units (n=9) and paired samples showing large differences in Recurrence Scores of >3 units (n=9). Oncotype DX group scores were calculated for each tumour, and changes in group scores between paired breast cancer samples were compared. The change in (B) Proliferation-group; (C) Estrogen-group; (D) HER2-group; and (E) Invasion-group scores between paired breast cancer samples. Results are presented as mean+SEM. Mean discordances were compared using unpaired t-test. Statistical significance was determined when $p<0.05$ * signifies $p<0.05$.

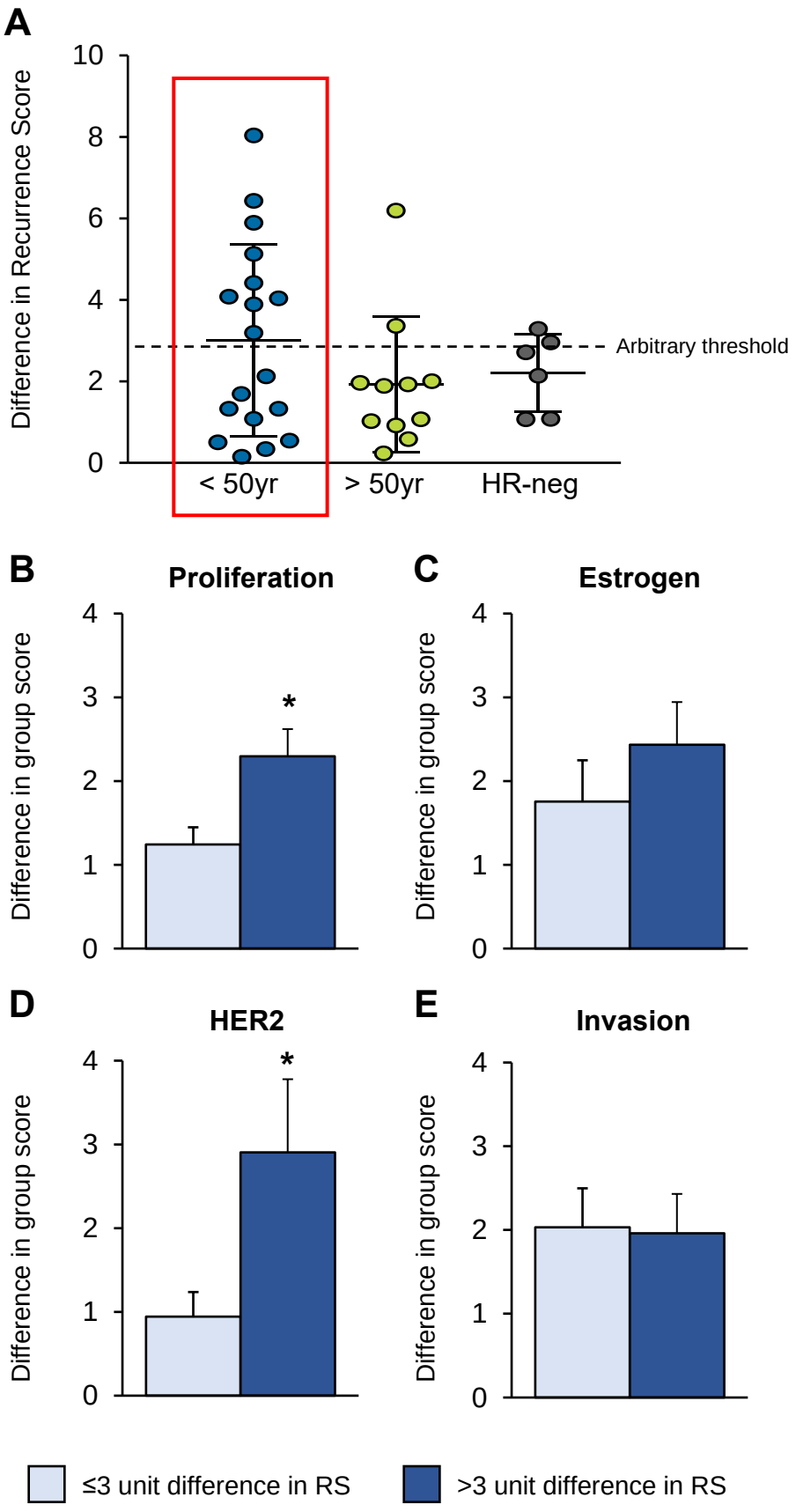

A
Difference in Recurrence Score
10
8
6
4
2
0
Arbitrary threshold
< 50yr
> 50yr
HR-neg
B
Proliferation
Difference in group score
*
C
Estrogen
Difference in group score
D
HER2
Difference in group score
*
E
Invasion
Difference in group score
≤3 unit difference in RS
>3 unit difference in RS

Table 15 Characteristics of patients aged <50 years with discordances in Recurrence Scores ≤3 units, compared to discordances >3 units.

Characteristics	≤ 3 unit change n (%)	> 3 unit change n (%)
Total Number		
	9	9
Median age at diagnosis (years; range)		
	47 (33 – 49)	43 (36 – 47)
Median days between samples (days; range)		
	21 (2 – 29)	15 (10 – 42)
Tumour Type		
IDC	8 (89)	6 (67)
ILC	1 (11)	2 (22)
Other	0 (0)	1 (11)
Tumour Grade		
1	3 (33)	2 (22)
2	4 (44)	3 (33)
3	2 (22)	3 (33)
Unknown	0 (0)	1 (1)
Tumour size		
≤5mm	1 (11)	0 (0)
6-10mm	1 (11)	0 (0)
11-20mm	4 (44)	4 (44)
21-50mm	2 (22)	4 (44)
>50mm	1 (11)	0 (0)
Unknown	0 (0)	1 (11)
Lymph node status		
Negative	8 (89)	4 (44)
Positive	1 (11)	4 (44)
Unknown	0 (0)	1 (11)
Lymphovascular invasion		
Present	2 (22)	3 (33)
Absent	7 (78)	6 (67)

3.2.8. A prospective analysis of the effects of menstrual cycling on the Oncotype DX 21-gene signature and Oncotype DX Recurrence Scores

To complement our retrospective analysis, we designed a prospective pilot study to further define the effects of menstrual cycle stage on the Oncotype DX 21-gene signature. This pilot study collected paired breast cancer core biopsies and corresponding surgical excisions from premenopausal women being treated at The Queen Elizabeth Hospital. At the time of breast cancer tissue collection, blood samples were also collected to quantify circulating concentrations of estrogen and progesterone, and patients completed a questionnaire detailing dates of their last menstrual period, menstrual cycle length, and pregnancy and oral contraceptive history.

However, due to unforeseen difficulties surrounding patient recruitment, as discussed later, we were only able to recruit one patient with invasive breast cancer into the study. A diagnostic core biopsy and corresponding surgical tumour specimen were collected from a 40 year old premenopausal woman. Samples were collected 20 days apart in the absence of neoadjuvant therapy and oral contraceptives. The patient's reported average cycle length was 26 days and samples were considered to be collected at different menstrual cycle stages. The core biopsy was collected on day 2 of her cycle (perimenstrual phase). Conversely, the tumour was surgically excised on day 22 of her cycle (luteal phase). Tumour characteristics were taken from the medical records at the time of diagnosis. The patient presented with a grade 3, 20mm, invasive ductal carcinoma. No lymph node metastasis was identified. The tumour was negative for expression of ER, PR and HER2, with a proliferative index of 70%.

To assess how gene expression varies between paired breast cancer samples, expression of the Oncotype DX gene signature was assessed through RT-PCR. Concordance in gene expression were compared between paired samples. Expression of each individual gene, normalised to the average of 5 housekeeping genes, was significantly correlated between core biopsies and surgical excisions (Spearman's correlation $r=0.98$; $p<0.0001$; Figure 15A). Similarly, there was good agreement in Oncotype DX group scores between paired samples (Figure 15B).

To determine how this variation in gene expression impacts Oncotype DX Recurrence Scores, Recurrence Scores were calculated for each sample. There was good agreement in Oncotype DX Recurrence Scores between paired samples. The Recurrence Score for the core biopsy was 87, while the Recurrence Score for the surgically excised tumour was 91 (Figure 15C). The tumour was classified into the Oncotype DX 'High Risk' group, which is expected as the cancer was diagnosed as a highly proliferative triple negative breast cancer subtype.

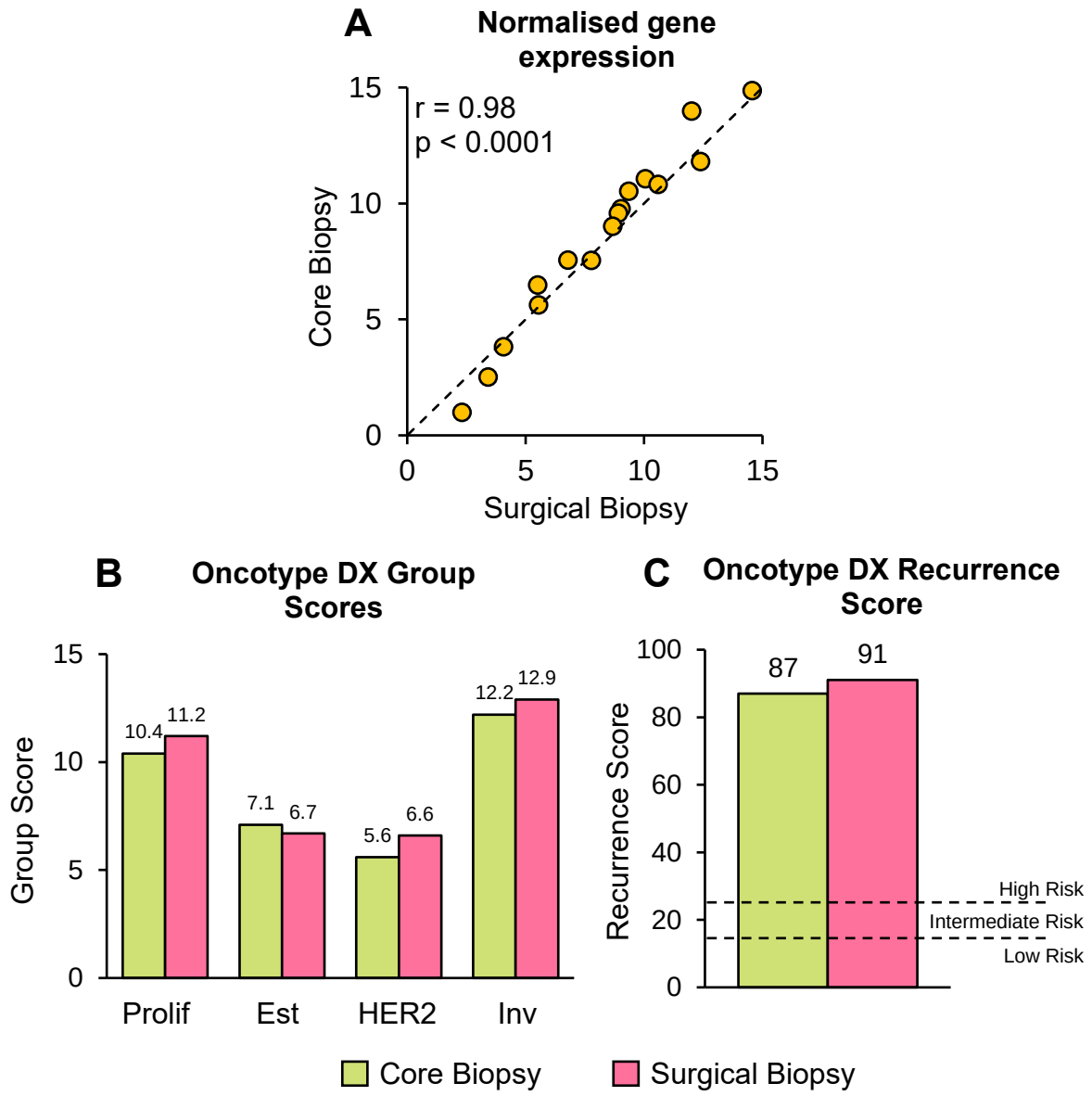


Figure 15 Concordance in the Oncotype DX 21-gene signature and Oncotype DX Recurrence Scores between a prospectively collected core biopsy and surgical breast cancer sample. (A) Correlations in the expression of Oncotype DX signature genes between paired core biopsy and surgical specimen (n=1). Each point represents an individual gene. Gene expression was normalised to the average of 5 reference genes (*ACTB, GAPDH, RPLPO, GUS, TFRC*), and are presented as arbitrary units. Spearman's correlations and p-values are presented. Agreement in (B) Oncotype DX group scores, and (C) Oncotype DX Recurrence Scores for the core biopsy and corresponding surgical specimen.

3.3. Discussion

The Oncotype DX 21-gene Recurrence Score is predictive of adjuvant chemotherapy benefit for women with early stage, HR-positive, HER2-negative breast cancer. In premenopausal women, fluctuations in estrogen and progesterone during the menstrual cycle impact gene expression in these hormone-responsive cancers. However, the extent to which hormonal fluctuations affect the Oncotype DX 21-gene signature and impact Oncotype DX Recurrence Scores remains unclear.

The experiments described in this chapter examined the extent to which the Oncotype DX 21-gene signature varies within the same tumour collected on different days. We show that discordances in Oncotype DX Recurrence Scores are more variable between paired samples collected from younger women, as compared to older women and women with HR-negative disease. In young women, discordances in Recurrence Scores were primarily driven by a variable expression of Proliferation- and HER2-group genes between paired samples. We propose that the increased variability in Oncotype DX Recurrence Scores is due to fluctuations in estrogen and progesterone during the menstrual cycle of premenopausal women, and the effects of estrogen and progesterone on expression of the Oncotype DX 21-gene signature.

3.3.1. Oncotype DX Recurrence Scores are more variable in young women and are driven by variable expression of Proliferation- and HER2-group genes

The observed variability in Proliferation- and HER2-group genes could be a factor of menstrual cycling, as previous studies report that tumour proliferation and HER2 gene expression fluctuate during the menstrual cycle, in accordance with concentrations of estrogen and progesterone. The highest proliferative activity of breast epithelium (11, 181), and breast cancer samples (152) are observed during the luteal phase, when circulating concentrations of progesterone peak. Additionally, *in vitro* and *in vivo* stimulation with estrogen and/or progesterone promotes proliferation of breast cancer cells, an effect that can be reversed with anti-estrogenic treatment (182-184). Similarly, HER2 expression fluctuates across the menstrual cycle, in accordance with concentrations of estrogen and progesterone. Previous studies suggest that HER2 expression is highest during the luteal phase (155), and that growth factor receptor signalling is increased in breast cancer cell lines following progesterone treatment (43).

While it has also been reported that expression of estrogen-regulated genes fluctuates across the menstrual cycle (5, 6), and that estrogen and progesterone affect the invasive properties of

premenopausal breast cancers (51-53), we did not observe variable expression of Estrogen or Invasion-group genes between paired breast cancer samples collected from younger women.

Together, these findings suggest that variable concentrations of estrogen and progesterone at the time of tissue collection could be affecting expression of Oncotype DX signature genes, primarily expression of genes involved in tumour proliferation and HER2 signalling, and contributing to the increased discordance in Oncotype DX Recurrence Scores. In further support of this possibility, postmenopausal breast cancers that are exposed to low circulating concentrations of estrogen and progesterone, and HR-negative tumours which are less responsive to fluctuations in estrogen and progesterone, exhibit lower variability in Oncotype DX Recurrence Scores.

However, there were several limitations within our study that must be considered. The patient's menopausal status was unknown, and women were defined as "premenopausal" or "postmenopausal" using the age of 50 years as a cut-off. While the age of 50 is suggested to be the optimal age-based proxy to distinguish premenopausal women from postmenopausal women when menopausal status is unknown (178), it may not be entirely accurate for distinguish between pre- and postmenopausal women, and does not take into account perimenopausal women. Critically, an incorrect classification of menopausal status could introduce significant variability into our results.

To address this issue, we also assessed variability in Oncotype DX Recurrence Scores with age as a continuous variable, finding a significant inverse relationship between patient age and discordance in Oncotype DX Recurrence Score. Younger women—who are likely to be cycling premenopausal women—exhibit a more variable discordance in Recurrence Scores between paired samples, compared to older non-cycling women. This further supports the possibility that variability in Oncotype DX Recurrence Scores are driven by fluctuations of estrogen and progesterone during the menstrual cycle of premenopausal women.

Our study was also limited by a lack of information on patient menstrual cycle stage, which prevented us from investigating the direct relationship between cycle stage (i.e, follicular versus luteal) and discordance in Oncotype DX Recurrence Scores. Similarly, the lack of information on circulating concentrations of estrogen and progesterone at the time of tissue collection prevented us from identifying a relationship between the specific hormonal environment and Oncotype DX Recurrence Scores. While we suggest Recurrence Scores are influenced by the timing of tissue collection in young women, the cycle stage and/or hormonal environment that

reflects the patient's true Oncotype DX Recurrence Score and predicted benefit from adjuvant chemotherapy is not yet known.

3.3.2. Discordances in Oncotype DX Recurrence Scores were limited to a subset of tumours collected from young women

While we suggest that Oncotype DX Recurrence Scores may be influenced by menstrual cycle stage, only a subset of young women showed large discordances in Recurrence Scores between biopsy and corresponding surgical breast cancer samples. As menstrual histories of women under the age of 50 were unknown, it is possible that tissue was collected at times of the cycle where concentrations of estrogen and progesterone did not differ significantly, or the woman was in a perimenopausal state with anovulatory cycles.

However, while the menstrual cycle stage was unknown, the time at which diagnostic and surgical biopsies were collected was documented. Paired samples were collected an average of 17 days apart in women under the age of 50, and therefore women were expected to be in a different menstrual phase at the time each sample collection. As such, the impact of menstrual cycle stage at the time of tissue collection on Oncotype DX Recurrence Scores might be limited, as only a dramatic change in circulating hormone concentrations might significantly impact Oncotype DX Recurrence Scores.

Additionally, biological differences between tumours may explain why discordances in Recurrence Scores were only observed in a subset of tumours collected from young women. There is significant heterogeneity within HR-positive breast tumours that contributes to their heterogeneous clinical outcomes and variable response to chemotherapy, and can also influence their responsiveness to ovarian hormones. A subset of tumours may be more sensitive to fluctuations in estrogen and progesterone during the menstrual cycle, and more prone to cycle-induced changes in Oncotype DX Recurrence Scores. The underlying tumour biology that drives this increased susceptibility, and whether it is possible to identify tumours that are more sensitive to fluctuations in estrogen and progesterone, warrants further investigation.

Similarly, the heterogeneous nature of HR-positive breast tumours may contribute to discordances in Oncotype DX Recurrence Scores. However, several studies have assessed the effects of tumour heterogeneity on gene expression profiling, finding that core biopsies are representative of the whole tumour area in terms of gene expression profiles (185) and Oncotype DX Recurrence Scores (185, 186). Furthermore, the inclusion of older non-cycling women and HR-negative tumours in our analysis allowed us to identify the degree of natural

variation between paired samples. Increased variability in younger women, compared to older women and HR-negative tumours, supports the notion that hormone variation could be driving discordances in Oncotype DX Recurrence Scores, as opposed to confounding extrinsic factors such as tumour heterogeneity.

Collectively, the results from this chapter suggest that Oncotype DX testing may be less reliable for premenopausal women, and Recurrence Scores might partially depend on the menstrual cycle stage at the time of tissue collection. This can have dramatic implications for the treatment of younger women, as Oncotype DX testing at a specific cycle stage may prompt unnecessary chemotherapy use or result in under-treatment. Further research is warranted, as the timing of Oncotype DX testing in accordance with a specific menstrual cycle stage, or identifying a panel of genes to be assessed alongside the 21-gene signature, may provide opportunities to improve the predictive power of Oncotype DX Recurrence Scores for premenopausal women.

3.3.3. Prospective analysis of paired biopsy and surgical breast cancer samples collected from premenopausal women

Limitations in our retrospective study are largely derived from the lack of information on patient's menstrual cycle history. The patient's menopausal status, cycle length, and menstrual cycle stage at the time of tissue collection was unknown, nor did we know whether women were taking hormonal contraceptives. Such factors can impact results if not accounted for, and caution strong conclusions.

To determine whether menstrual cycle stage significantly impacts Oncotype DX Recurrence Scores, a study that prospectively collects paired samples from premenopausal women at different menstrual cycle stages is required. In this chapter, we have presented preliminary data using a prospectively collected core biopsy and matched surgical breast cancer sample. Information regarding the patient's last menstrual period was collected, and blood samples were taken to measure circulating hormone concentrations at the times of tissue collection. We also considered confounding factors, including irregular menses, oral contraceptive use, and adjuvant therapy.

While we found generally good concordance in Oncotype DX Recurrence Scores between the single paired sample collected in this study, the samples were obtained from a HR-negative tumour. This result is consistent with our retrospective analysis where good concordance in Oncotype DX Recurrence Scores between paired HR-negative breast cancer samples were observed. The effects of menstrual cycling are likely to be limited to hormone responsive

tumours and therefore we do not expect to observe cycle-induced changes in gene expression or Recurrence Scores between these paired HR-negative samples.

This pilot prospective study revealed a number of ethical and practical complexities that will help inform development of a larger study. Approval from The Queen Elizabeth Hospital Human Ethics Committee took well over a year due to the intricacies of obtaining informed consent from patients prior to their diagnostic biopsy. Due to internal hospital issues around coordinating the vast number of clinical units involved in the project (i.e., surgery, oncology, radiology, pathology, nursing), it took over 6 months to obtain governance approval. Further complexities arose due to the scarcity of paired premenopausal breast cancer samples. Many premenopausal women presented to private clinics prior to being referred to The Queen Elizabeth Hospital for surgery, preventing us from collecting the initial diagnostic biopsy. Additionally, premenopausal women who had received neoadjuvant therapy, who presented with benign disease, or who had prior oophorectomy were excluded from the study, further limiting the number of women we could enrol.

The knowledge gained from establishment of ethics and governance, as well as the successful collection of paired breast cancer samples from a premenopausal woman at diagnosis and surgery, can be used to develop a future large prospective trial. Increasing the number of hospitals and clinics participating in this study will enable an increased sample size. Additionally, longer follow-up times would provide insight into the long-term survival outcomes to identify which cycle stage shows the best prognostic ability. Knowledge of an optimal time of the month to obtain tumour samples to conduct Oncotype DX testing, and identifying women who are more prone to cycle-induced changes in Oncotype DX Recurrence Scores, is urgently required to improve treatment outcomes for young women.

3.4. Conclusion

In summary, using paired breast cancer samples collected on different days, we have shown that discordances in Oncotype DX Recurrence Scores are more variable between paired samples collected from younger women compared to older women, and that this discordance is driven by variable expression of Proliferation- and HER2-group genes. We propose that the increased variability observed in younger women is due to fluctuations in estrogen and progesterone that occur during the menstrual cycle. In the following chapter, we use a mouse model to explore whether the greater variability in Oncotype DX Recurrence Scores is due to ovarian cycling, and further define the effects of specific cycle stages on the Oncotype DX 21-gene signature.

Chapter Four

The effect of ovarian cycling on the Oncotype DX 21-gene signature

4.1. Introduction

HR-positive breast cancers are highly responsive to the fluctuations in ovarian hormones that occur across the menstrual cycle. Studies in premenopausal women have shown that the highest proliferative activity of breast cancer is observed during the luteal phase, when circulating concentrations of progesterone peak (152). Similarly, growth factor receptor expression is increased at the luteal phase of the menstrual cycle (155). Expression of estrogen-regulated genes also varies with menstrual cycle stage, with reduced expression observed during the luteal phase (5, 6). Given the impact of ovarian hormones on breast cancer cell biology, it is possible that the Oncotype DX 21-gene signature and Recurrence Score are also affected by the menstrual cycle in HR-positive breast cancers.

In chapter three, analysis of archival paired breast cancer samples demonstrated that Oncotype DX Recurrence Scores are more variable between paired HR-positive breast cancer samples collected from younger women compared to older women. It is possible that this increased variability is due to menstrual cycling in premenopausal women. However, this previous retrospective study lacked information on the patient's menstrual cycle stage at the time of tissue collection, which prevented investigation of a direct relationship between cycle stage (i.e, follicular versus luteal) and discordance in Oncotype DX Recurrence Scores.

The experiments described in this chapter investigate the impact of ovarian cycle stage on Oncotype DX Recurrence Score using a naturally cycling mouse model of breast cancer. The use of a mouse model addresses some of the limitations of the retrospective study; tumours can be dissected from naturally cycling mice at a known ovarian cycle stage and in the absence of any confounding clinical factors, such as exogenous hormones introduced by oral contraceptive use. This enables a more robust analysis on how a specific ovarian cycle stage might contribute to discordances in Oncotype DX Recurrence Scores.

Transgenic mice harbouring the polyomavirus middle T-antigen oncogene, driven by the mammary gland specific mouse mammary tumour virus promoter (MMTV-PyMT) were used in this study. MMTV-PyMT transgenic mice are an excellent model of human breast cancer (187) as mammary tumours arise with 100% penetrance, are HR-positive, and are a heterogeneous cell population similar to human breast cancer.

As increased tumour proliferation and growth factor receptor expression, and reduced HR-signalling, are all associated with increased Oncotype DX Recurrence Scores (104), it is possible that Oncotype DX Recurrence scores are increased with increasing concentrations of progesterone. In support of this possibility, previous *in vitro* studies have reported that the co-

treatment of HR-positive breast cancer cell lines with estrogen and progesterone increases Oncotype DX Recurrence Scores, compared to estrogen treatment alone (43). In mice, the estrus phase of the ovarian cycle is characterised by low circulating concentrations of progesterone and high estrogen, and the diestrus phase is characterised by high circulating concentration of progesterone and moderate estrogen (188-190). Based on these observations, we hypothesised that tumours collected from MMTV-PyMT transgenic mice at the diestrus phase of the ovarian cycle would exhibit increased Oncotype DX Recurrence Scores compared to tumours dissected during the estrus phase.

HR-positive mammary tumours were dissected from MMTV-PyMT transgenic mice at either the estrus or diestrus phase of the ovarian cycle. The Oncotype DX 21-gene signature was assessed through quantitative RT-PCR and Recurrence Scores were calculated using the Oncotype DX Recurrence Score algorithm (104). Results from this chapter show that ovarian cycle stage indeed affects expression of the Oncotype DX 21-gene signature in this mouse model. Mammary tumours dissected from mice at diestrus exhibit increased expression of proliferative and HER2-associated genes, and increased Oncotype DX Recurrence Scores, compared to tumours dissected at estrus. Furthermore, clustering analysis identified a subset of tumours collected at diestrus that may be more sensitive to hormonal fluctuations during the ovarian cycle, and more prone to cycle-induced changes in Recurrence Scores.

4.2. Results

4.2.1. Assessment of tumour clinical features for inclusion in study

Histopathology and immunohistochemistry were performed on primary tumours dissected from MMTV-PyMT transgenic mice to confirm that tumours met the study inclusion criteria. Tumours were included in the study if they were of mammary epithelial cell origin and HR-positive. Additionally, it was required that mice exhibited normal estrous cycles, defined as 4 to 5 days between one estrus phase and the next estrus phase. To assess this, estrous cycles were tracked by daily vaginal smearing for at least 2 complete cycles prior to tissue collection (189).

Hematoxylin and eosin stains were performed on primary tumours collected from a total of 60 mice, and tumours were assessed and graded by a veterinary pathologist. Examples of hematoxylin and eosin stained mammary tumours are presented in Figure 16A-C. Mammary tumours were also assessed for HR positivity through immunohistochemical analysis of ER expression. All tumours exhibited positive nuclear staining of ER, and were therefore defined as HR-positive tumours. Furthermore, as ER translocates to the nucleus upon activation, the detection of nuclear ER staining suggests that these tumours are estrogen-responsive. An example of ER staining is presented in Figure 16D.

Of the 60 mice in the study, 1 mouse was excluded from analysis as the tumour was salivary gland epithelial cell in origin. Two mice were excluded from analysis as smearing was performed for less than 2 full cycles. These mice were culled for ethical reasons before 2 cycles could be tracked, as the primary tumour size exceeded the 2000mm^3 tumour burden threshold set by the animal ethics committee. A further 4 mice were excluded from analysis as they were not cycling normally.

A total of 53 mice were included in analysis (estrus n=25; diestrus n=28). Tumour and mouse characteristics are presented in Table 16. The clinical features of the mammary tumours collected from mice at estrus compared to diestrus were similar and not likely to confound results.

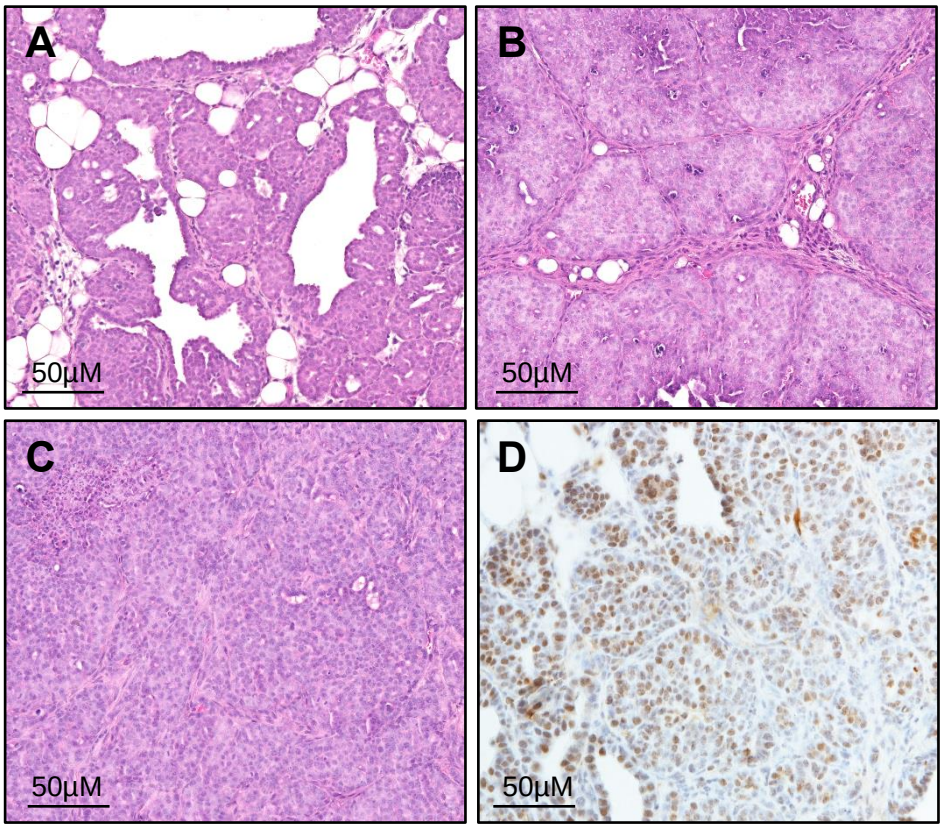


Figure 16 Histological and immunohistochemical analysis of MMTV-PyMT mammary tumours. Representative images of haematoxylin and eosin stained MMTV-PyMT mammary tumours of different grades. Tumours were assessed and graded by a veterinary pathologist. (A) grade 2, (B) grade 3, and (C) grade 4 tumours. (D) Representative images of ER staining in MMTV-PyMT mammary tumours. Bars represent 50µM.

Table 16 Clinical features of primary mammary tumours collected from MMTV-PyMT mice that met the inclusion criteria for the study. Mammary tumours were collected from MMTV-PyMT transgenic mice at either estrus or diestrus phase of the ovarian cycle. Ovarian cycle stage was determined by cytological analysis of vaginal smears, performed for at least two weeks. All tumours are hormone receptor positive as determined through immunohistochemical staining for the estrogen receptor. No differences in tumour features were observed between ovarian cycle stages.

Characteristics	Estrus n (%)	Diestrus n (%)
Total number	25	28
Tumour latency (median days; range)	53 (20 - 89)	49.0 (33 - 85)
Total tumour number (median; range)	4 (1 - 7)	3 (1 - 6)
Primary tumour weight (mg; mean±stdev)	450 ± 30	470 ± 25
Primary tumour size		
<10mm	6 (24)	6 (21)
10 – 15mm	9 (36)	10 (36)
16 – 20mm	8 (32)	7 (25)
>21mm	2 (8)	5 (18)
Tumour Grade		
2	4 (16)	2 (7)
3	7 (28)	7 (25)
4	14 (56)	19 (68)
Oncotype DX Recurrence Score		
≤10	7 (28)	4 (14)
11-25	14 (56)	15 (54)
26-30	2 (8)	4 (14)
≥31	2 (8)	5 (18)

4.2.2. The effect of ovarian cycle stage on the Oncotype DX 21-gene signature in HR-positive mouse mammary tumours

To determine whether ovarian cycling affects the Oncotype DX 21-gene signature, gene expression was assessed through quantitative RT-PCR and compared between tumours collected at estrus and diestrus phases of the ovarian cycle. Expression of 6 Oncotype DX signature genes were differentially expressed. Tumours collected from mice at diestrus exhibited a significant increase in the expression of genes *Mki67* (p=0.05), *Ccnb1* (p=0.02), *Erbb2* (p=0.03), *Grb7* (p=0.02), and *Bag1* (p=0.05), compared to tumours collected at estrus. Conversely, expression of *Esr1* (p=0.02) was significantly reduced in tumours collected at diestrus compared to estrus (Figure 17).

We next sought to determine how changes in the Oncotype DX 21-gene signature with ovarian cycle stage might impact Oncotype DX Recurrence Scores. Using reference-normalised gene expression, Recurrence Scores were calculated for each tumour. Tumours collected from mice at diestrus show a significant increase in Oncotype DX Recurrence Score (21.8±2.4; mean±SEM) compared to tumours collected at estrus (15.5±1.9; p=0.039; Figure 18).

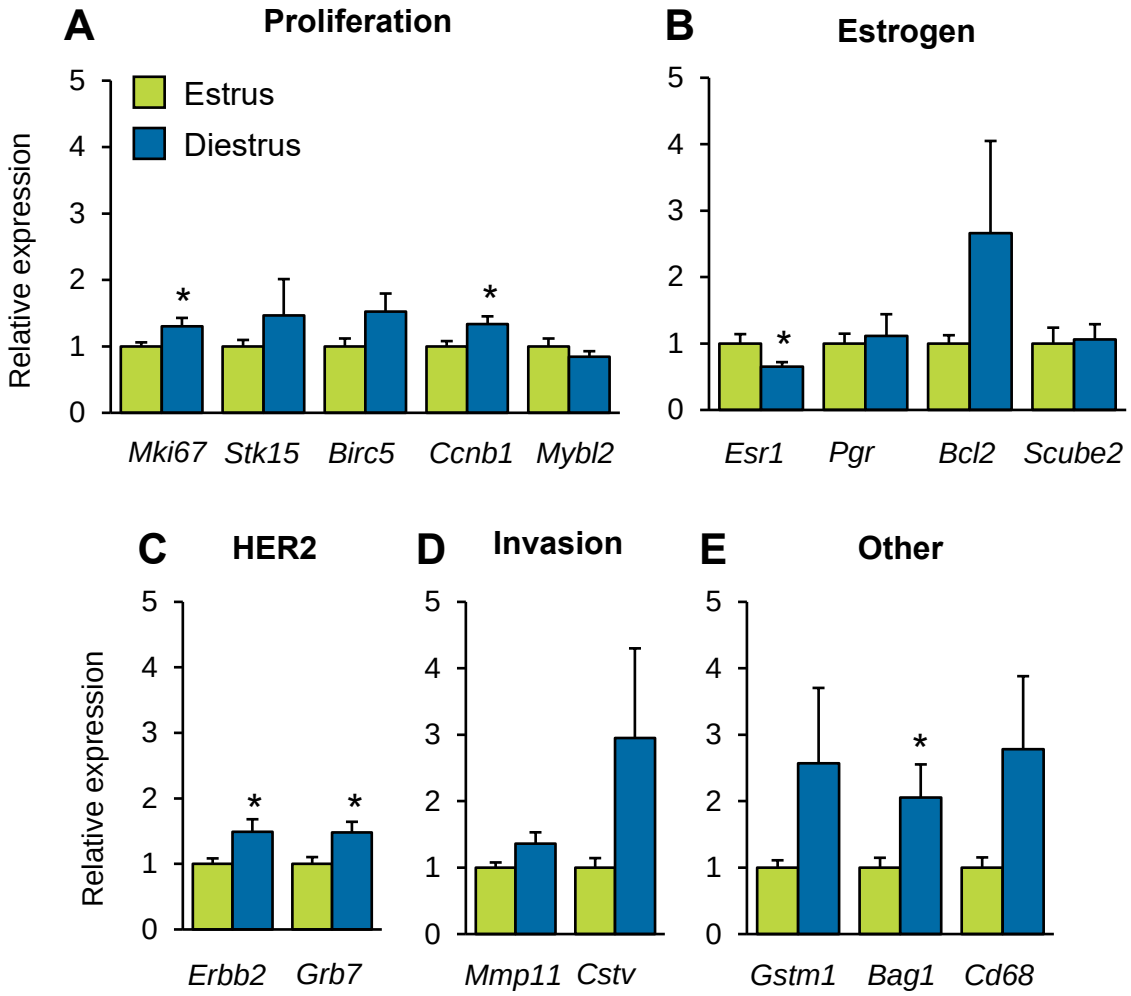


Figure 17 The effect of ovarian cycle stage on the Oncotype DX 21-gene signature. HR-positive mammary tumours were collected from MMTV-PyMT transgenic mice at either the estrus (n=25) or diestrus (n=28) phase of the ovarian cycle. The Oncotype DX 21-gene signature was assessed through real-time PCR; including (A) 5 Proliferation (*Mki67, Stk15, Birc5, Ccnb1, Mybl2*); (B) 4 Estrogen (*Esr1, Pgr, Bcl2, Scube2*); (C) 2 HER2 (*Erbb2, Grb7*); (D) 2 Invasion (*Mmp11, Cstv*); and (E) 3 Other genes (*Gstm1, Bag1, Cd68*). Gene expression was normalised to the average of 5 reference genes (*Actb, Gapdh, Rplpo, Gus, Tfrc*). Results are presented relative to tumours collected at estrus. Data are presented as mean+SEM. Statistical significance was determined when $p<0.05$ using Student's t-test. *signifies $p<0.05$

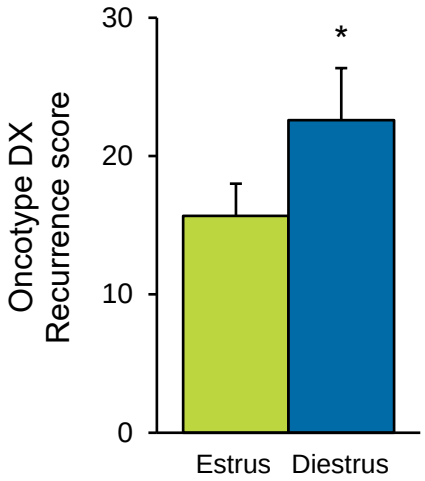


Figure 18 The effect of ovarian cycle stage on Oncotype DX Recurrence Scores. HR-positive mammary tumours were collected from MMTV-PyMT transgenic mice at either the estrus (n=25) or diestrus (n=28) phase of the ovarian cycle. Oncotype DX Recurrence Scores were calculated from reference-normalised gene expression using the published algorithm (Paik *et al.*, 2004). Data are presented as mean+SEM. Statistical significance was determined when $p<0.05$ using Student's t-test. *signifies $p<0.05$

4.2.3. Analysis of the variability in gene expression between mammary tumours collected at estrus compared to diestrus

To investigate the relationship between mammary cancer gene expression and circulating estrogen and progesterone, serum was collected from each mouse and analysed for estradiol and progesterone. However, the concentration of these hormones could not be determined in this cohort of mice. Many previous studies have employed a radioimmunoassay (RIA) approach to investigate mouse estradiol and progesterone in serum from naturally cycling mice. The commercial RIA kits DSL-4800 and DSL-3400 (Diagnostic Systems Laboratories, Webster, TX, USA) that measure estradiol and progesterone respectively have been well-validated and are considered the industry standard. Unfortunately, these kits were recently discontinued.

In collaboration with Professor Sarah Robertson, we sought to assess the specificity of commercial enzyme-linked immunosorbent assays (ELISA) using IBL-International (IB79105 and IB79183) and CalBiotech (ES180S-100) kits, and compared them to results obtained by RIA using archived serum samples described previously (189). Unfortunately, none of the ELISA kits were shown to be specific for estradiol or progesterone and were highly variable between serum samples of known cycle stage (Personal communication C. Dorian, data not shown).

In order to investigate the potential role of estradiol and progesterone in driving the gene expression changes observed in diestrus compared to estrus mice, a novel approach was sought. It is known that circulating concentration of progesterone is more variable during the diestrus phase of the ovarian cycle compared to the estrus phase (mean±standard deviation, 4.8±1.2 ng/ml compared to 1.5±0.1 ng/ml for diestrus and estrus mice respectively). On the other hand, estradiol concentration exhibits less fluctuation (34.3±3.0 pg/ml and 41.3±3.3 pg/ml for diestrus and estrus mice respectively) (188-190). If variable concentration of progesterone affects the Oncotype DX 21-gene signature, then gene expression is expected to be more variable in tumours collected at diestrus, where concentrations of progesterone vary more dramatically.

Variability in gene expression was assessed using the Levene's test for equality of variances. We found that there was significantly more variation in expression of *Mki67* (p=0.009), *Birc5* (p=0.043), *Ccnb1* (p=0.031), *Cstv* (p=0.048), *Bag1* (p=0.017), and *Cd68* (p=0.037) in tumours collected at diestrus, compared to tumours collected at estrus. Conversely, there was reduced variation in expression of *Esr1* (p=0.005) in tumours collected at diestrus (Table 17). Variability in Oncotype DX Recurrence Scores were not different between tumours collected at diestrus, compared to estrus (p=0.68; Table 17).

Table 17 The variability in gene expression with ovarian cycle stage. HR-positive mammary tumours were collected from MMTV-PyMT transgenic mice at either the estrus (n=25) or diestrus (n=28) phase of the ovarian cycle. Gene expression was assessed through quantitative RT-PCR, and results are presented in Figure 17. Variability in expression of each gene was compared between ovarian cycle stages using Levene's test for equality of variances. Data are mean±stdev. Data were considered heterogeneously distributed when $p<0.05$.

	Mean ± Stdev		Variability
	Estrus	Diestrus	Levene's test sig.
Mki67	1.0 ± 0.30	1.30 ± 0.68	0.009
Birc5	1.0 ± 0.60	1.52 ± 1.44	0.043
Ccnb1	1.0 ± 0.40	1.34 ± 0.62	0.031
Esr1	1.0 ± 0.71	0.65 ± 0.37	0.005
Cstv	1.0 ± 0.72	2.95 ± 7.15	0.048
Bag1	1.0 ± 0.74	2.06 ± 2.64	0.017
Cd68	1.0 ± 0.77	2.78 ± 5.82	0.037
Oncotype DX RS	15.5 ±9.91	21.8 ± 12.50	0.681

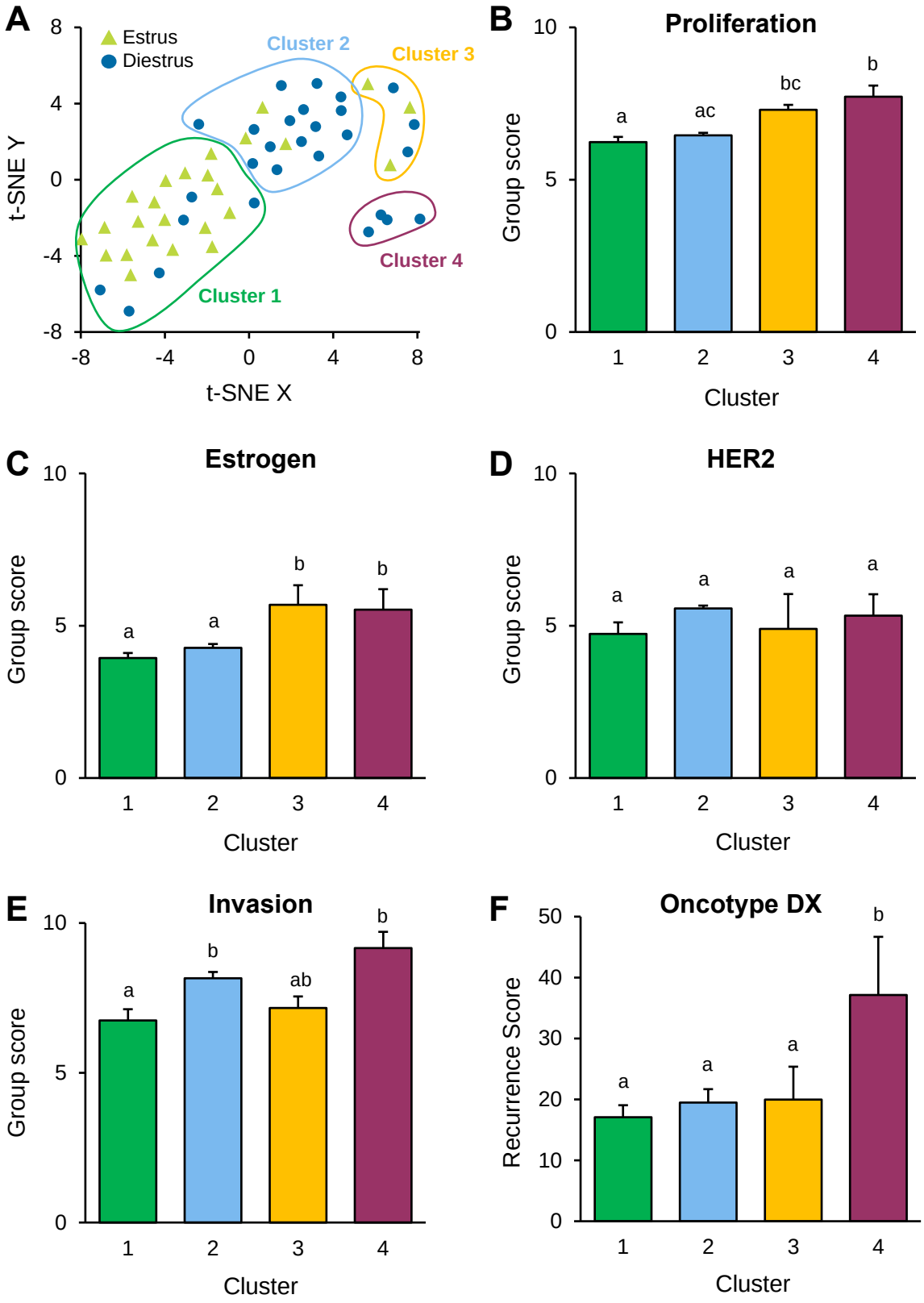
4.2.4. The effect of ovarian cycle stage on clustering analysis of HR-positive mouse mammary tumours

To further investigate how ovarian cycling affects the gene expression profile, t-Distributed Stochastic Neighbour Embedding (t-SNE) analysis was applied to our gene expression data. The t-SNE technique of dimensionality reduction allows for multi-gene datasets to be visualised in a 2-dimentional plot (191). Consequently, this allows us to visualise similarities in gene expression profiles between individual tumours.

Tumours grouped into 4 main clusters, each of which exhibited distinct gene expression profiles (Figure 19A). To highlight the differences in gene expression between clusters, Oncotype DX group scores were calculated for each cluster using the algorithm published in Paik *et al.* (2004) (Figure 19B-E). Clusters 1 and 2, which predominantly correspond to tumours collected at estrus and diestrus phases of the ovarian cycle respectively, show similarities in expression of Estrogen-, Proliferation-, and HER2-group genes; with the difference between clusters being driven primarily by increased expression of Invasion-group genes in Cluster 2, compared to Cluster 1 ($p=0.005$). In respect to the remaining clusters, Cluster 1 and Cluster 2 showed reduced expression of Estrogen-group genes, compared to Cluster 3 ($p<0.001$, $p=0.001$ respectively) and Cluster 4 ($p=0.001$, $p=0.009$ respectively).

We next compared average Oncotype DX Recurrence Scores for each cluster. Interestingly, tumours in Cluster 4 showed a significant increase in Oncotype DX Recurrence Scores (37.1±9.5; mean±SEM), compared to Cluster 1 (17.1±2.0; $p=0.001$), Cluster 2 (19.5±2.2; $p=0.004$) and Cluster 3 (20.0±3.8; $p=0.014$). No other differences in Recurrence Scores were observed between clusters ($p>0.05$; Figure 19F). The increased Oncotype DX Recurrence Scores of Cluster 4 were driven by an increased expression of Estrogen-, Proliferation- and Invasion-group genes, as compared to Cluster 1 ($p=0.001$, $p<0.001$, $p=0.006$ respectively), and Cluster 2 ($p=0.009$, $p=0.002$, $p=0.25$ respectively). No differences in gene expression were observed between Cluster 3 and Cluster 4 ($p=0.77$, $p=0.33$, $p=0.06$ respectively).

Figure 19 Clustering of mammary tumours based on similarities in gene expression profiles. (A) HR-positive mammary tumours collected from MMTV-PyMT transgenic mice at either estrus (n=25) or diestrus (n=28) stages of the ovarian cycle were clustered using t-Distributed Stochastic Neighbour Embedding (t-SNE) analysis. Each cluster was characterised by a unique gene expression signature. Presented are the average Oncotype DX group scores for each cluster: (B) Proliferation-, (C) Estrogen-, (D) HER2-, and (E) Invasion-group. (F) The average Oncotype DX Recurrence Score for each cluster. Group scores and Oncotype DX Recurrence Scores were calculated using the algorithm described in Paik *et al.* (2004). All results are presented as mean+SEM. Significance was assessed using ANOVA. Different letters indicate statistical significance; data with the same letters do not differ significantly ($p>0.05$), while data with different letters show significant differences ($p<0.05$).


A
Estrus
Diestrus
Cluster 1
Cluster 2
Cluster 3
Cluster 4
t-SNE Y
t-SNE X
B
Proliferation
Group score
Cluster
C
Estrogen
D
HER2
E
Invasion
F
Oncotype DX
Recurrence Score

4.2.5. The relationship between ovarian cycle stage and proliferative activity of MMTV-PyMT mammary tumours

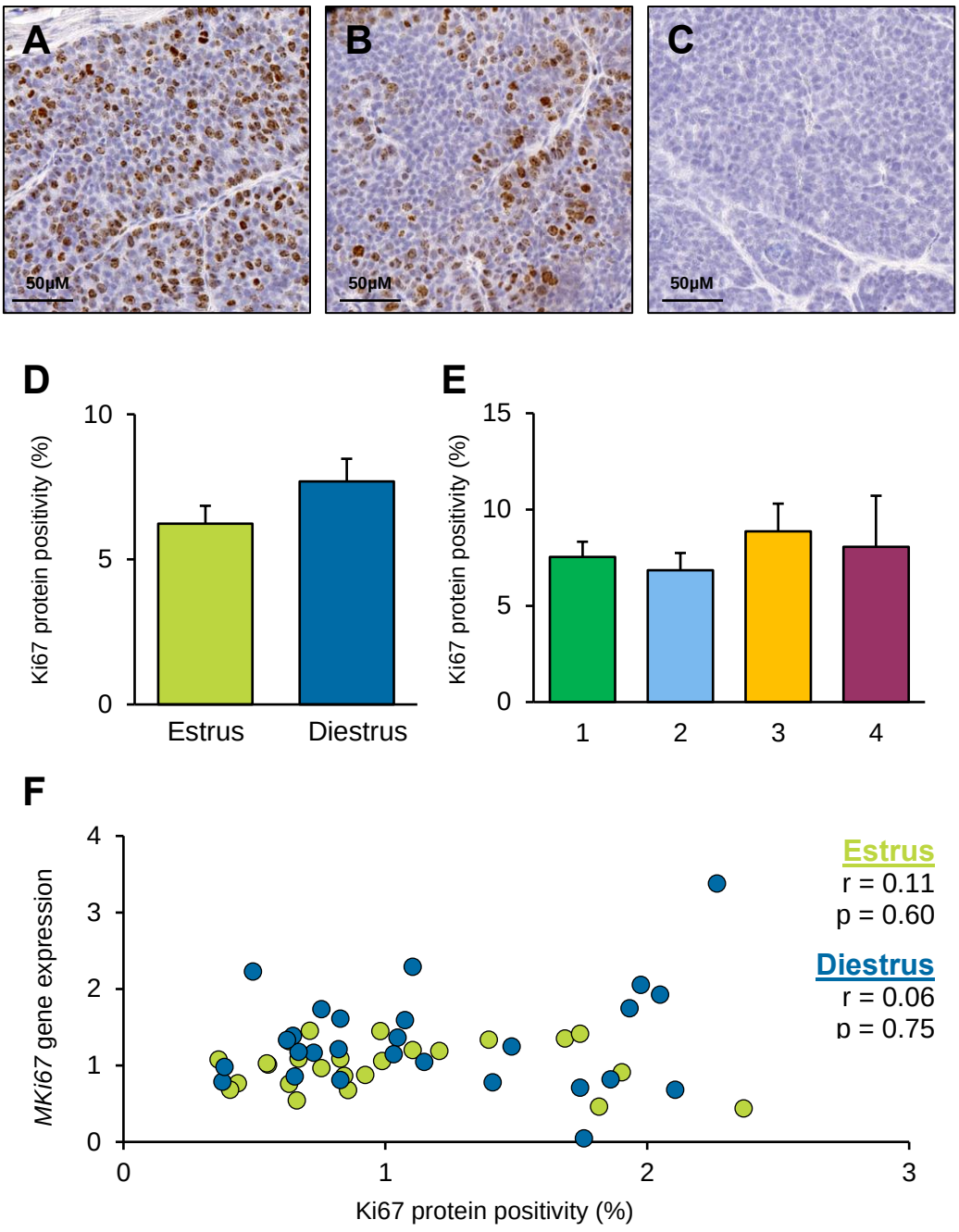
The Oncotype DX Recurrence Score algorithm weights the Proliferation-group score the heaviest, meaning that expression of proliferative genes contribute most strongly to Oncotype DX Recurrence Scores. Having found that the ovarian cycle stage affects expression of proliferation-associated genes in mouse mammary tumours, we sought to further define the effects of ovarian cycling on mammary tumour cell proliferation.

Tumour cell proliferation was assessed through immunohistochemical analysis of Ki67 protein. Abundance of Ki67 was highly heterogeneous and exhibited significant variability within different areas of the same mammary tumour (Figure 20A,B). To determine the average proliferation across the entire tumour, Ki67 protein expression was assessed across a whole section of the tumour. This provided a result that can be compared to gene expression analysis.

In our gene expression analysis, expression of the gene encoding Ki67 (*Mki67*) was reduced in HR-positive mammary tumours collected at diestrus (Figure 17A). However, there were no significant differences in the percent of cells positive for Ki67 protein in tumours collected at diestrus (8.1±0.78%; mean±SEM), compared to tumours collected at estrus (6.9±0.73%; $p=0.26$; Figure 20D). Similarly, there were no significant differences in Ki67 positivity between Cluster 1 (7.5±0.79%), Cluster 2 (6.5±0.90%), Cluster 3 (8.9±1.4%), and Cluster 4 (8.0±2.6; $p>0.05$; Figure 20E).

Given that we identified reduced *Mki67* gene expression but not protein expression in tumours from diestrus mice, we next assessed the concordance between gene and protein expression of Ki67 using Spearman's correlations. We found generally poor correlation between *Mki67* gene expression and Ki67 protein expression in mammary tumours collected at both diestrus ($r=0.06$; $p=0.75$), and estrus ($r=0.11$; $p=0.60$) phases of the ovarian cycle (Figure 20F).

Figure 20 Proliferation of mammary tumours with ovarian cycle stage. HR-positive mammary tumours were collected from MMTV-PyMT mice at either estrus (n=25) or diestrus (n=28) stages of the ovarian cycle. Proliferation was assessed through immunohistochemical staining for Ki67 protein expression. Ki67 staining was variable within different areas of the mammary tumour. The images presented do not represent the entire mammary tumour, but rather reflect areas of (A) low and (B) high proliferation. (C) Secondary antibody only was used as a negative control. Ki67 staining across a section of the whole tumour was quantified using ImageJ plugin ImmunoRatio. Proliferation is presented as a percentage of (DAB stain)/(haematoxylin stain) for (D) tumours collected at different ovarian cycle stages, and for (E) t-SNE identified clusters. Data are presented as mean+SEM with statistical analysis conducted using ANOVA. No data were statistically significant ($p>0.05$). (F) Correlation in expression of *Mki67* (gene) against Ki67 (protein) for each tumour. Spearman's correlations and p-values are presented.


A
50µM
B
50µM
C
50µM
D
Ki67 protein positivity (%)
10
5
0
Estrus
Diestrus
E
Ki67 protein positivity (%)
15
10
5
0
1
2
3
4
F
MKi67 gene expression
4
3
2
1
0
0
1
2
3
Ki67 protein positivity (%)
Estrus
r = 0.11
p = 0.60
Diestrus
r = 0.06
p = 0.75

4.3. Discussion

In the previous chapter, discordances in Oncotype DX Recurrence Scores were shown to be more variable in premenopausal women, compared to postmenopausal women. We proposed that variability in Recurrence Scores were being driven by premenopausal factors; primarily fluctuations in circulating concentrations of estrogen and progesterone that occur during the menstrual cycle of premenopausal women. However, this previous study was limited by a lack of information surrounding the patient's menstrual cycle stage, and this prevented the investigation of a direct relationship between cycle stage and discordance in Oncotype DX Recurrence Scores.

The experiments described in this chapter used a naturally cycling mouse model of HR-positive breast cancer to define how ovarian cycle stage affects the Oncotype DX 21-gene signature. Mammary tumours dissected from mice at diestrus exhibit increased expression of Proliferation- and HER2-group genes, and increased Oncotype DX Recurrence Scores, compared to tumours collected from mice at estrus. Furthermore, clustering analysis identified a subset of diestrus-collected tumours that may be more sensitive to cycle-induced changes in Oncotype DX Recurrence Scores.

4.3.1. Ovarian cycling affects the Oncotype DX 21-gene signature in HR-positive mouse mammary tumours

Mammary tumours dissected from MMTV-PyMT transgenic mice at diestrus show a significant increase Oncotype DX Recurrence Scores, compared to tumours dissected at estrus. We propose that the effect of the ovarian cycle stage on Oncotype DX Recurrence Scores is likely due to fluctuating concentrations of estrogen and progesterone during the ovarian cycle, and their effect on the Oncotype DX 21-gene signature.

Previous studies by our and other groups have established that the estrus phase of the ovarian cycle is characterised by low circulating concentrations of progesterone and high estrogen. Conversely, the diestrus phase is characterised by high concentrations of progesterone and moderate estrogen (188-190, 192). As circulating concentrations of progesterone vary significantly during the ovarian cycle, while concentrations of estrogen do not fluctuate as dramatically, it is likely that variable concentrations of progesterone at the time of tissue collection are the main driver affecting the Oncotype DX 21-gene signature.

If variable concentrations of progesterone are contributing to the observed gene expression changes, we expect to see more variable gene expression in tumours collected at diestrus. Indeed, 3 of the 5 proliferative genes (*Mki67*, *Birc5*, *Ccnb1*) included in the Oncotype DX 21-gene signature showed more variable expression in tumours dissected at diestrus. Furthermore, two of these genes (*Ki67*, *Ccnb1*) showed increased expression at diestrus. Together with the knowledge that concentrations of progesterone peak during diestrus, these findings suggest a role for progesterone in driving tumour cell proliferation.

Tumours collected at diestrus also exhibited reduced expression, and significantly less variation, in *Esr1* expression; suggesting that progesterone may have pronounced inhibitory effect on *Esr1* expression. Indeed, previous studies support this role of progesterone, reporting that progesterone supresses the actions of estrogen by downregulating the synthesis of ER, and opposing ER-mediated gene expression (7, 36).

In parallel with the loss of *Esr1* expression, we found that expression of genes involved in HER2 signalling were increased at diestrus, including *Erbb2* and *Grb7*. Consistent with these findings, previous studies report an inverse relationship between hormone receptor and growth factor receptor signalling in breast cancers (43, 156, 193), which is driven primarily by progesterone signalling (43).

Interestingly, we also show that tumours collected at diestrus exhibit more variable expression of stromal and immune-associated genes, *Cstv* and *Cd68*. While the primary target for progesterone is the mammary epithelium, progesterone also affects the activity of surrounding stromal and immune cells during the ovarian cycle, through both direct and indirect mechanisms (189, 190, 194). Critically, it is important to consider the effects of ovarian cycling on both tumour epithelium and the surrounding microenvironment, as variability in their expression has the potential to impact Oncotype DX Recurrence Scores.

Together, our findings suggest that breast cancer gene expression fluctuates across the ovarian cycle of mice, and that differences in gene expression are likely mediated by the increased concentrations of progesterone during diestrus. It is possible that progesterone-induced changes in the expression of Oncotype DX signature genes contribute to the increased Oncotype DX Recurrence Scores observed in tumours collected at diestrus. For premenopausal women, Oncotype DX testing may produce a higher Recurrence Score for a tumour assessed during the luteal phase, when concentrations of progesterone are high, compared to the same breast tumour sampled during the follicular phase, when concentrations of progesterone are low.

4.3.2. Increased Oncotype DX Recurrence Scores at diestrus are being driven primarily by an increased expression of Proliferation-group genes

In premenopausal women, previous studies report that fluctuations in ovarian hormones across the menstrual cycle direct the mammary gland epithelium to undergo sequential waves of proliferation, where highest proliferative activity is observed during the luteal phase when concentrations of progesterone peak (152, 195). Similarly in mice, we have shown that proliferative gene expression is increased at diestrus, which is analogous to the luteal phase in humans.

As the Oncotype DX Recurrence Score algorithm weights proliferative genes the heaviest, meaning that their expression contributes most strongly to Oncotype DX Recurrence Scores, we suggest that cycle-induced changes in expression of genes associated with proliferation are driving the increase in Oncotype DX Recurrence Scores observed in diestrus-collected tumours. However, as Oncotype DX uses a panel of genes to calculate Recurrence Scores, any changes in gene expression may accumulate to significantly impact the Recurrence Score.

Indeed, tumours collected at diestrus exhibited increased expression of Proliferation- and HER2-group genes, and reduced expression of Estrogen-group genes; all of which are associated with an increased Oncotype DX Recurrence Score. While we suggest that cycle-induced changes in expression of Proliferation-group genes is the main driver affecting Oncotype DX Recurrence Scores, minor changes in the expression of Invasion, HER2 and Estrogen-group genes may accumulate and also affect Oncotype DX Recurrence Scores.

Furthermore, although we found significant differences in expression of Proliferation-group genes in mammary tumours, proliferation as assessed through Ki67 protein positivity did not differ with ovarian cycle stage. It is important to appreciate that reduced mRNA expression does not necessarily lead to reduced expression of the biologically active protein. Gene and protein expression may not be directly comparable, as the effects of ovarian hormones might be more pronounced on gene expression—and by extension gene expression profiling tests—compared to traditional protein-based methods.

4.3.3. The effects of ovarian cycle stage on Oncotype DX Recurrence Scores are limited to a subset of HR-positive tumours

There is significant inter-tumour and intra-tumour heterogeneity within HR-positive breast tumours, and this contributes to their heterogeneous clinical outcomes, variable response to

chemotherapy, and also how these tumours respond to ovarian hormones. Therefore, certain tumours might be more sensitive to fluctuations in circulating concentrations of ovarian hormones during the menstrual cycle. Indeed, in mice, we identified a subset of HR-positive tumours collected at diestrus (Cluster 4) that showed a significant increase in Oncotype DX Recurrence Scores, compared to the remaining tumour clusters. Tumours in Cluster 4 may be more sensitive to fluctuations in estrogen and progesterone during the ovarian cycle, and consequently, more prone to cycle-induced changes in Recurrence Scores.

Increased expression of genes downstream of estrogen signalling reflects tumour responsiveness to ovarian hormones. Tumours exhibiting high expression of estrogen-regulated genes are more dependent on hormone signalling for growth, and therefore may be more sensitive to fluctuations in estrogen and progesterone during the ovarian cycle. Indeed, Cluster 4, which showed highest Oncotype DX Recurrence Scores, also exhibited highest expression of estrogen-regulated genes, consistent with the possibility that these tumours are more sensitive to hormonal fluctuations. Additionally, this cluster was characterised by increased expression of proliferative genes, which we have previously described as being affected by ovarian cycle stage. We suggest that Cluster 4 is more sensitive to estrogen and progesterone, and an increase in expression of Proliferation-group genes at diestrus is the key driver behind their increased Oncotype DX Recurrence Scores.

In parallel with this possibility, it is likely that for a majority of HR-positive tumours ovarian cycle stage does not affect Oncotype DX Recurrence Scores. However, the underlying tumour biology that defines an increased sensitivity to cycle-induced changes is not well defined, and whether it is possible to identify tumours that are more sensitive to fluctuations in estrogen and progesterone warrants further investigation. Furthermore, while we report that the ovarian cycle stage can impact Oncotype DX Recurrence Scores, the cycle stage and hormonal environment that reflects the true tumour biology and the likely response from adjuvant chemotherapy has not been defined.

4.3.4. Limitations and future directions

There were several limitations in this study that must be considered. Firstly, as Oncotype DX Recurrence Scores were calculated in-house, differences in RT-PCR protocols could affect our findings. Furthermore, as we used mouse mammary tumours, differences between mouse and human gene sequences could influence the calculated Oncotype DX Recurrence Scores. For example, the human genome encodes for two cathepsin proteases, which show different

expression patterns in different tissues. Oncotype DX assesses expression of *CTSL*, encoding the cathepsin L protein, which is expressed in normal breast epithelium and breast cancers (196). Conversely, the mouse genome encodes for only one cathepsin, *Cstv*, which is ubiquitously expressed.

However, despite differences in RT-PCR protocols and gene sequences, we noted a distinct effect of ovarian cycle stage on the Oncotype DX 21-gene signature. By applying the Oncotype DX Recurrence Score algorithm to our gene expression data, we can model the likely effects of ovarian cycle stage on true Oncotype DX Recurrence Scores. Future prospective studies using breast cancer samples from premenopausal women will confirm the effect of menstrual cycling on the Oncotype DX 21-gene signature and Oncotype DX Recurrence Scores.

Another limitation of this study was the lack of information on circulating hormone concentrations at the time of tissue collection. Serum was collected from mice at the time of tissue collection, with the intention of quantifying concentrations of estrogen and progesterone. However, there was no feasible method to quantify hormone concentrations. We trialled ELISA kits, which use antibodies to detect estrogen and progesterone; however, due to low-specificity and high background these kits were unable to accurately quantify concentrations of estrogen and progesterone in serum.

Several groups have suggested that mass spectrometry is a promising approach to quantify serum hormone concentrations (197, 198). The use of mass spectrometry may provide a means to measure serum concentrations of estrogen and progesterone for this study. However, the use of mass spectrometry in hormone analysis has not yet been validated, and several aspects of this assay—such as the amount of serum required and the cost of performing mass spectrometry—suggest that it may not be practical for use with serum collected from mice.

Ideally, expression of the Oncotype DX 21-gene signature and Oncotype DX Recurrence Scores should be analysed in light of the specific hormonal environment at the time of tissue collection. However, although this study lacked information on circulating concentrations of estrogen and progesterone, classification of ovarian cycle stage on the basis of vaginal smear cytology revealed a distinct effect of cycle stage on gene expression and Oncotype DX Recurrence Scores.

4.4. Conclusion

We found that ovarian cycle stage significantly affects expression of the Oncotype DX 21-gene signature and Oncotype DX Recurrence Scores in mouse mammary tumours. We also identified a subset of HR-positive tumours that were more sensitive to cycle-induced changes in their Recurrence Score. This could be due to an increased sensitivity to hormonal fluctuations during the ovarian cycle. We propose that differences in Oncotype DX Recurrence Scores with ovarian cycle stage are likely being driven by variable concentrations of progesterone at the time of tissue collection, and the effects of progesterone on the Oncotype DX 21-gene signature. However, the absence of information on the concentration of progesterone at the time of tissue collection limits conclusions. In the following chapter, we define the effects of exogenous progesterone on the Oncotype DX 21-gene signature and Recurrence Scores using HR-positive breast cancer cell lines *in vitro* and *in vivo*.

Chapter Five

The effects of progesterone on the Oncotype DX 21-gene signature

5.1. Introduction

Previous studies have shown that progesterone modulates gene expression in HR-positive breast cancers. Progesterone promotes tumour proliferation (152, 195), and increases growth factor receptor signalling (155) in HR-positive premenopausal breast cancer samples. Similarly, progesterone reduces HR expression and downstream signalling (5, 6, 151, 175).

Results from the previous chapters demonstrated that discordances in Oncotype DX Recurrence Scores are more variable between paired samples from younger women compared to older women, and that ovarian cycle stage affects Oncotype DX Recurrence Scores in HR-positive mouse mammary tumours. We propose that fluctuations in the circulating concentration of progesterone across the menstrual cycle contributes to the observed variability in Oncotype DX Recurrence Scores. However, these previous studies were limited by an absence of information on the circulating concentration of progesterone at the time of tissue collection. Therefore, while we have shown that ovarian cycle stage can impact the Oncotype DX 21-gene signature, the precise effects of progesterone on Oncotype DX Recurrence Scores remains unclear.

Previous studies report that gene expression is more sensitive than its protein counterparts (199), and suggest that changes in gene expression are not necessarily mirrored by changes in protein expression (200, 201). It is possible that the effects of ovarian cycle stage, and variable concentrations of progesterone, are more pronounced on gene expression assays compared to traditional protein-based methods. However, no study has addressed this.

In this chapter, the effect of progesterone on the Oncotype DX 21-gene signature is investigated, and compared to the effect of progesterone on biomarker protein expression. Human breast cancer cell lines T-47D and ZR-75-1 were used for this study. These cell lines are HR-positive, HER2-negative human breast cancer cell lines, characteristic of Luminal A and Luminal B subtypes respectively (202). As Oncotype DX was developed for use in HR-positive, HER2-negative tumours, these cell lines are ideal for studying the effects of ovarian hormones on breast cancer gene expression, and the subsequent influence on Oncotype DX Recurrence Scores.

These breast cancer cell lines were studied *in vitro* and *in vivo* using mammary fat pad-xenografted nude mice. This mouse model is suitable for this study as it enables investigation of human HR-positive breast cancer cell lines with administration of exogenous estrogen and progesterone. If progesterone is driving changes in the Oncotype DX 21-gene signature, then we anticipate that progesterone treatment of breast cancer cell lines will affect expression of

the Oncotype DX gene signature and increase Oncotype DX Recurrence Scores, compared to estrogen treatment alone.

The effects of progesterone on human HR-positive breast cancer cell lines *in vitro* were examined by co-treating cells with estrogen with or without progesterone. The effects of ovarian hormones on breast cancer cell lines *in vivo* were examined in mammary fat pad-xenografted BALB/c nude mice, treated with exogenous estrogen with or without exogenous progesterone. The Oncotype DX 21-gene signature was assessed through quantitative RT-PCR and Recurrence Scores were calculated using the Oncotype DX Recurrence Score algorithm (104). Expression of breast cancer protein biomarkers, including ER, PR and marker of proliferation Ki67, were assessed through immunohistochemistry. Results from this chapter show that estrogen and progesterone co-treatment modulates breast cancer gene and protein expression *in vitro* and *in vivo*. However, despite the effect of progesterone on the expression of Oncotype DX signature genes, such changes in gene expression were not sufficient to significantly impact Oncotype DX Recurrence Scores.

5.2. Results

5.2.1. Estrogen and progesterone co-treatment of HR-positive breast cancer cell lines *in vitro*

To investigate the effects of ovarian hormones on breast cancer gene expression, hormone-responsive human breast cancer cell lines (T-47D and ZR-75-1) were treated with estrogen with or without progesterone. In parallel, cells were treated with the vehicle control (EtOH) alone. Hormone treatments were performed in triplicate, and gene expression was normalised to vehicle-treated controls. This normalised gene expression data was then averaged between triplicates, and defined as a single experiment. Hormone treatment experiments were repeated 5 times, to provide a sample size of 5 per treatment group for statistical analysis.

Co-treatment of T-47D cells with estrogen and progesterone resulted in reduced gene expression encoding the estrogen receptor (*ESR1*; p=0.002), and progesterone receptor (*PGR*; p<0.001), and increased epidermal growth factor receptor gene expression (*EGFR*; p=0.008), compared to estrogen treatment alone (Figure 21). No differences in gene expression encoding HER2 (*ERBB2*; p=0.06*)* between treatment groups were observed. Consistent with the reduced hormone receptor gene expression, expression of estrogen-regulated genes was also reduced in estrogen and progesterone co-treated cells; including expression of genes encoding forkhead box protein A1 (*FOXA1*; p=0.01), and B-cell lymphoma 2 (*BCL2*; p=0.003; Figure 21). Furthermore, gene expression encoding the androgen receptor (*AR*; p=0.006) and claudin-3 (*CLDN3*; p<0.0001) were reduced following progesterone treatment.

The effects of estrogen and progesterone co-treatment were largely reproduced in ZR-75-1 cells. Co-treatment of ZR-75-1 cells with estrogen and progesterone resulted in reduced *ESR1* (p=0.007), *PGR* (p=0.01), and *BCL2* (p=0.02) gene expression, and increased *EGFR* (p=0.008) gene expression, compared to estrogen treatment alone (Figure 22). In contrast with T-47D cells, *FOXA1* (p=0.81), *AR* (P=0.16) and *CLDN3* (p=0.43) expression were not significantly reduced following estrogen and progesterone co-treatment.

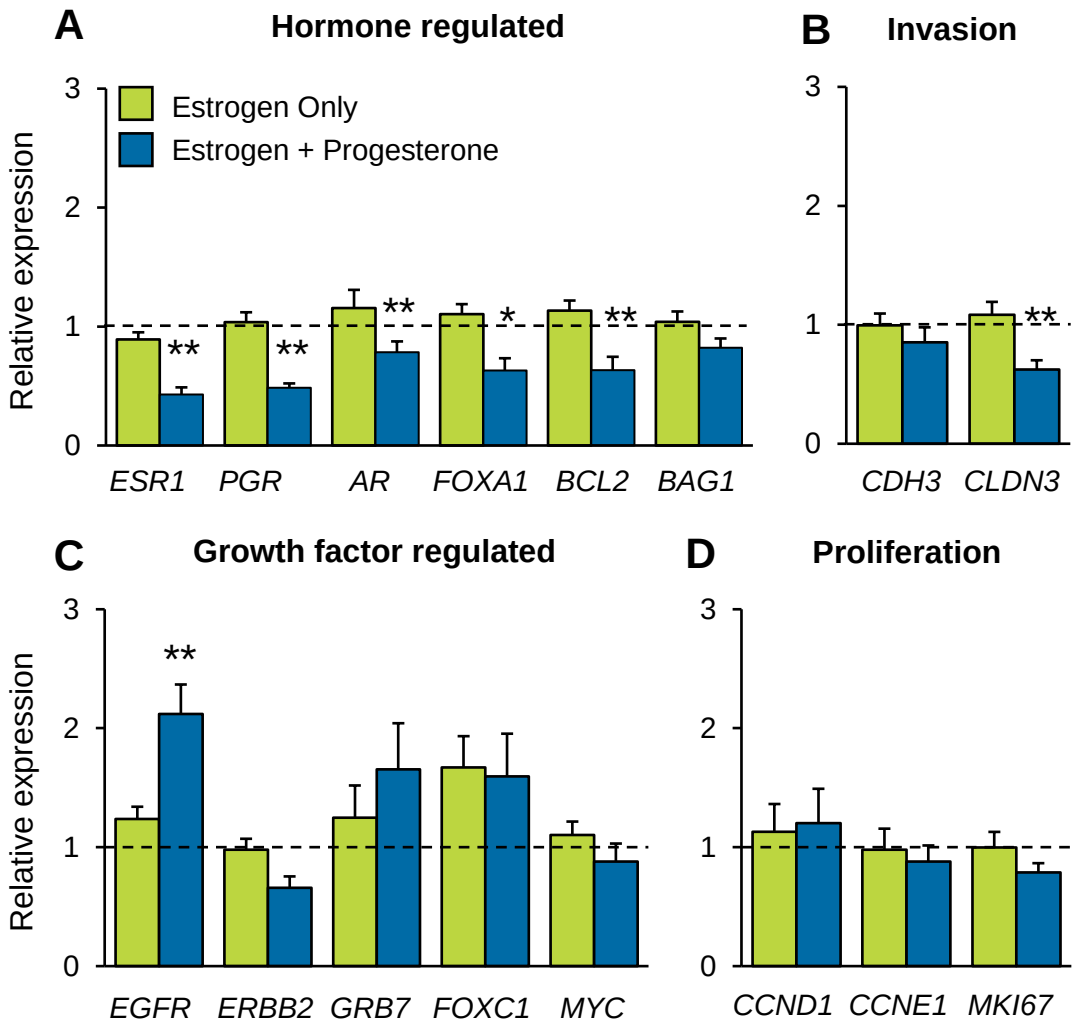


Figure 21 Gene expression in human HR-positive breast cancer cell line T-47D *in vitro*. Cells were treated with either 10nM estrogen only (n=5 experiments), or 10nM estrogen+10nM progesterone (n=5 experiments). In parallel, cells were treated with ethanol as a vehicle control (n=5 experiments). For each experiment, hormone treatments were performed in triplicate, averaged, and normalised to vehicle-treated controls (dotted line). Results are an average of 5 experiments, and presented as mean+SEM. Expression of (A) hormone regulated genes; (B) invasion genes; (C) growth factor regulated genes; and (D) proliferation genes were measured by quantitative RT-PCR and normalised to housekeeping gene *MRPL19*. Statistical significance was determined when $p<0.05$ using Student's t-test. *signifies $p<0.05$, **signifies $p<0.01$.

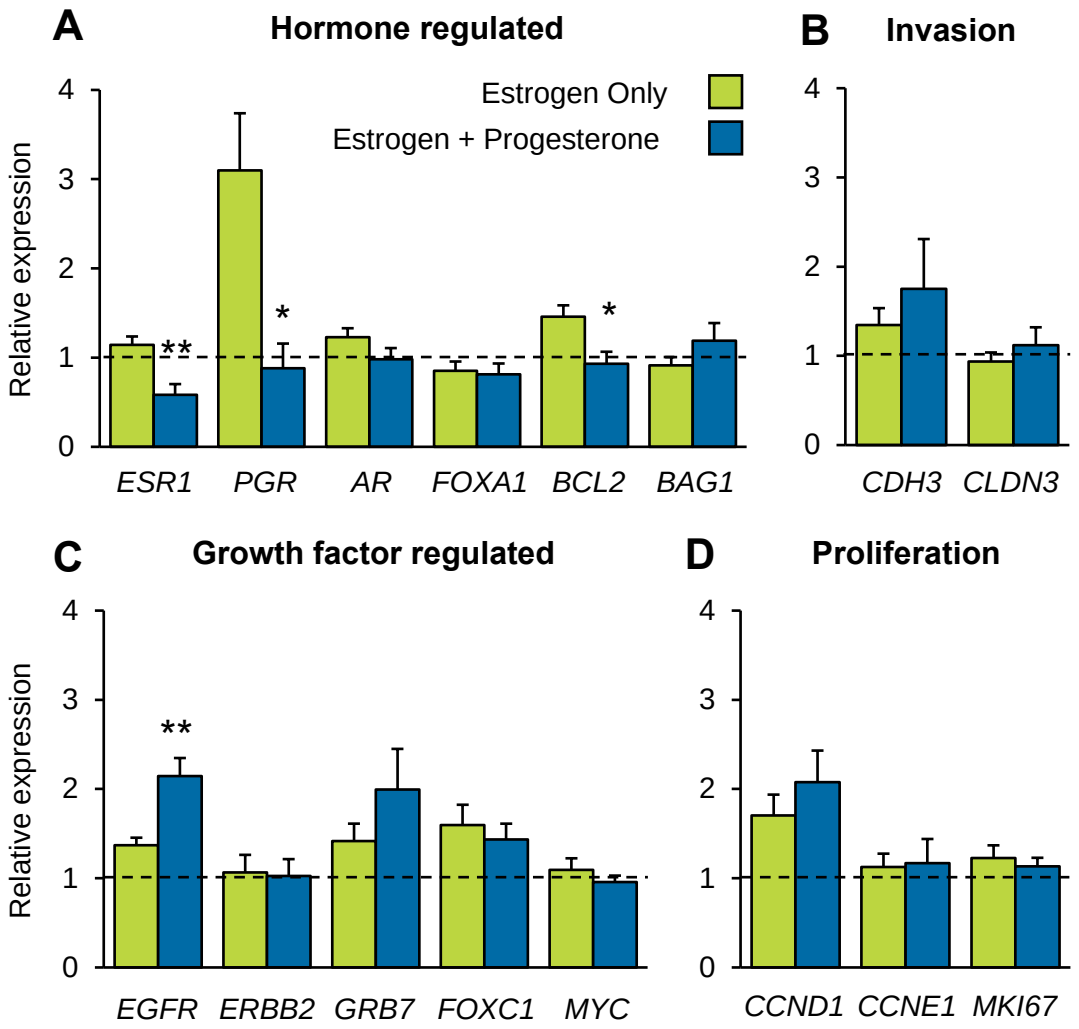


Figure 22 Gene expression in human HR-positive breast cancer cell line ZR-75-1 *in vitro*. Cells were treated with either 10nM estrogen only (n=5 experiments), or 10nM estrogen+10nM progesterone (n=5 experiments). In parallel, cells were treated with ethanol as a vehicle control (n=5 experiments). For each experiment, hormone treatments were performed in triplicate, averaged, and normalised to vehicle-treated controls (dotted line). Results are an average of 5 experiments, and presented as mean+SEM. Expression of (A) hormone regulated genes; (B) invasion genes; (C) growth factor regulated genes; and (D) proliferation genes were measured by quantitative RT-PCR and normalised to housekeeping gene *MRPL19*. Statistical significance was determined when $p<0.05$ using Student's t-test. *signifies $p<0.05$, **signifies $p<0.01$.

5.2.2. Xenograft models to investigate the effects of progesterone on HR-positive breast cancer cell lines *in vivo*

To further define the effect of ovarian hormones on breast cancer biomarker expression, HR-positive breast cancer cell lines (ZR-75-1 and T-47D) were grown orthotopically in the mammary fat pad of immunocompromised mice. As HR-positive breast cancer cell lines require estradiol supplementation for cells to establish and tumours to develop, mice were implanted with a slow release estradiol pellet prior to tumour cell inoculation. As a result, mice were supplemented with consistent concentrations of estradiol for the duration of the experiment.

Supplementation with a slow release estradiol pellet is expected to disrupt the normal ovarian cycle of mice. To confirm that mice were no longer cycling, estrous cycles were evaluated by cytological analysis of vaginal smears performed for at least two weeks. All mice exhibited a continual presence of cornified epithelial cells in vaginal smears indicating that they were not cycling, and therefore fluctuations in endogenous concentrations of estrogen or progesterone would not interfere with the experiment.

Breast cancer cell lines were inoculated directly into the mammary fat pad in a 1:1 ratio with Matrigel. For T-47D tumours, 1×10^6 cells were inoculated into the mammary fat pad, and took approximately 12 weeks for tumours to reach an appropriate size for treatment (Figure 23A). Of these mice, 2 mice did not develop tumours and were excluded from analysis. A further 5 mice were excluded as an insufficient amount of RNA was extracted from the tumour, and gene expression was not detected during quantitative RT-PCR (i.e, CT value > 50). A total of 23 mice were included in the analysis (n=11 estrogen only treated; n=12 estrogen + progesterone treated)

To increase the efficacy of tumour development, the number of cancer cells inoculated was increased 5-fold (to 5×10^6 cells) for the ZR-75-1 cell line. One mouse did not develop a tumour, and one mouse was excluded from analysis due to an unexpected death. All remaining mice developed tumours by 6 weeks post inoculation, and all tumours had sufficient tissue for analysis (Figure 23B). A total of 28 mice were included in the analysis (n=14 estrogen only treated; n=14 estrogen + progesterone treated)

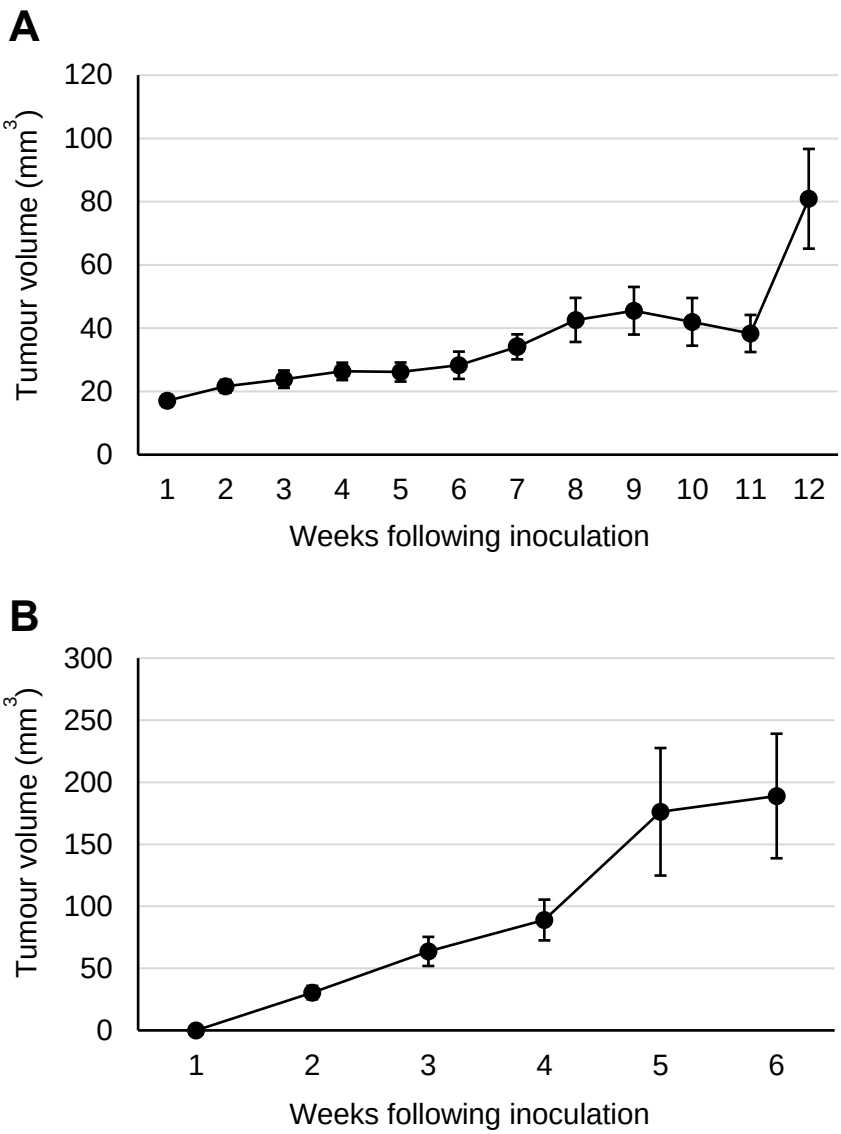


Figure 23 Growth curves of breast cancer cell line xenografts in immunocompromised mice. Breast cancer cell lines T-47D (1×10^6 cells; n=30 mice) or ZR-75-1 (5×10^6 cells; n=30 mice) were inoculated into the mammary fat pad of immunocompromised BALB/c nude mice supplemented with estrogen through slow release pellets. Tumour size was measured weekly for all mice, and volumes calculated as ($\frac{\boldsymbol{length \times width^2 \times \pi}}{\boldsymbol{6}}$) for (A) T-47D and (B) ZR-75-1 tumours. Results are presented as mean±SEM.

5.2.3. The effect of progesterone on breast cancer gene expression and Oncotype DX Recurrences Scores *in vivo*

To define the effect of progesterone on breast cancer gene expression *in vivo*, the Oncotype DX 21-gene signature was assessed through quantitative RT-PCR. In T-47D tumours, significant differences were observed in the expression of 4 Oncotype DX signature genes following progesterone treatment. Gene expression encoding *MKI67* (p=0.03), *STK15* (p=0.04), *PGR* (p=0.03), and *GSTM1* (p=0.05) was significantly reduced in tumours collected from estrogen and progesterone co-treated mice, compared to estrogen only treated mice. Expression of *ESR1* trended to decrease, but did not reach significance (p=0.08). No other changes in gene expression were observed between treatment groups (Figure 24).

Consistent with *in vitro* findings, ZR-75-1 tumours showed fewer gene expression changes following progesterone treatment, compared to the T-47D cell line. Differences in gene expression were observed in one Oncotype DX signature gene. Progesterone receptor gene expression was significantly reduced in tumours collected from estrogen and progesterone co-treated mice (*PGR;* p=0.05), compared to estrogen only treated mice. No other changes in gene expression were observed (Figure 25).

As Oncotype DX calculates Recurrence Scores on the basis of gene expression, we next sought to determine how progesterone-mediated changes in the Oncotype DX gene signature impact Oncotype DX Recurrence Scores. In T-47D xenograft tumours, no significant differences were observed in Oncotype DX Recurrence Scores in tumours collected from estrogen and progesterone co-treated mice (16.3±5.3; mean±SEM), compared to estrogen only treated mice (18.2±5.0; p=0.7; Figure 26A). Similarly, in ZR-75-1 xenograft tumours, no significant differences were observed in tumours collected from estrogen and progesterone co-treated mice (20.0±3.6) compared to estrogen only treated mice (18.3±3.2, p=0.7; Figure 26B).

As T-47D and ZR-75-1 are human breast cancer cell lines, gene expression was assessed using primers that recognised human genes. However, *CD68* expression reflects macrophage infiltration, therefore, it would be expected that any recruited macrophages would be of mouse origin. Using the ZR-75-1 cell line, we re-assessed *CD68* expression using primers that recognise the mouse CD68 gene. We found no differences in *CD68* expression when assessed using mouse primers, compared to human primers (Figure 27A). Furthermore, re-calculation of Oncotype DX Recurrence Scores using this gene expression data showed no significant differences in Oncotype DX Recurrence Scores depending on the primers used (Figure 27B).

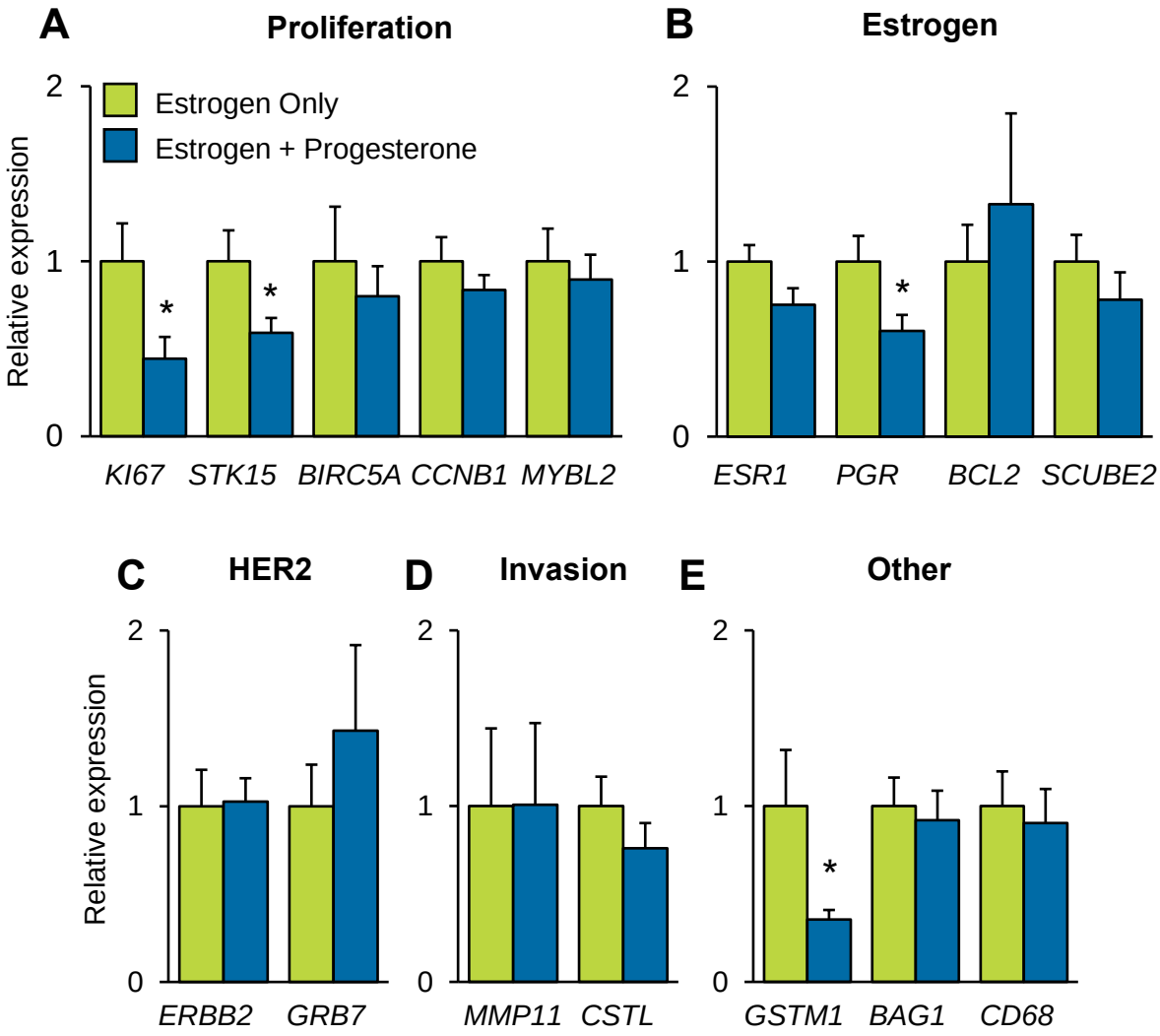


Figure 24 The effect of progesterone on the Oncotype DX 21-gene signature in T-47D xenograft tumours. Human HR-positive breast cancer cell line T-47D (1×10^6 cells) was grown orthotopically in BALB/c nude mice supplemented with 0.36mg estrogen through slow release pellets. Mice were treated with either 1mg progesterone (n=12) or a vehicle control (n=11) daily for 3 days prior to tissue collection. The Oncotype DX 21-gene signature was assessed through real-time PCR; including (A) 5 Proliferation (*KI67, STK15, BIRC5, CCNB1, MYBL2*); (B) 4 Estrogen (*ESR1, PGR, BCL2, SCUBE2*); (C) 2 HER2 (*ERBB2, GRB7*); (D) 2 Invasion (*MMP11, CSTL*); and (E) 3 Other genes (*GSTM1, BAG1, CD68*). Results are presented as mean+SEM. Statistical significance was determined when $p<0.05$ using Student's T-test. *signifies $p<0.05$.

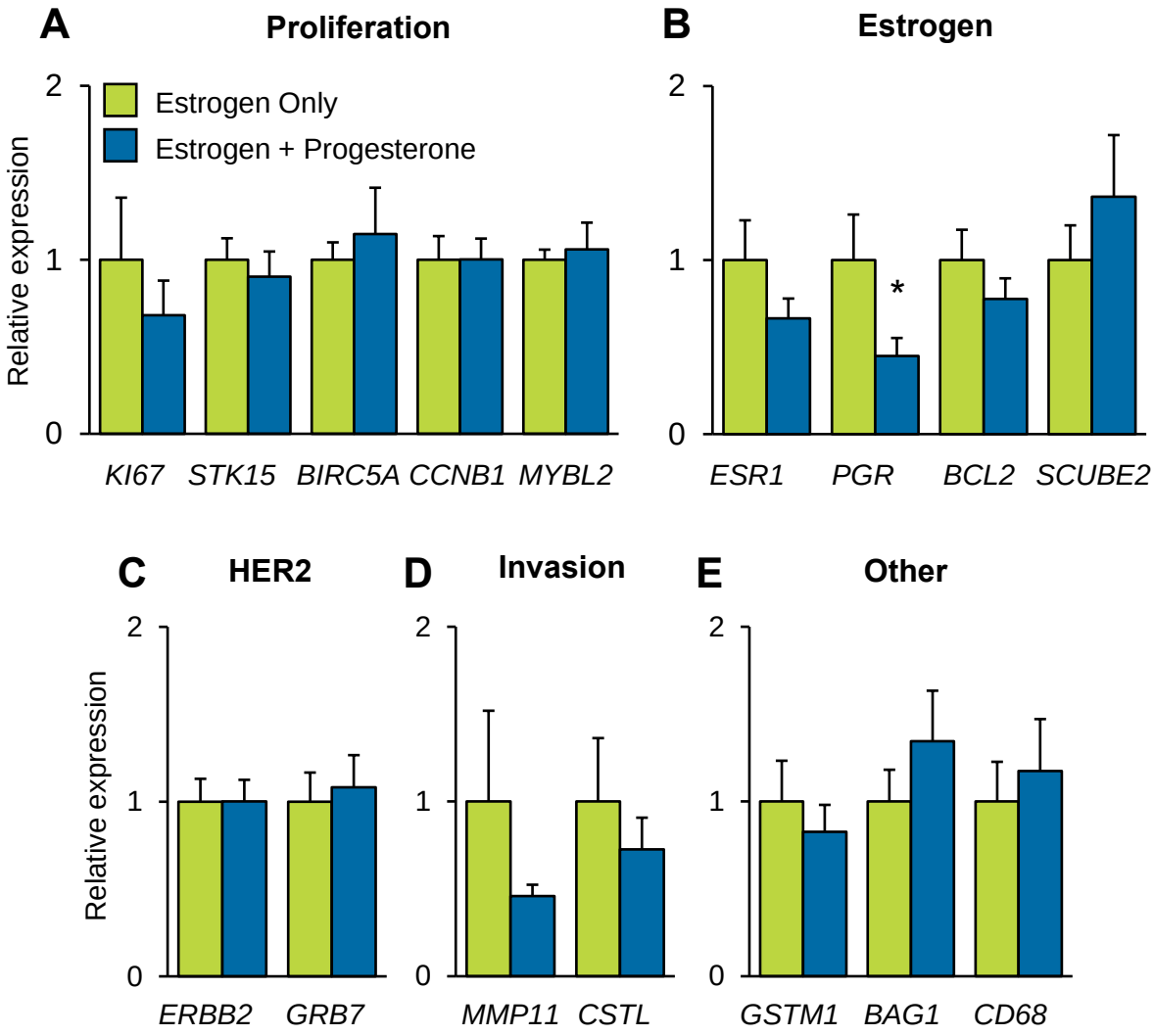


Figure 25 The effect of progesterone on the Oncotype DX 21-gene signature in ZR-75-1 xenograft tumours. Human HR-positive breast cancer cell line ZR-75-1 (5×10^6 cells) was grown orthotopically in BALB/c nude mice supplemented with 0.36mg estrogen through slow release pellets. Mice were treated with either 1mg progesterone (n=14) or a vehicle control (n=14) daily for 3 days prior to tissue collection. The Oncotype DX 21-gene signature was assessed through real-time PCR; including (A) 5 Proliferation (*KI67*, *STK15*, *BIRC5*, *CCNB1*, *MYBL2*); (B) 4 Estrogen (*ESR1*, *PGR*, *BCL2*, *SCUBE2*); (C) 2 HER2 (*ERBB2*, *GRB7*); (D) 2 Invasion (*MMP11*, *CSTL*); and (E) 3 Other genes (*GSTM1*, *BAG1*, *CD68*). Gene expression was normalised to the average of 5 reference genes (*ACTB*, *GAPDH*, *RPLPO*, *GUS*, *TFRC*). Results are presented as mean+SEM. Statistical significance was determined when $p<0.05$ using Student's T-test. * signifies $p<0.05$.

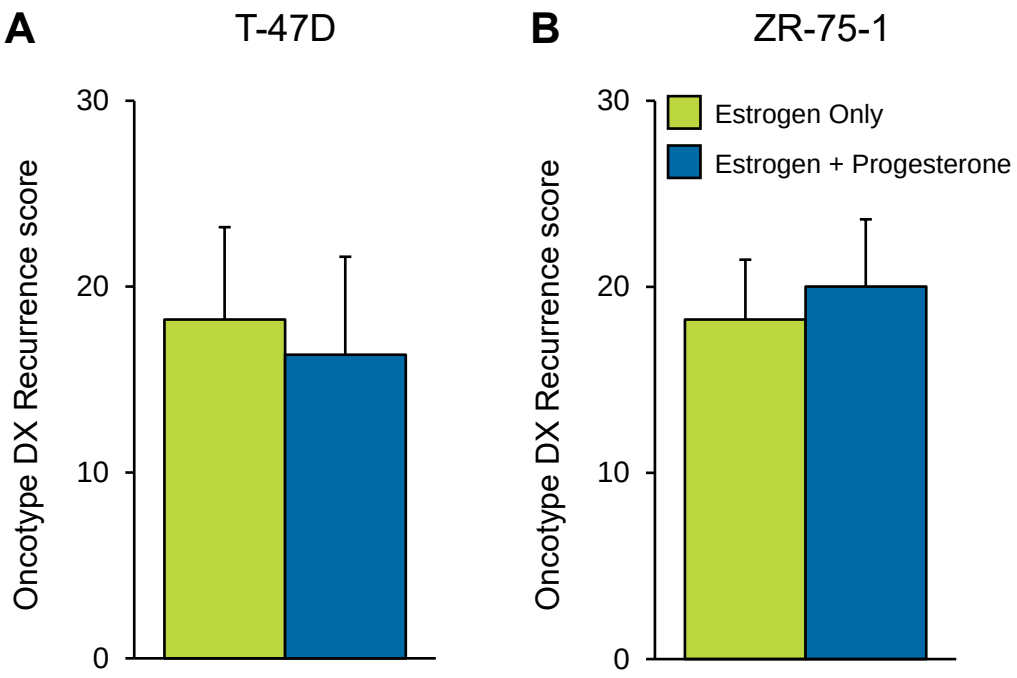


Figure 26 The effect of progesterone on Oncotype DX Recurrence Scores in xenograft tumours. Human HR-positive breast cancer cell line T-47D (1×10^6 cells) or ZR-75-1 (5×10^6 cells) was grown orthotopically in BALB/c nude mice supplemented with 0.36mg estrogen through slow release pellets. Mice were treated with either 1mg progesterone (T-47D n=12; ZR-75-1 n=14) or a vehicle control (T-47D n=11; ZR-75-1 n=14) daily for 3 days prior to tissue collection. Oncotype DX Recurrence Scores for (A) T-47D and (B) ZR-75-1 xenograft tumours were calculated from reference-normalised gene expression using the published algorithm (Paik *et al.*, 2004). Results are presented as mean+SEM. Statistical significance was determined when $p<0.05$ using Student's T-test. No data were significant.

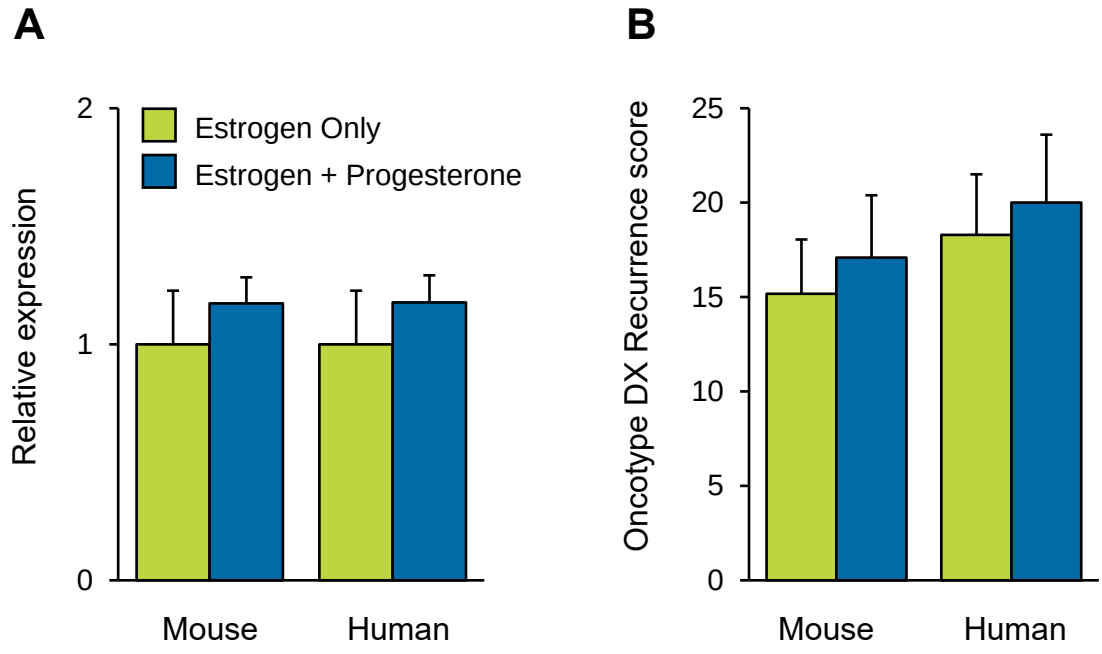


Figure 27 Assessing CD68 gene expression using primers that recognise mouse versus human gene sequences. Human HR-positive breast cancer cell line ZR-75-1 (5×10^6 cells) was grown orthotopically in BALB/c nude mice supplemented with 0.36mg estrogen through slow release pellets. Mice were treated with either 1mg progesterone (n=14) or a vehicle control (n=14). (A) Expression of CD68 was assessed through real-time PCR, using primers that recognised the mouse or human CD68 gene sequence. CD68 gene expression was normalised to the average of 5 reference genes (*ACTB, GAPDH, RPLPO, GUS, TFRC*), and presented relative to tumours collected from estrogen only treated mice. (B) Oncotype DX Recurrence Scores were calculated from gene expression data generated using either mouse or human primers. Data are presented as mean+SEM.

5.2.4. The effect of progesterone treatment on breast cancer protein expression *in vivo*

In the clinic, the assessment of ER, PR, HER2 and Ki67 protein expression is the current gold standard used to inform breast cancer management. To investigate how ovarian hormones affect the expression of traditionally used breast cancer biomarkers, xenografted-tumours were assessed for protein expression of ER, PR, and Ki67 through immunohistochemistry. Although HER2 expression is routinely assessed in breast cancer diagnosis, HER2 expression was not quantified in this study, as expression was not detected through immunohistochemistry. This is consistent with the anticipated immunohistochemical profile of T-47D and ZR-75-1 cell lines as Luminal A and Luminal B subtypes respectively.

Normal human breast tissue was included as a positive control when assessing protein expression in xenograft tumours, as it has been well established that normal breast tissue stains positive for ER, PR and Ki67 protein expression. The application of the secondary antibody only was used for a negative control. Examples of ER, PR and Ki67 staining in normal breast tissue are presented in Figure 28.

In T-47D xenograft tumours, the percentage of cells expressing ER was not significantly different between estrogen and progesterone co-treated mice (49.2±3.8%; p=0.3), compared to estrogen only treated mice (42.7±5.5%; mean±SEM). In contrast, PR positivity was significantly reduced following estrogen and progesterone co-treatment (27.1±4.1%) compared to estrogen treatment alone (44.2±5.3%, p=0.02). Similarly, tumour proliferation as measured through Ki67, was significantly reduced in estrogen and progesterone co-treated mice (8.3±1.1%) compared to estrogen only treated mice (12.2±1.3%, p=0.02; Figure 29). The changes observed in protein expression with estrogen and progesterone co-treatment mirrored the changes observed in *ESR1, PGR,* and *KI67* gene expression (presented in section 5.2.3).

In ZR-75-1 tumours, percent positivity of ER (23.7±4.2%) and PR (21.9±2.4%) in estrogen and progesterone co-treated mice showed no significant differences, compared to estrogen only treated mice (ER 25.3±4.1%, p=0.8; PR 27.5±4.0%, p=0.2). Furthermore, there were no significant differences in proliferation between estrogen and progesterone co-treated mice (20.2±1.6%; p=0.4), compared to estrogen only treated mice (22.6±2.6%; Figure 30), as measured through Ki67 expression. The lack of changes in ER and Ki67 protein expression following estrogen and progesterone co-treatment mirrored the lack of changes observed in *ESR1* and *KI67* gene expression (as presented in 5.2.3). However, the reduced *PGR* gene expression observed following estrogen and progesterone co-treatment was not observed in PR protein expression.

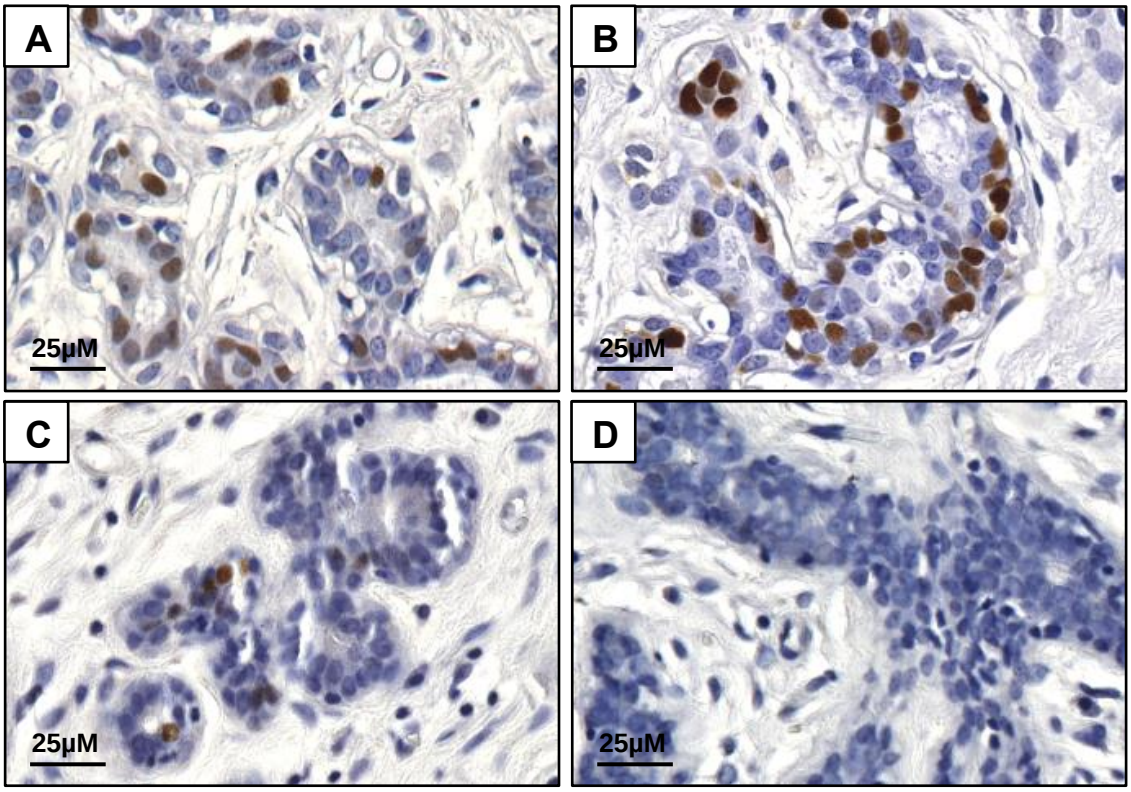


Figure 28 Normal breast tissue as a positive control for investigation of ER, PR, and Ki67 protein by immunohistochemistry. Representative images of (A) estrogen receptor; (B) progesterone receptor; and (C) Ki67 (proliferation marker) stained normal human breast tissue. Positive cells are identified by brown staining. (D) Application of the secondary antibody only was used for a negative control. Bars represent 25µM.

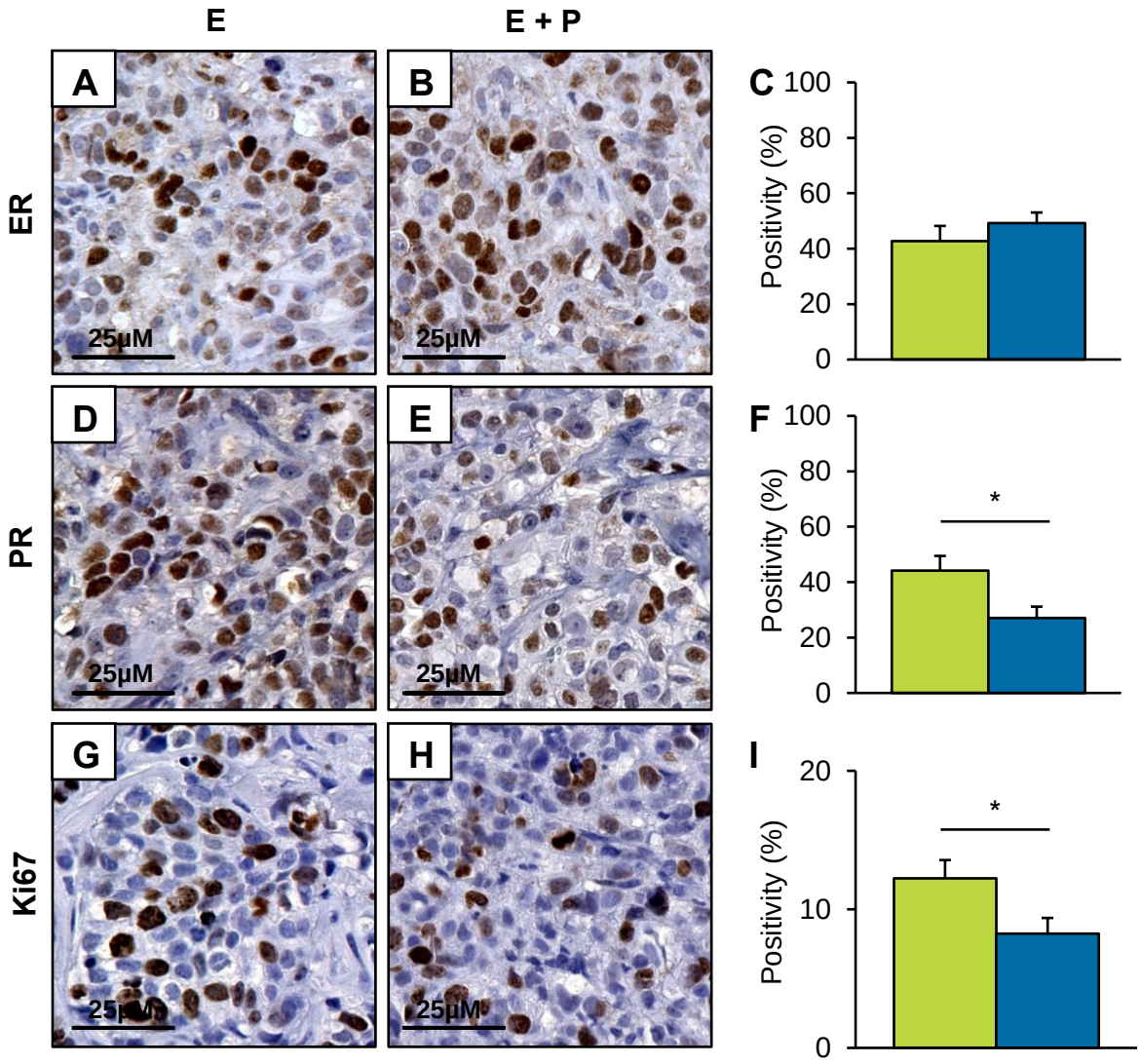


Figure 29 The effect of progesterone on ER, PR, and Ki67 protein expression in T-47D xenograft tumours. T-47D mammary fat pad-xenografted tumours were collected from estrogen only treated mice (E; n=11) and estrogen and progesterone co-treated mice (E + P; n=12). Tumours were stained with antibodies to detect (A, B) estrogen receptor (ER); (D, E) progesterone receptor (PR); and (G, H) Ki67 protein expression. Bars represent 25μM. Positive cells are identified by brown staining. Staining was analysed using ImageJ software, and the ImmunoRatio plugin. The number of positive cells was quantified using ImmunoRatio and presented as a percentage of (positive stained area)/(total nuclear stained area). The graphs show percent positivity for (C) ER, (F) PR, and (I) Ki67. Data are presented as mean+SEM. Statistical significance was determined when $p<0.05$ using Student's T-test. *signifies $p<0.05$.

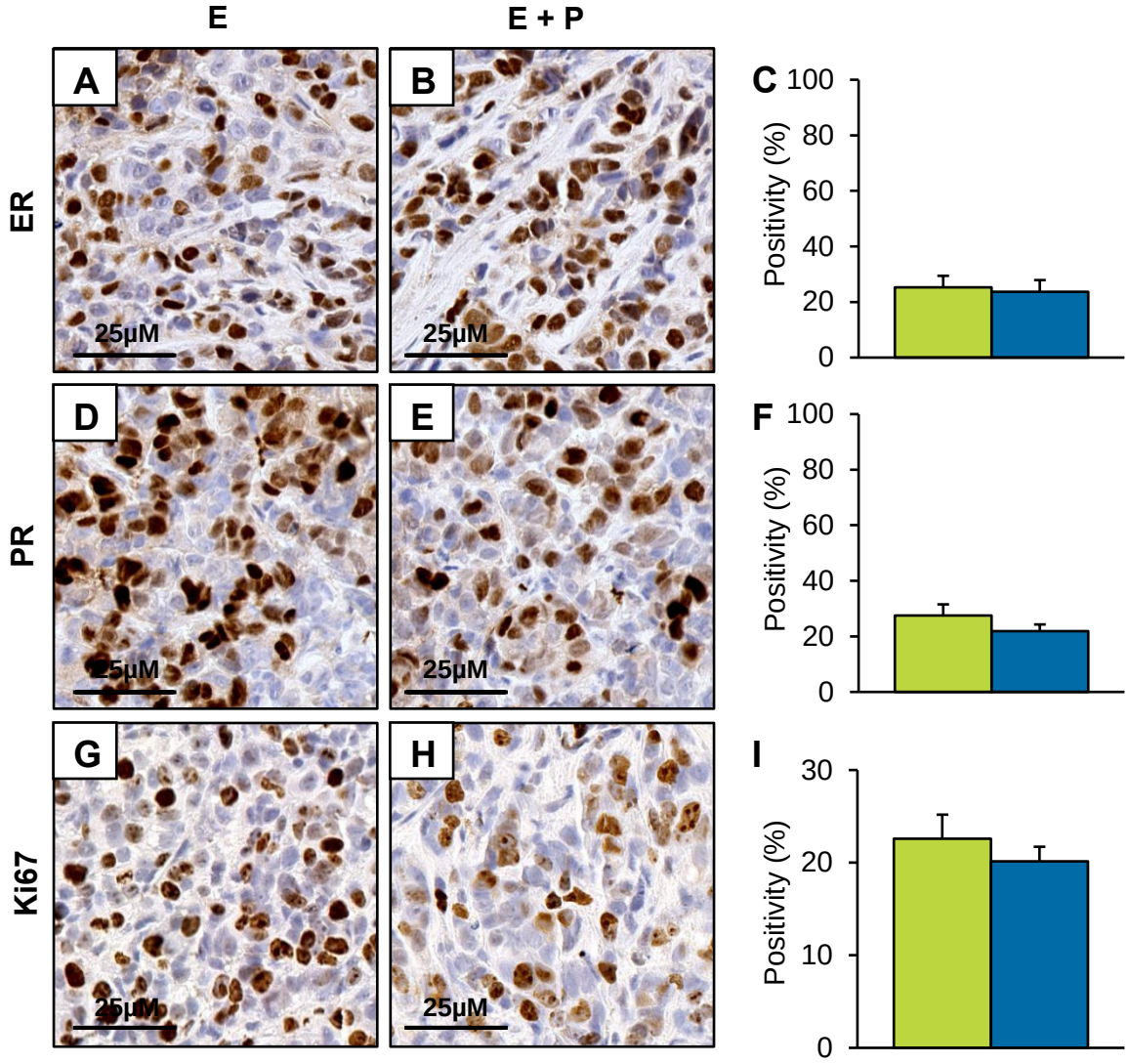


Figure 30 The effect of progesterone on ER, PR, and Ki67 protein expression in ZR-75-1 xenograft tumours. ZR-75-1 mammary fat pad-xenografted tumours were collected from estrogen only treated mice (E; n=14) and estrogen and progesterone co-treated mice (E + P; n=14). Tumours were stained with antibodies to detect (A, B) estrogen receptor (ER); (D, E) progesterone receptor (PR); and (G, H) Ki67 protein expression. Bars represent 25µM. Positive cells are identified by brown staining. Staining was analysed using ImageJ software, and the ImmunoRatio plugin. The number of positive cells was quantified using ImmunoRatio and presented as a percentage of (positive stained area)/(total nuclear stained area). The graphs show percent positivity for (C) ER, (F) PR, and (I) Ki67. Data are presented as mean+SEM. Statistical significance was determined when $p<0.05$ using Student's T-test. *signifies $p<0.05$.

5.3. Discussion

In the previous chapters, discordances in Oncotype DX Recurrence Scores are shown to be more variable in younger women, and the ovarian cycle stage influences Oncotype DX Recurrence Scores in HR-positive mouse mammary tumours. This suggests that variable concentration of progesterone at the time of tissue collection affects the expression of genes included in the Oncotype DX 21-gene signature, and impacts Oncotype DX Recurrence Scores. The experiments described in this chapter investigated whether progesterone is a driver of the alterations in Oncotype DX Recurrence Scores associated with the menstrual cycle.

Experiments reported in this chapter show that estrogen and progesterone co-treatment modulates breast cancer gene and protein expression *in vitro* and *in vivo*. However, the changes in gene expression in response to progesterone were not sufficient to significantly impact Oncotype DX Recurrence Scores. In the literature, there is controversy regarding the role of progesterone on breast cancer gene expression. *In vivo* studies using breast cancer cell lines and xenograft models report different findings compared to studies using premenopausal breast cancer samples, therefore the model used could affect the results obtained.

5.3.1. Estrogen and progesterone modulate breast cancer gene expression *in vitro*

Estrogen and progesterone co-treatment of HR-positive breast cancer cell lines *in vitro* reduced HR gene expression, and the expression of genes downstream of ER signalling, compared to estrogen treatment alone. Simultaneously, EGFR gene expression was increased following estrogen and progesterone co-treatment. This suggests that signalling in breast cancer cell lines may be switched from ER-driven to EGFR-driven tumour growth, when concentrations of progesterone are high. Previous studies support this possibility and provide preclinical evidence for an inverse relationship between HR and growth factor receptor signalling in breast cancers (43, 156, 193).

The inverse relationship between hormone signalling and growth factor signalling—likely mediated by progesterone—could have implications for the subtyping of premenopausal breast cancers. It is possible that cancers diagnosed during the follicular phase, when concentrations of progesterone are low, may be more likely to have a gene expression profile consistent with luminal cancers; tumours that exhibit hormone dependent growth. In contrast, cancers diagnosed during the luteal phase when concentrations of progesterone are high may be more likely to have a gene expression profile consistent with basal-like cancers, due to progesterone-

mediated upregulation of growth factor signalling. Indeed, previous work by our group suggests that estrogen and progesterone co-treatment of breast cancer cell lines *in vitro* results in the switching from a Luminal A to Basal-like intrinsic subtype (43). This could have implications for treatment decisions for premenopausal breast cancers.

Furthermore, the inverse relationship between HR and growth factor receptor expression may have implications for the treatment response of premenopausal breast cancers. In HR-positive breast cancer samples, *ESR1* expression is a predictor of tamoxifen resistance. Increased expression of *ESR1* is predictive of an increased benefit from tamoxifen; conversely, reduced expression of *ESR1* is associated with tamoxifen resistance (203). Similarly, increased growth factor receptor expression is associated with reduced *ESR1* expression and a resistance to tamoxifen; while blocking growth factor signalling upregulates ER signalling (61) and restores tamoxifen sensitivity (60).

These findings implicate the cross-talk between growth factor receptor and HR signalling pathways as a mechanism involved in resistance to tamoxifen; however the nature of this relationship is not well-understood. If variable concentrations of progesterone during the menstrual cycle mediate this cross-talk and contribute to tamoxifen resistance, then there is the potential to concurrently target PR to increase tumour sensitivity to hormonal- or targeted-therapies. However, future studies are required to elucidate the relationship between HR and growth factor receptor signalling, and the role progesterone plays in mediating this relationship.

5.3.2. Progesterone-mediated changes in the Oncotype DX 21-gene signature do not affect Oncotype DX Recurrence Scores in HR-positive breast cancer cell lines *in vivo*

Changes in gene expression, and a switching between ER and EGFR driven signalling, in accordance with fluctuating concentrations of estrogen and progesterone during the menstrual cycle could impact Oncotype DX Recurrence Scores. However, in contrast with earlier *in vitro* findings by our group, which demonstrate that progesterone treatment of breast cancer cells increases Oncotype DX Recurrence Scores (43), we found that progesterone treatment did not affect Recurrence Scores *in vivo*. Despite finding that progesterone treatment affects the Oncotype DX 21-gene signature in HR-positive breast cancer cell line xenografts *in vivo*, such changes in gene expression were not sufficient to impact Oncotype DX Recurrence Scores.

In T-47D xenograft tumours, progesterone treatment reduced expression of PR, which has the potential to reduce the signalling capacity of progesterone, and therefore impede tumour

proliferation. Indeed, the loss of PR expression following progesterone treatment was coupled with a reduced expression of proliferative genes. Importantly, these changes in gene expression have opposite effects on Oncotype DX Recurrence Scores. Reduced expression of *PGR* is associated with an increased Oncotype DX Recurrence Score, while reduced expression of proliferation-associated genes are associated with a decreased Recurrence Score. Thus the changes in Estrogen-group genes are offset by the changes in Proliferation-group genes, resulting in no net change in Oncotype DX Recurrence Score. Consequently, in this model of breast cancer, the panel of genes used in the Oncotype DX algorithm enables a robust Recurrence Score result despite fluctuating progesterone.

5.3.3. The effects of progesterone on gene expression vary depending on the model used

There is significant controversy in the literature as to the effects of progesterone on gene expression. Studies in normal mammary epithelium and premenopausal breast cancer samples report different findings compared to *in vivo* studies using breast cancer cell lines and xenograft models. These discrepancies between models limit us from drawing strong conclusions, as the extent to which our *in vivo* findings can be applied to premenopausal women, and the extent to which progesterone impacts the Oncotype DX 21-gene signature, remains unclear.

Results from this chapter have shown that progesterone treatment reduced proliferation of T-47D xenograft tumours, while no effect of progesterone was observed on proliferation of ZR-75-1 xenograft tumours. However, the role that progesterone plays in regulation of proliferation is not well defined. While there is evidence that progesterone stimulates proliferation of normal mammary epithelium and premenopausal breast cancer samples, where highest proliferative activity is observed during the luteal phase when concentrations of progesterone peak (152, 195); other *in vivo* studies, including our findings, report that progesterone either decreases or has no effect on proliferation in breast epithelium and breast cancer cells (204).

Furthermore, studies using HR-positive breast cancer cell lines report that progesterone stimulates a short-lived increase in proliferation, followed by a long-term anti-proliferative effect (23), and that the proliferative effects of progesterone depend on whether treatment is transient or continuous (205). Consequently, cyclical fluctuations in progesterone during the natural 4-day ovarian cycle of mice might result in a different proliferative effect of progesterone, compared to our model using a 3-day exogenous progesterone treatment of breast cancer cell lines *in vivo*.

Similarly, the impact of progesterone treatment on HR expression is conflicting. In normal breast epithelium and premenopausal breast cancer samples, several studies have suggested that ER and PR expression fluctuates across the menstrual cycle in accordance with fluctuating concentrations of progesterone (149, 151, 175); while subsequent studies found no association between ovarian hormone concentrations and HR expression (206, 207). In parallel with the findings reported in this chapter, studies using breast cancer cell lines have shown that progesterone treatment decreases HR expression (208, 209). Conversely, other studies have found no relationship between progesterone treatment and HR expression *in vivo* (204).

There are several factors that may explain the discordances between studies. Firstly, the absence of paracrine signalling from the surrounding stroma or immune microenvironment in xenograft breast cancer cell lines, compared to premenopausal breast cancer samples, could significantly impact our findings. Different stromal phenotypes are associated with different clinical outcomes (210), and the absence of cross-talk between tumour and stromal cells can influence how tumours respond to external stimuli.

Oncotype DX testing assesses expression of *MMP11*, *CSTL*, and *CD68* to identify the risk of disease recurrence and likely benefit from adjuvant chemotherapy. While these genes are expressed by epithelial cells at low levels, they are expressed predominantly by stromal- or immune-related cells, and are used to assess the invasive ability and immune infiltration of breast cancers. Xenograft tumours grown in immunocompromised mice do not exhibit abundant stroma and therefore do not reflect the effect of estrogen and progesterone on stromal and immune cell gene expression.

The use of homogenous breast cancer cell lines, and the treatment of mice with high concentrations of estrogen and progesterone that are not comparable to natural concentrations during the ovarian cycle could also contribute to discrepancies. The growth of breast cancer cell lines *in vivo* requires supplementation with high concentrations of estrogen for tumours to establish. Estrogen itself is a potent proliferative agent, and stimulates expression of PR (211), which can further confound the role of progesterone in proliferation and HR expression.

Using mouse models enables control of the hormonal environment and the timing of tissue collection, to better define the impact of progesterone on the Oncotype DX 21-gene signature at the time of diagnosis; a feat not feasible in breast cancer patients. However, given the conflicting results reported between *in vivo* xenograft studies, compared to studies that used premenopausal breast cancer samples, there needs to be caution when interpreting results.

Therefore, the different effect of progesterone on gene expression depending on the model significantly impacts conclusions drawn from the study. This is discussed further in chapter six.

5.3.4. Progesterone-mediated changes in protein expression do not affect protein-based treatment recommendations

Having found that progesterone treatment affects protein expression in HR-positive breast cancer xenografts, we sought to understand whether such changes could impact traditional protein-based treatment recommendations. Previous studies have reported that the expression of ER and PR fluctuates across the menstrual cycle (6, 151, 175). Pujol *et al.* (175) compared HR expression across the menstrual cycle in 575 premenopausal breast cancer patients. The authors measured protein expression of ER and PR using the dextran-coated charcoal (DCC) method, where concentrations of cytosolic protein >10fmoL/mg were considered positive. The authors reported that HR expression fluctuates in accordance with fluctuating concentrations of estrogen and progesterone. Expression of ER was highest in the follicular phase (30 fmol/mg) of the menstrual cycle, compared to ovulatory (20 fmol/mg) and the luteal phases (25 fmol/mg). Similarly, PR expression was highest in the ovulatory phase (177 fmol/mg) compared to follicular (134fmol/mg) and luteal phases (92 fmol/mg). However, despite protein expression fluctuating with menstrual cycle stage, tumours remained ER and PR positive regardless of menstrual cycle stage, as concentrations of receptors were consistently greater than the 10fmoL/mg threshold. Subsequent studies have reported similar findings (6, 151). Based on these observations, it is unlikely that fluctuations in ER and PR protein expression with menstrual cycle stage would significantly impact chemotherapy treatment decision-making.

Consistent with this, the changes in HR expression in breast cancer cell lines *in vivo* following progesterone treatment reported here are unlikely to have a significant effect on treatment recommendations. Current guidelines state that tumours expressing ER and PR in >1% of cells are classified as HR-positive (76, 172), and our results show that HR expression in xenografted tumours remained well above this threshold.

In the clinic, Ki67 is another marker used to guide chemotherapy treatment decisions, however its use is controversial due to the subjective nature of assessing Ki67 expression. While several guidelines have recommended the use of Ki67 for guiding chemotherapy treatment decisions (76, 172), other guidelines do not recommend the use of Ki67 (94) due to the lack of standardised procedure for assessing Ki67 expression (212). Therefore, while we observed reduced Ki67 expression in T-47D xenografts following progesterone treatment, which has the

potential to influence treatment recommendations, whether such changes in Ki67 expression in fact affect chemotherapy treatment decisions is debatable.

In ZR-75-1 tumours, progesterone treatment did not affect percent positivity of ER, PR, or Ki67. Similarly, fewer changes in gene expression were observed in ZR-75-1 cell lines compared to T-47D cell lines following progesterone treatment. These findings suggest that the ZR-75-1 cell line may be less responsive to ovarian hormones, and that the effects of progesterone on the Oncotype DX 21- gene signature may be limited to a subset of HR-positive breast cancers. This effect could be attributed to the lower PR positivity observed in ZR-75-1 tumours, compared to T-47D tumours (213-215). Consequently, the extent to which menstrual cycling and variable concentrations of progesterone impact the Oncotype DX 21-gene signature may be influenced by the tumour's PR percent positivity, and the capacity for progesterone signalling.

5.3.5. Limitations and future directions

The limitations of this study stem from the use of homogenous breast cancer cell lines, which are unable to capture the complexity of human breast cancers. Future studies that use different approaches are required to investigate the effects of progesterone on breast cancer gene expression and Oncotype DX Recurrence Scores. Patient derived xenografts (PDX) are an option to further explore the effects of ovarian hormones on heterogeneous, patient derived breast tumours, while still being able to control the hormonal environment and timing of tissue collection. PDX involve the transfer of breast tumour tissue excised from a patient during surgery into an immunodeficient mouse model. However, while this model can identify the impact of progesterone on heterogeneous patient derived tumours, there still remains no interplay between the tumour and the immune environment, as tumours are implanted into immunocompromised mice.

Ideally, future prospective studies should collect breast cancer samples from premenopausal women at a known menstrual cycle stage, and analyse results in light of circulating concentrations of estrogen and progesterone at the time of tissue collection. These studies would provide the strongest translational evidence on the direct effects of ovarian hormones on Oncotype DX Recurrence Scores, and overcome the limitations in using cell lines and animal models.

5.4. Conclusion

In summary, using HR-positive breast cancer cell lines, these studies demonstrate that ovarian hormones modulate breast cancer gene and protein expression *in vitro* and *in vivo*, and that the relative effects of progesterone are consistent between both gene and protein expression. However, although we show that progesterone affects expression of Oncotype DX signature genes, changes in gene expression did not translate into changes in Oncotype DX Recurrence Scores. This was possibly due to the lack of stromal, immune, and epithelial interplay within this breast cancer model, and the unphysiological concentrations of estrogen and progesterone used.

Chapter Six

General Discussion and Conclusions

6.1. Introduction

Emerging in the clinic are new assays that utilise biomarker gene expression profiling to predict breast cancer response to treatment. The Oncotype DX 21-gene Recurrence Score assay is recommended in clinical guidelines for guiding adjuvant chemotherapy treatment decisions for women with early stage, HR-positive, HER2-negative breast cancer (76, 94, 172, 173), and results in a net reduction in chemotherapy use (4). However, despite its use in the clinic, Oncotype DX has been developed predominantly in postmenopausal women, and there is a scarcity of literature on whether Oncotype DX is suitable for use in premenopausal women.

This thesis aimed to identify how Oncotype DX Recurrence Scores fluctuate in HR-positive breast cancers in response to fluctuating concentrations of estrogen and progesterone during the menstrual cycle. We have employed a variety of approaches to address this, including using paired human breast cancer samples, transgenic mouse models, and human breast cancer cell line xenografts. Results from this thesis have shown that Oncotype DX Recurrence Scores are more variable between paired breast cancer samples collected from younger women, compared to older women, and our mouse models support the notion that this is due to menstrual cycling. We suggest that Oncotype DX Recurrence Scores may be critically affected by the menstrual cycle stage at the time of tissue collection; and recommend that physicians should exercise caution when incorporating Oncotype DX Recurrence Scores in treatment decisions for premenopausal breast cancer patients.

The results in this thesis are the first to identify how menstrual cycling affects the Oncotype DX 21-gene signature. In this chapter, we highlight the clinical implications that our findings have for the use of genomic assays in premenopausal women, and we present opportunities for future research to improve the accuracy of these tests for young women. Key considerations required to improve the management of breast cancer in premenopausal women are also discussed.

6.2. Menstrual cycling critically affects the Oncotype DX 21-gene signature

The Oncotype DX 21-gene Recurrence Score assay is used to guide adjuvant chemotherapy treatment decisions for women with early stage, HR-positive, HER2-negative breast cancer. For women over the age of 50 years with Oncotype DX Recurrence Scores $\leq$26, endocrine therapy alone is equivalent to chemoendocrine therapy in terms of disease free and overall survival. However, women under the age of 50 with Oncotype DX Recurrence Scores between

16 and 26 still exhibit some benefit from chemotherapy treatment (77). When clinical information is integrated with Oncotype DX Recurrence Scores, the prediction of which premenopausal patients would benefit from chemotherapy was not improved (114). The biological basis for this age-related difference in chemotherapy benefit is not well defined.

Results from this thesis suggest that Oncotype DX Recurrence Scores are affected by the menstrual cycle stage at the time of tissue collection, which may contribute to the differences in Oncotype DX Recurrence Score thresholds between younger and older women. For premenopausal women, changes in Oncotype DX Recurrence Scores with menstrual cycle stage could result in Recurrence Scores not accurately reflecting the patient's true benefit from chemotherapy, and explain why chemotherapy benefit for women with Recurrence Scores between 16 to 26 is less clear. Critically, this uncertainty could lead to young women receiving a suboptimal or unnecessary chemotherapy treatment for their breast cancer; being exposed to the toxic side-effects of adjuvant chemotherapy without deriving significant benefit, or not receiving this potentially life-saving therapy.

6.3. Different mouse models of HR-positive breast cancer describe a different effect of progesterone on the Oncotype DX 21-gene signature

The experiments detailed in this thesis used a variety of approaches, including paired premenopausal breast cancer samples, transgenic mouse models, and human breast cancer cell line xenografts, to identify how menstrual cycling affects the Oncotype DX 21-gene signature. The use of patient breast cancer samples provides the strongest evidence on how Oncotype DX Recurrence Scores fluctuate within the same tumour collected on different days of the menstrual cycle. However, the lack of information surrounding the patient's menstrual history prevented us from investigating a direct relationship between the menstrual cycle stage and hormonal environment, and discordance in Oncotype DX Recurrence Scores.

Therefore, we complemented our findings using mouse models of HR-positive breast cancer, including naturally cycling MMTV-PyMT transgenic mice and xenograft breast cancer cell line models. The use of mouse models enabled us to manipulate the hormonal environment and the timing of tissue collection, to better explore how menstrual cycle stage and the hormonal environment affect Oncotype DX Recurrence Scores. However, we found that the effects of progesterone on the Oncotype DX 21-gene signature differed depending on the mouse model used, as summarised in Figure 31.

Homogenous xenograft breast cancer cell lines do not capture the heterogeneity of human breast cancer, and the interplay between tumour epithelial cells and the surrounding stromal and immune microenvironment is absent in these models. In contrast, MMTV-PyMT mice develop heterogeneous murine mammary tumours, with complete stromal and immune elements. These components of the tumour microenvironment play key roles in breast cancer progression, and are assessed by the Oncotype DX 21-gene assay to generate Recurrence Scores. Importantly, in MMTV-PyMT transgenic mice, we have shown that ovarian cycle stage influences expression of stromal and immune-associated genes, and that variability in their expression can affect Oncotype DX Recurrence Scores. Therefore, the lack of stromal and immune elements in the xenograft model may cloud the effects of progesterone on the Oncotype DX 21-gene signature and contribute to the discrepancies between mouse models.

Furthermore, breast cancer cell lines do not capture the complex signalling networks and cell cross-talk that is observed in heterogeneous breast tumours. In MMTV-PyMT transgenic mice, we found an inverse relationship between HR and HER2 signalling. Mammary tumours collected at diestrus when concentrations of progesterone are expected to be high exhibited reduced HR gene expression, coupled with an increase in *Errb2* expression and a subsequent increase in HER2-driven tumour proliferation. Conversely, the HR-positive cell line xenografts do not overexpress HER2. Therefore, the loss of HR expression in these tumours were not coupled with an increase in *ERBB2* expression, nor increase in HER2-driven tumour growth. This likely contributes to the observed anti-proliferative effect of progesterone, as following the loss of HR expression and HR-driven tumour growth in xenograft tumours, there was no substitute signalling pathway to promote tumour proliferation.

Our findings using premenopausal breast cancer samples suggest that menstrual cycle stage has a significant impact on the expression of genes associated with proliferation, and that this contributes to variability in Oncotype DX Recurrence Scores. While our mouse models are consistent with these findings, showing that progesterone affects the Oncotype DX 21-gene signature, we found that the effect of progesterone on tumour proliferation differed depending on the model used.

However, regardless of the proliferative effect of progesterone—either promoting or inhibiting tumour cell proliferation—changes in the expression of Proliferation-group genes could impact Oncotype DX Recurrence Scores. Taken together, the results from this thesis provide strong evidence that menstrual cycling can affect the Oncotype DX 21-gene signature; however, the direct effect of progesterone on Oncotype DX Recurrence Scores are less clear.

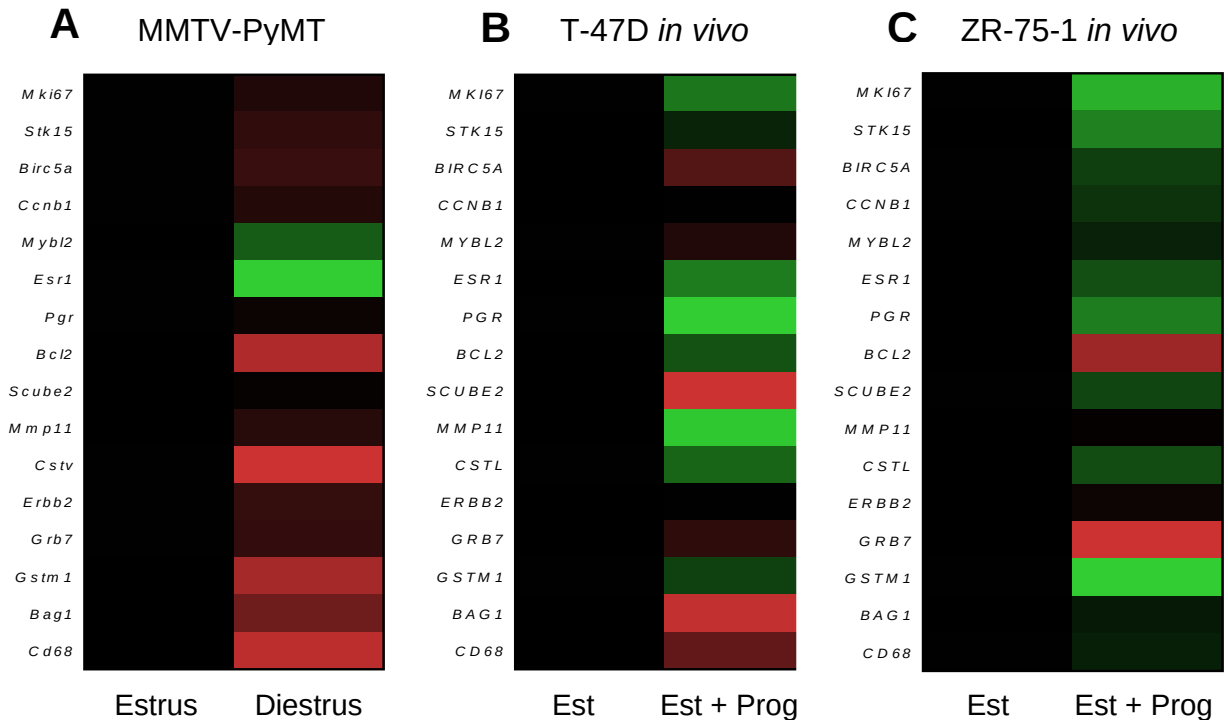


Figure 31 Heat-map visualisation summarising the different effects of progesterone on the Oncotype DX 21-gene signature in different mouse models. (A) Expression of the Oncotype DX 21-gene signature in mammary tumour collected at estrus and diestrus phases of the ovarian cycle from naturally cycling MMTV-PyMT transgenic mice. Expression of the Oncotype DX 21-gene signature in mammary fat pad-xenografted (B) T-47D or (C) ZR-75-1 breast cancer cell lines treated with estrogen only (Est) or estrogen with progesterone (Est + Prog). Data are normalised to tumours collected when concentrations of progesterone are absent (i.e, normalised to estrus in MMTV-PyMT transgenic mice and normalised to estrogen only treated mice in *in vivo* cell line studies). The scale of gene expression changes is presented. Red indicates increased expression of genes following progesterone treatment, green indicates reduced expression of genes following progesterone treatment, black indicates no change in gene expression.

6.4. Tailoring the Oncotype DX 21-gene Recurrence Score assay to improve its efficacy for premenopausal women

Although our findings suggest that Oncotype DX Recurrence Scores are affected by the menstrual cycle stage at the time of tissue collection, and are less effective for guiding treatment decisions in premenopausal women, there is the potential to tailor the Oncotype DX 21-gene assay for use in these women. The timing of Oncotype DX testing in accordance with a specific menstrual cycle stage would be a simple approach for improving the accuracy of Oncotype DX Recurrence Scores. However, this may not be entirely feasible in practice, as it may not be appropriate to delay a woman's biopsy collection or surgery for up to a month. Furthermore, although we have shown that Recurrence Scores are influenced by ovarian cycle stage, the cycle stage that reflects the patient's true Oncotype DX Recurrence Score and benefit from adjuvant chemotherapy is not known. Further research is warranted, as knowledge of an optimal time of the month to perform Oncotype DX testing provides a simple approach to improve the accuracy of Oncotype DX Recurrence Scores for premenopausal women.

Additionally, measuring circulating concentrations of estrogen and progesterone at the time of tissue collection, and modifying Oncotype DX Recurrence Scores in light of this information is another approach to improve the predictive capabilities of Oncotype DX Recurrence Scores for premenopausal women. However, there are limitations with this approach, and further research is required to define whether this is a valid option. Studies have suggested that the proliferative effects of progesterone—either promoting or supressing tumour proliferation—depend on the length of time the tumour is exposed to progesterone (23, 205). The effects of progesterone on tumour proliferation, and therefore on Oncotype DX Recurrence Scores, may be different depending on whether tumours are collected during early- or late-luteal phase, independent of the concentration of progesterone. Circulating concentrations of progesterone at the time of tissue collection would therefore need be considered in parallel with the patients reported last menstrual period, date of ovulation, and cycle length.

Another consideration is that concentrations of estrogen and progesterone can be highly variable between patients of the same cycle stage, and this does not necessarily affect the extent to which the tumours responds to hormonal stimuli. In mice, we identified a cluster of tumours dissected at the diestrus stage that show increased Oncotype DX Recurrence Scores, driven by an increase expression of Proliferation- and Estrogen-group genes. Expression levels of estrogen-regulated genes reflect a tumours responsiveness to hormonal stimuli. Consequently, these tumours may be more sensitive to fluctuations in estrogen and progesterone during the

ovarian cycle, and more prone to cycle-induced changes in Oncotype DX Recurrence Scores compared to other tumours dissected at the diestrus stage.

If the relative expression level of Estrogen-group genes can identify tumours which are more sensitive to fluctuations in ovarian hormones, then there is the potential to identify a panel of genes which can be assessed alongside the Oncotype DX 21-gene signature to improve the predictive power of Oncotype DX Recurrence Scores for premenopausal women. However, while we found that expression of Estrogen-group genes were associated with increased Oncotype DX Recurrence Scores in mice, we did not see a variable expression of such genes in paired breast cancer samples collected from younger women. However, other studies have reported that expression of estrogen-regulated genes fluctuate across the menstrual cycle, and support the possibility that their expression could be used as a test for endocrine responsiveness (5, 6).

Further research on the effects of menstrual cycling on Oncotype DX Recurrence Scores and chemotherapy treatment recommendations are required to improve treatment outcomes for premenopausal women. Defining the specific menstrual cycle stage that reflects the true Oncotype DX Recurrence Score and patients benefit from chemotherapy, and identifying tumours that are more sensitive to fluctuations in estrogen and progesterone warrants further investigation. Additionally, there is the possibility to identify a unique panel of genes and/or develop new algorithms that are more appropriate for guiding treatment decisions in premenopausal women.

6.5. The effects of ovarian cycling are not limited to the Oncotype DX 21-gene Recurrence Score assay

The effects of menstrual cycle stage are likely not limited to the Oncotype DX 21-gene assay. Currently in clinical guidelines, four other tests are recommended for guiding treatment decisions in breast cancer; Prosigna, EndoPredict, MammaPrint, and the Breast Cancer Index. However, there is an absence of literature surrounding the effects of fluctuating concentrations of estrogen and progesterone on their risk prediction. While several studies have compared concordance in these tests between biopsy and surgical breast cancer samples, finding generally high concordance, such studies were performed predominantly in postmenopausal women (164, 179), and whether these tests are appropriate for use in premenopausal women remains unknown.

Prosigna is a genomic assay that classifies tumours into intrinsic subtypes based on their gene expression profile. Each intrinsic subtype displays a distinct gene expression profile predictive of overall and relapse-free survival; and can therefore be used to inform risk prediction and help guide treatment decisions. The HR-positive intrinsic subtypes include Luminal A and Luminal B tumours, which show high similarity in their expression of estrogen-regulated genes. However, in contrast to Luminal A tumours, Luminal B tumours are distinguished by an increased in expression of proliferative genes, and therefore possess a more aggressive phenotype, higher proliferative index, and worse prognosis. Consequently, while Luminal A tumours can be treated with endocrine therapy alone, Luminal B tumours often require a more aggressive chemotherapy treatment.

We have previously described that progesterone plays an important role in modulating tumour cell proliferation, and that cycle-induced changes in genes associated with proliferation can influence tumour clustering. For premenopausal women, subtyping of breast tumours during the luteal phase of their menstrual cycle, when concentrations of progesterone are high, might classify a tumour into the more proliferative Luminal B subtype. Conversely, if the same breast cancer were assessed during the follicular phase, when concentrations of progesterone are low, the tumour may have a gene expression profile consistent with a Luminal A subtype. This could have dramatic implications for Prosigna-based treatment recommendations for premenopausal women, as treatment decisions could be skewed by the cycle stage at the time of tissue collection.

EndoPredict is another gene expression profiling test used to guide adjuvant chemotherapy and extended endocrine treatment decisions for HR-positive, HER2-negative breast cancer. EndoPredict measures the expression of eight proliferation and HR-associated genes to guide treatment decisions; genes whose expression can be impacted by the ovarian cycle stage. In 2012, Muller *et al.* (179) compared EndoPredict scores between core biopsies and corresponding surgical sections, finding high concordance. However, the age of women enrolled in this study was undefined, and whether EndoPredict scores fluctuate between paired breast cancer samples collected from premenopausal women remains unclear.

Similarly, the Breast Cancer Index depends heavily on the expression of proliferative genes; and MammaPrint assesses the expression of 70 genes associated with metastasis, proliferation, invasion, survival, and angiogenesis to guide chemotherapy treatment decisions. Given the dependence of these tests on genes associated with proliferation, they may also be susceptible to ovarian cycle-induced changes in treatment recommendations. While we primarily focused on the effects of ovarian cycling on Oncotype DX Recurrence Scores, as the Oncotype DX

21-gene assay is the leading genomic test in Australia, further research into the effects of ovarian cycling on other genomic tests is urgently needed, given their dependence on proliferative and estrogen-regulated genes and their current use in clinical practice.

6.6. Future Directions

6.6.1. A prospective analysis of paired breast cancer samples collected from premenopausal women

Further study is required to identify the extent to which menstrual cycle stage affects Oncotype DX Recurrence Scores, and to define the specific cycle stage that reflects the patient's true benefit from chemotherapy. A large scale study that prospectively collects paired breast cancer samples from premenopausal women at the time of biopsy and surgery, with precise information on menstrual cycle stage and circulating concentrations of estrogen and progesterone at the time of tissue collection, will further define the impact of menstrual cycling on breast cancer gene expression and identify the downstream impact on genomic tests and treatment recommendations. Importantly, such studies must adjust for confounding factors including irregular menses, the use of oral contraceptives, and neoadjuvant therapy.

We have presented preliminary data using a prospectively collected core biopsy and matched surgical breast cancer sample. However, there were limitations to this study that affected the sample size. Firstly, this was a single-centred study. A majority of premenopausal women were presenting to private clinics prior to being referred to The Queen Elizabeth Hospital for surgery, preventing us from collecting the initial diagnostic biopsy and accompanying blood sample. Additionally, this study excluded premenopausal women receiving neoadjuvant therapy, who presented with benign disease, or who had prior oophorectomy, which further limited our sample size. By increasing the number of participating hospitals and clinics, the number of women enrolled can be increased. A large-scale, long-term prospective study will provide further evidence on the effects of menstrual cycling on Oncotype DX Recurrence Scores, which is required for the Oncotype DX 21-gene assay to be tailored for use in premenopausal women.

6.6.2. Defining the effect of menstrual cycling on treatment outcomes for premenopausal breast cancer patients

Premenopausal women with breast cancer often present with more aggressive disease and have worse clinical outcomes compared to postmenopausal women. Young women show higher

rates of disease recurrence (216, 217) and reduced overall survival (218, 219) when the cancer is diagnosed at an early stage. However, there is a limited understanding of the underlying biology that contributes to these age-related differences in clinical outcomes. There is some evidence that, in addition to the effects on treatment-decision making, menstrual cycle stage may also impact surgical outcomes or the tumour response to therapy, and contribute to these poorer survival outcomes for younger women.

A relationship between menstrual cycle stage at the time of breast cancer surgery and clinical outcomes has been proposed. During this thesis, an in-depth literature review exploring this potential relationship between menstrual cycle stage at the time of surgery and patient clinical outcomes was conducted, and the manuscript is presented in Appendix A. To summarise briefly, studies have suggested that changes in the characteristics of the tumour and tumour microenvironment with menstrual cycle stage might influence the metastatic potential of tumour cells, and affect surgical treatment outcomes for premenopausal women. If the hormone milieu at a specific phase of the menstrual cycle results in a more favourable outcome, then the timing of breast cancer surgery to this phase might be a possible means of improving treatment outcomes for young women.

However, while several studies suggest that surgery performed during the luteal phase results in favourable outcomes in terms of metastatic incidence, disease free survival, and overall survival; other studies report the follicular phase is more favourable, and other studies show no association. Currently, there is significant controversy in the literature surrounding the impact of menstrual cycle phase at the time of surgery on breast cancer outcomes, and whether there is an optimal time of the month to perform surgery remains unclear.

Menstrual cycle stage might also directly affect the tumour response to adjuvant therapy. Oncotype DX Recurrence Scores reflect how a tumour will likely respond to adjuvant chemotherapy. However, if Oncotype DX Recurrence Scores change with menstrual cycle stage, does this also reflect a change in the tumour's response to chemotherapy? A retrospective analysis by Di Cosimo *et al.* (220) suggests that the impact of adjuvant chemotherapy may indeed vary depending on the menstrual cycle stage at the time of therapy commencement. Consequently, the timing of chemotherapy in accordance with a specific menstrual cycle stage could be a simple approach to improve treatment outcomes for premenopausal women. Similarly, if variable concentrations of progesterone are affecting the Oncotype DX 21-gene signature, and potentially influencing the tumours responsiveness to chemotherapy, then concurrently targeting PR may also be an approach to improve treatment outcomes for younger women.

Additionally, menstrual cycle stage may also influence the tumour response to hormonal therapies. In premenopausal women, breast cancer HR expression fluctuates across the menstrual cycle. Breast cancer tissue samples are more likely to be ER positive, and exhibit greater ER positivity when taken during the follicular phase compared to the luteal phase (150, 151). Furthermore, breast cancer samples exhibit greater PR positivity during the ovulatory phase, compared to either follicular or luteal phases (150). The percentage of ER and PR positive cells in a tumour is a predictor of the response to therapy, where increasing HR expression is associated with an increased benefit to endocrine therapy (157, 158). Therefore, changes in HR expression with menstrual cycle stage might influence the extent to which the tumour responds to treatment.

Currently, the extent to which menstrual cycling affects treatment outcomes remains largely understudied. Further research in this area is warranted, as knowledge of an optimal time of the month to conduct surgery would be a simple, cost-effective, and non-toxic approach to reduce the morbidity and mortality for young breast cancer patients. Similarly, defining whether the menstrual cycle stage affects a tumours response to therapy could help to identify new therapeutic approaches to improve the responsiveness to breast cancer therapy.

6.6.3. Understanding how menstrual cycling affects the development of specific breast cancer subtypes in premenopausal women

Premenopausal women often present with more advanced and aggressive types of breast cancer (2, 3), which exhibit unique gene expression profiles and signalling signatures (221), compared to breast cancer in postmenopausal women. However, there is a limited understanding of the underlying biology of premenopausal breast cancer, and the mechanisms that contribute to the differences between premenopausal and postmenopausal breast cancer are not well defined.

Given the role of estrogen and progesterone in breast cancer development (222, 223), it is possible that the specific hormonal environment at the time of tumour development might influence the cell-fate decisions of early breast cancer cells to promote the development of specific tumour subtypes. This might explain why premenopausal women, who are exposed to higher circulating concentrations of estrogen and progesterone, are more likely to develop HER2 and basal-like breast cancers, compared to postmenopausal women, who are more likely to develop luminal breast cancers.

Supporting a role for estrogen and progesterone in the development of specific breast cancer subtypes, a recent meta-analysis has suggested that women using oral contraceptives, and

therefore exposed to combinations of estrogen and progesterone, are more likely to develop triple negative breast cancer subtypes, compared to women not on oral contraceptives (224). Similarly, pregnancy associated breast cancers, which are breast cancers diagnosed during pregnancy or within five years of delivery, are more likely to be triple negative, compared to breast cancers in nulliparous women (225, 226). As pregnancy is characterized by increased circulating concentrations of estrogen and progesterone, these hormones may play a key role in the development of specific of breast cancer subtypes in young women.

There is some mechanistic evidence that supports a role for estrogen and progesterone in the development of specific breast cancer subtypes. In addition to the direct effects of estrogen and progesterone on breast epithelial cell gene expression (5, 6), estrogen and progesterone modulate angiogenesis and vascular invasion (51-53, 227), influence immune cell abundance and activity (188, 190, 194, 228-231), and can alter the cytokine environment (189, 232). These effects on tumour epithelial cells and the surrounding immune and stromal environment may play a role in determining tumour cell lineage fate, by providing a supportive environment to drive the development of a specific breast cancer subtype.

The results reported in this thesis, which describe an inverse relationship between growth factor receptor and HR-signalling driven by progesterone, also support the possibility that the hormonal environment may drive cell-fate decisions. In premenopausal women, high concentrations of progesterone during the luteal phase may promote growth factor-driven tumour growth and result in the development of more aggressive HER2 or basal-like breast cancer subtypes; subtypes that depend strongly on growth factor signalling. Conversely, low progesterone concentrations could promote HR-driven tumour growth and result in the development of luminal breast cancer subtypes.

However, the effects of estrogen and progesterone on the early events in breast tumour development are not well defined, and further research in this area is warranted. Understanding how menstrual cycling affects the development of breast cancers in premenopausal women will aid in developing new approaches to treat and prevent breast cancer in young women. Current studies in our lab are exploring this; identifying how menstrual cycling and immune-associated factors are involved in the development of breast cancer, with outcomes aimed at identifying new approaches to prevent breast cancer and develop new therapies to treat breast cancer in premenopausal women.

6.7. Recommendations for improving treatment outcomes for young breast cancer patients

Improving the management of premenopausal breast cancer is crucial for improving the survival outcomes and quality of life for these young women. However, there is a limited understanding of the underlying biology of premenopausal breast cancer, and the effects of menstrual cycling on breast cancer development, progression, and treatment outcomes are not well defined.

Often, studies using retrospectively collected data are the first approach to provide preliminary evidence to address these paucities in knowledge. However, retrospective studies are often limited by missing, inconsistent or inaccurate data. Studies in premenopausal women are further limited by poorly recorded or missing information on patient's menstrual histories; an issue that stems from the fact that the patient's menstrual cycle stage and oral contraceptive use are not routinely recorded at the time of breast cancer diagnosis or surgery. This has implications for current research, as studies that explore the role of menstrual cycling in premenopausal breast cancer are limited to finding small cohorts of women where this information is available.

This was a limitation for our study, which retrospectively collected paired breast cancer samples from women to investigate how menstrual cycling affects the Oncotype DX 21-gene signature. The patient's menopausal status and menstrual cycle stage at the time of tissue collection (i.e follicular versus luteal) was not known. Furthermore, we were unable to control for women on oral contraceptive or irregular cycling women. An absence of this information cautions strong conclusions, and prevented us from identifying a direct association between menstrual cycling and discordance in Oncotype DX Recurrence Scores.

However, an absence of this information is a common limitation for most retrospective studies in premenopausal women. We have reviewed the literature, and described that there is significant controversy surrounding the effect of menstrual cycle stage at time of surgery on the prognosis of premenopausal breast cancer (Appendix A). Missing and inconsistent information surrounding patient's menstrual histories likely contributed to the disagreements between studies. Many studies were limited to small retrospective cohorts that relied on the patients' recall of their last menstrual period. For the few patients whose menstrual cycle stage was known, methods were not standardised; there were variable approaches and different day cut-offs used to distinguish between follicular and luteal phases. Furthermore, for many studies, information on the patient's menstrual cycle length, oral contraceptive use and recent pregnancies were not available, which can further confound results if not accounted for.

Understanding how menstrual cycling impacts the development, progression, and treatment outcomes of premenopausal breast cancer is crucial for improving the quality of life for young women with breast cancer. However, currently available data sets are underpowered and are missing vital information on confounding factors. This missing and inconsistently recorded data raises concerns when comparing results between studies, and leaves currently available datasets inadequate at addressing these issues.

There is a pressing need to record information on the patient's menstrual cycle stage and cycle length, as well as identifying confounding factors, such as recent pregnancies, irregular menses and the use of oral contraceptives, at the time of breast cancer diagnosis and surgery. This will allow large-scale data sets to be collated, and well-controlled studies can provide significant power to address current and emerging issues. A better understanding of the role that menstrual cycling plays in breast cancer development, progression, and treatment outcomes is vital for improving the care of premenopausal breast cancer patients.

6.8. Conclusions

Genomic biomarker assays such as Oncotype DX were largely developed in postmenopausal women, and whether menstrual cycling in premenopausal women affects genomic biomarker expression has been a remarkably underappreciated research question. Results from this thesis demonstrate that menstrual cycling critically affects the Oncotype DX 21-gene signature, and suggests that Oncotype DX Recurrence Scores might partially depend on the menstrual cycle stage at the time of tissue collection. Therefore, the Oncotype DX 21-gene Recurrence Score assay may be less effective for guiding chemotherapy treatment decisions for cycling premenopausal women, compared to non-cycling postmenopausal women. Critically, incorporation of the Oncotype DX assay into treatment decision-making could lead to a suboptimal or unnecessary chemotherapy treatment for some premenopausal women. There is a pressing need for large-scale, long-term prospective studies to investigate the effects of menstrual cycling on genomic predictive biomarkers, to tailor assays such as Oncotype DX for use in premenopausal women. A better understanding of the role that menstrual cycling plays in breast cancer development, progression, and treatment outcomes is vital for improving the care of premenopausal breast cancer patients.

Chapter Seven

Appendices

APPENDIX A

Breast cancer surgery – is there an optimal time of the month?

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Abstract

An intriguing relationship between menstrual cycle phase at the time of breast cancer surgery and clinical outcomes was first proposed in the late 1980s. Some studies have suggested that surgery performed during the luteal phase results in the most favourable outcome, other studies report the follicular phase is more favourable, and others show no association. Despite meta-analyses and systematic reviews of these studies, there remains insufficient evidence to determine whether there is an optimal time of the month to perform surgery. This issue has dogged breast cancer surgery for decades; knowledge of an optimal time of the month to conduct surgery would be a simple approach to improving patient outcomes. A significant problem with current clinical studies is the lack of insight from mechanistic research that would elucidate the important variables. There are variable approaches to defining the menstrual cycle phase and hormone receptor status of the tumour and few studies controlled for prognostic factors such as tumour size and stage, or addressed the impact of adjuvant treatments. In this review, we discuss why study findings are so variable and explore the potential biological mechanisms through which the hormonal milieu might contribute to differences in prognosis. Elucidation of the specific confounding factors, as well as biological mechanistic pathways that could explain the potential relationship between timing of surgery and survival, will greatly assist in designing robust well-controlled clinical studies to evaluate this paradigm.

Introduction

In premenopausal women, fluctuations in circulating estrogen and progesterone occur across the course of the menstrual cycle. Breast tissue is highly responsive to ovarian hormones, and the cellular and molecular changes that occur in the breast over the course of the menstrual cycle affect breast development and function. These hormones also affect the activity of breast cancer cells, both directly through ligand-receptor binding to hormone receptor positive cancer cells, and indirectly through effects on cells within the cancer cell microenvironment.

An intriguing association between the timing of surgery in relation to menstrual cycle phase and breast cancer clinical outcomes was first proposed in the late 1980s. Mouse studies supported by small retrospective clinical studies suggested that changes in the characteristics of the tumour and tumour microenvironment across the menstrual cycle might influence the metastatic potential of tumour cells, and affect clinical outcomes in premenopausal women. This concept provides a potential approach to improving survival outcomes for premenopausal women. If the hormone milieu at a specific phase of the menstrual cycle results in a more favourable outcome, then the timing of breast cancer surgery to this phase might be a non-toxic and cost-effective means of reducing morbidity and mortality for young breast cancer patients.

However, there is significant controversy in the literature surrounding the impact of menstrual cycle phase at the time of surgery on breast cancer outcomes. While some studies suggest that surgery performed during the luteal phase results in favourable outcomes in terms of metastatic incidence, disease free survival, and overall survival; other studies report the follicular phase is more favourable, and other studies show no association. Currently, there is insufficient evidence to support a change in surgery scheduling for premenopausal breast cancer patients.

The lack of consistency in studies is likely due to a number of differences in study design and the small sample sizes used. There are variable approaches to defining the menstrual cycle phase and hormone receptor status of the tumour. Few studies controlled for prognostic factors such as tumour size and stage, or addressed the impact of adjuvant treatments such as chemotherapy and hormonal therapy. There are a number of potential biological mechanisms that might affect surgical outcomes, and currently no causal mechanisms have been demonstrated. To fully address this lack of clear evidence, prospective, well-controlled studies are required, supported by research on animal models that link biological mechanisms with clinical findings. Here, we review the current evidence for a relationship between the menstrual cycle phase at the time of surgery on breast cancer outcomes, and explore the biological mechanisms that may contribute to a phase-specific prognosis.

Current evidence of an impact of menstrual cycle phase at time of surgery on breast cancer metastasis

In 1988, Ratajczak *et al.* (233) published a study showing a relationship between the incidence of postoperative pulmonary metastasis, and the rodent estrous cycle phase at which the mammary tumour was removed. Using a hormone receptor-positive murine mammary carcinoma, the authors showed that tumours resected from mice around the time of ovulation (designated 'near estrus') showed fewer incidences of pulmonary metastasis 4 weeks after surgery compared to tumours resected at a time further away from the time of ovulation (designated 'post-estrus'). The study used the cytology of vaginal smears to classify the phases of the estrous cycle and did not assess circulating ovarian hormones in the mice. However, this classification system would have resulted in the mice exhibiting high circulating concentration of estrogen and low progesterone at 'near estrus', and high circulating concentration of progesterone and mid-range estrogen at 'post-estrus'. The authors show that the incidence of lung metastasis, as assessed by gross morphology and bioassay, was significantly reduced in 'near estrus' mice (44 of 60 mice; 73%) compared to 'post-estrus' mice (64 of 78 mice; 82%).

The authors proposed that the hormonal environment at the time of surgery can influence the metastatic potential of a cancer cell, through direct effects on the tumour, or indirect effects on the cancer microenvironment or the host immune system. Different hormonal environments may either facilitate or impede the metastasis of breast cancer cells, and therefore explain the observed differences in pulmonary metastasis with estrous cycle phase.

However, a subsequent study by Ben-Eliyahu *et al.* (234) suggested that rats are instead more susceptible to mammary carcinoma metastasis during the proestrus phase of the estrous cycle. The authors investigated lung metastasis in rats injected intravenously with hormone receptor-negative cancer cells, and reported that metestrus and diestrus stages of the cycle, which are characterised by high circulating concentrations of progesterone and mid-range estrogen, were protective against metastasis. Similarly, the authors demonstrated that treatment with estrogen increased the metastatic burden in the lung, an effect which was attenuated by progesterone treatment (234).

In 1989, Hrushesky and colleagues (235) published the first retrospective review in premenopausal women, investigating the effects of the timing of breast cancer surgery on disease recurrence and metastasis. The review included 44 premenopausal women, with both hormone receptor-positive and -negative disease. The authors found that patient outcomes varied significantly depending on the day of the menstrual cycle that surgery was performed.

In agreement with their earlier mouse study, the authors found that women operated on close to the time of menstruation showed poorer disease free and overall survival outcomes, and a greater incidence of metastasis, compared to women operated on during other phases of the cycle. This suggests that premenopausal women might have an increased risk of metastasis and poorer survival outcomes if surgery is performed during the perimenstrual phase of their menstrual cycle.

However, later studies have found conflicting results (236, 237), and there is significant controversy in the literature surrounding the effects of the menstrual cycle stage at the time of surgery on the survival outcomes of premenopausal breast cancers. A meta-analysis of 37 published studies (n = 10,476) suggested favourable prognosis when surgery was performed during the luteal phase (238). Similarly, a meta-analysis of 5353 premenopausal women demonstrated an overall survival benefit for women operated on during the luteal phase of the menstrual cycle (239). Conversely, two meta-analyses of 19 published studies (240, 241), found no significant relationship between menstrual cycle stage and patient prognosis.

The discrepancies between meta-analyses are likely associated with differences in their methodology. Different meta-analyses had different defining criteria for study inclusion; restricting their analysis to studies based on only one specific type of menstrual cycle stage classification, using one combined prognostic outcome, or limiting analysis to cohorts of women residing solely in Italy (240) or the United States (239). The four systematic reviews to date (241-244), which examined the relationship between the menstrual cycle stage at the time of surgery and patient outcomes, reported that there is insufficient evidence to determine if one phase of the menstrual cycle provides a more favourable outcome.

Confounding factors that could affect the relationship between timing of surgery and patient prognosis

Despite the large number of existing studies, there remains significant controversy in the literature surrounding how the menstrual cycle stage at time of surgery affects breast cancer outcomes. Disagreement between published studies could be due to a number of confounding factors including how menstrual cycle stage was classified for the study, variability in circulating hormone profiles between women, tumour stage at the time of surgery, and how psychological stress can affect ovarian hormone secretion and menstrual cycling.

Differences in classification methods can introduce significant variability into results, and may provide some explanation for the differences in results between different studies (150, 207, 235-237, 245-261)(Table 18). Other factors include inaccuracies in menstrual cycle data, as there

Table 18 Methods for classifying the menstrual cycle stage. Menstrual cycle stage classification was based on either serum hormone concentrations, days since the patients last menstrual period, or both. Abbreviations; DFS = disease free survival; OS = overall survival; E2 = estrogen; P4 = progesterone.

				Classification of menstrual cycle using serum hormone concentrations			Classification of menstrual cycle using patient reported last menstrual period (LMP)			
Author	**Number of women**	**Favourable outcome**	**Variable measured**	**Follicular**	**Luteal**	**Ovulatory**	**Follicular**	**Luteal**	**Midcycle**	**Peri-menstrual**
Hrushesky et al. (1989)	44	Follicular	DFS, OS	x	x	x	x	x	7-20	0-6, 21-32
Senie et al. (1991)	283	Luteal	DFS	x	x	x	0-14	>14	7-20	0-6, 21-32
Badwe et al. (1991)	249	Luteal	DFS, OS	x	x	x	3-12	13-32, 0-2	x	x
Wobbes et al. (1994)	89	No relationship	DFS	E2 100-990 pmol/L, P4 < 1.3 nmol/L	E2 330-1500pmol/L, P4 14-81 nmol/L	E2 86-1700pmol/L, P4 1.5-9.7 nmol/L	0-9	13-24	10-12	x
Badwe et al. (1994)	271	No relationship	DFS, OS	P4<1.5ng/mL	P4 >1.5ng/mL	x	3-12	13-28, 0-2	x	x
Corder et al. (1994)	157	Follicular	DFS, OS	x	x	x	3-12	0-2, 13-32	x	x
Veronesi et al. (1994)	1175	Luteal	DFS	x	x	x	0-14	15-36	x	x
Saad et al. (1994)	84	Luteal	DFS, OS	x	x	x	1-12	>12	x	x
Saad et al. (1994)	96	Luteal	DFS, OS	x	x	x	1-12	>12	x	x
Minckwitz et al. (1995)	266	Luteal	DFS, OS	x	x	x	3-12	13-35, 0-2	x	x
Holli et al. (1995)	267	No relationship	OS	x	x	x	1-14	15-28	x	x
Mohr et al. (1996)	289	Luteal	DFS, OS	P4 <4ng/mL	P4 >4ng/mL		0-12	13-30	x	x
Vanek et al. (1997)	150	Perimenstrual	DFS, OS	x	x	x	x	x	7-20	0-6, 21-32
Milella et al. (1999)	248	Luteal	DFS, OS	x	x	x	0-14	15-37	x	x
Nomura et al. (1999)	721	No relationship	DFS, OS	x	x	x	3-12 or 0-14	0-2, 13-32 or 15-36	x	x
Holmburg et al. (2001)	774	No relationship	OS	E2 <440pmol/L	200-700pmol/L	500-1300pmol/L	x	x	x	x
Pujol et al. (2001)	360	No relationship	DFS, OS	Everything else	P4 > 2.5ng/mL	E2 > 100pg/mL, 10mIU/mL	x	x	x	x
Takeda et al. (2001)	28	Perimenstrual	DFS	x	x	x	3-12 or 0-14	13-28, 0-2 or 15 -36	7-20	0-6, 21-32
Thorpe et al. (2008)	412	No relationship	DFS, OS	Not defined	Not defined	Not defined	0-14 or 3-12	15-36 or 0-12, 13-12	x	x
Grant et al. (2009)	834	No relationship	DFS, OS	P4 <3ng/mL	P4 >5ng/mL	x	0-21	21-35	x	x
Kucuk et al. (2012)	90	Luteal	DFS, OS	Everything else	P4 >2.5ng/mL	E2 > 100pmol/L, 10mIU/mL	0-14	15-28	x	x
Liu et al. (2015)	554	Follicular	DFS, OS	x	x	x	1-14	15-31	x	x

can be significant variability in cycle length (i.e, 22–36 days) between women (262); and other factors, such as irregular menses, use of oral contraceptives, recent pregnancies, or differing hormonal and chemotherapy treatment regimens may impact circulating ovarian hormones and menstrual cycle phase. McGuire *et al.* suggested that by changing the cut-off days used to classify the menstrual cycle phase, a significant number of patients can be shifted into a different phase, and this could influence the significance and outcomes of published results (263, 264).

Differences in the definition of surgery could also contribute to discordances between findings (Table 19). The majority of studies that found an association between menstrual cycle stage at the time of surgery and patient prognosis defined surgery as the time of first intervention. It is possible that the menstrual cycle stage when the tumour is first manipulated, through excision or incision biopsies or fine needle aspiration (FNA) has the greatest effect on patient prognosis, regardless of the total number of surgeries. Indeed, a study by Corder *et al.* (1994)(249) reported that FNAs performed during the follicular phase were associated with an improved patient prognosis, but there was no association between menstrual cycle stage at the time of first surgical intervention and patient prognosis. On the other hand, Vanek *et al.* (1997)(237) found that the menstrual cycle stage at the time of both biopsy and surgery correlated with patient disease free survival, suggesting that any time the tumour is manipulated, through either biopsies or surgeries, might influence patient prognosis.

To date, the majority of human studies have suggested that menstrual cycle stage at the time of surgery does indeed affect breast cancer outcomes; however, have disagreed on what stage of the cycle is optimal. It is unclear whether these observed effects of menstrual cycling are due to menstrual cycle phase per se, or due to biological effects of circulating hormones on breast cancer metastasis. Serum concentrations of estrogen and progesterone vary significantly between women of the same menstrual cycle stage. There is evidence that it is the elevated concentration of circulating progesterone during the luteal phase that exerts a protective effect against metastatic incidence (248, 258). If favourable outcomes are associated with higher concentration of circulating progesterone, then treatment with progesterone prior to surgery may be a feasible approach to improving breast cancer outcomes. Indeed, it has been reported that the injection of hydroxyprogesterone prior to surgery is associated with improved disease free survival for node positive breast cancer patients (265). However, there is controversy in the literature on the beneficial effects of progesterone on prognosis, and not all studies found a beneficial relationship between progesterone concentrations and survival outcomes (150).

Table 19 Criteria in studies investigating the relationship between menstrual cycle stage and patient prognosis. The survival outcomes measured for each study are highlighted. Surgery was defined as either the first intervention (i.e, FNAs or biopsies), the first surgical intervention (i.e, excisional or incisional biopsies, breast conserving surgery, partial mastectomies, or mastectomies), or the definitive surgery (i.e, re-excisions, mastectomies, or axillary node dissections). In many cases, the date of first surgical intervention corresponded with the date of definitive surgery. All studies included malignant disease only, while some studies only included invasive disease (denoted by *). Abbreviations; DFS = disease free survival; OS = overall survival.

Author	Number of women	Favourable outcome	Variable measured	Surgery definition
Hrushesky et al. (1989)	44	Follicular	DFS, OS	First intervention
Senie et al. (1991)	283	Luteal	DFS	First surgical intervention
Badwe et al. (1991)	249	Luteal*	DFS, OS	First intervention
Wobbes et al. (1994)	89	No relationship*	DFS	First surgical intervention
Badwe et al. (1994)	271	No relationship*	DFS, OS	First surgical intervention
Corder et al. (1994)	157	Follicular	DFS, OS	Analysed both initial and definitive procedures
Veronesi et al. (1994)	1175	Luteal	DFS	Definitive surgery
Saad et al. (1994)	84	Luteal	DFS, OS	First surgical intervention
Saad et al. (1994)	96	Luteal	DFS, OS	Analysed both initial and definitive procedures
Minckwitz et al. (1995)	266	Luteal	DFS, OS	First surgical intervention
Holli et al. (1995)	267	No relationship	OS	Undefined
Mohr et al. (1996)	289	Luteal*	DFS, OS	First intervention
Vanek et al. (1997)	150	Perimenstrual	DFS, OS	Analysed both initial and definitive procedures
Milella et al. (1999)	248	Luteal	DFS, OS	Definitive surgery
Nomura et al. (1999)	721	No relationship	DFS, OS	Definitive surgery
Holmburg et al. (2001)	774	No relationship*	OS	Definitive surgery
Pujol et al. (2001)	360	No relationship	DFS, OS	First intervention
Takeda et al. (2001)	28	Perimenstrual*	DFS	First surgical intervention
Thorpe et al. (2008)	412	No relationship	DFS, OS	First surgical intervention
Grant et al. (2009)	834	No relationship	DFS, OS	First surgical intervention
Kucuk et al. (2012)	90	Luteal	DFS, OS	First surgical intervention
Liu et al. (2015)	554	Follicular	DFS, OS	Undefined

Alternatively, it may be that high luteinsing hormone (LH) or follicle-stimulating hormone (FSH) concentrations, which peak prior to ovulation, are responsible for poorer rates of disease free and overall survival independent of estrogen and progesterone concentrations. FSH and LH can increase the invasive ability of breast cancer cells *in vitro* and *in vivo* (266, 267); and in breast cancer patients LH expression is increased in breast tumour tissue compared to normal breast tissue (268). However, the roles of LH and FSH in breast cancer initiation and progression are not well defined, and how they may contribute to metastasis warrants further investigation.

Several studies have shown that the effects of menstrual cycle phase at the time of surgery on prognosis is more pronounced in lymph node positive patients (Table 20). Lymph node positive tumours operated on during the luteal phase (245, 246, 250, 253), or when circulating concentrations of progesterone were high (248, 258), showed improved outcomes; however, these differences were less pronounced, or not observed, in node negative tumours. The more pronounced effect may be due to lymph node positive tumours already showing the potential for metastasis, and the hormonal environment at the time of surgery may further facilitate tumour cell metastasis in lymph node positive disease. However, not all studies have found a relationship between menstrual cycle phase and outcomes in lymph node positive patients (247, 269).

Another confounding factor in these studies may be the acute psychological impact of a breast cancer diagnosis on ovarian hormones and menstrual cycle length. Stressful life events affect the hypothalamo-pituitary-ovarian axis through catecholamine-induced inhibition of gonadotropin-releasing hormone, suppressing ovulation and progesterone secretion (270). The impact of stress on circulating estrogen, progesterone and menstrual cycle length (271) is difficult to address in retrospective studies on timing of surgery with menstrual cycle phase.

Impact of menstrual cycle phase at time of surgery on adjuvant therapy

Hormone receptor expression in breast cancer directs decision-making around use of adjuvant therapies, and influences the extent to which a tumour responds to treatment. The majority of studies investigating the effect of cycle phase on breast cancer outcomes did not take into account the percent positivity of hormone receptors (Table 21), nor the treatment regimen given to patients (Table 22). However, as hormone receptor expression and adjuvant therapy use are independent predictors of improved survival, differences in treatment regimens and treatment responses between menstrual cycle phases could confound results if not accounted for.

Table 20 Nodal status of patients involved in studies which examined the relationship between menstrual cycle stage and patient prognosis. a = studies where the effect more pronounced in lymph node positive cases; b = studies where the effect was limited to lymph node positive cases; x = nodal status wasn't stated.

			Nodal status		
Author	**Number**	**Favourable outcome**	**Pos**	**Neg**	**Ukn**
Hrushesky et al. (1989)	44	Follicular	16	28	0
Senie et al. (1991)	283	Luteal[a]	117	166	0
Badwe et al. (1991)	249	Luteal[a]	126	123	0
Wobbes et al. (1994)	89	No relationship	46	39	4
Badwe et al. (1994)	271	No relationship	119	151	1
Corder et al. (1994)	157	Follicular	66	91	0
Veronesi et al. (1994)	1175	Luteal[a]	436	739	0
Saad et al. (1994)	84	Luteal	45	39	0
Saad et al. (1994)	96	Luteal[b]	50	46	0
Minckwitz et al. (1995)	266	Luteal[a]	146	120	0
Holli et al. (1995)	267	No relationship	78	89	100
Mohr et al. (1996)	289	Luteal[b]	140	149	0
Vanek et al. (1997)	150	Perimenstrual	59	80	11
Milella et al. (1999)	248	Luteal	155	93	0
Nomura et al. (1999)	721	No relationship	329	392	0
Holmburg et al. (2001)	774	No relationship	x	x	x
Pujol et al. (2001)	360	No relationship	137	220	3
Takeda et al. (2001)	28	Perimenstrual	15	13	0
Thorpe et al. (2008)	412	No relationship	208	193	11
Grant et al. (2009)	834	No relationship	328	500	6
Kucuk et al. (2012)	90	Luteal	44	46	0
Liu et al. (2015)	554	Follicular	214	340	0

Table 21 Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) expression. x = receptor status not defined or measured; a = ER or PR status was not given, however 'hormone receptor' expression was given; b = The intensity of staining was measured; c = receptor status was measured by the DCC method, using cut-off as <10fmol/mg to define negative, and >10fmol/mg to define positive; d = measured by the DCC method using cut-off as <20fmol/mg to define negative, and >20fmol/mg to define positive; e = measured by the DCC method using cut-off as <25fmol/mg to define negative, and >25fmol/mg to define positive

			Receptor expression								
Author	**Number**	**Favourable outcome**	**ER+**	**ER-**	**Ukn**	**PR+**	**PR-**	**Ukn**	**HER2+**	**HER2-**	**Ukn**
Hrushesky et al. (1989)	44	Follicular	27[c]	17[c]	0	27[c]	17[c]	0	x	x	x
Senie et al. (1991)	283	Luteal	126	88	69	x	x	x	x	x	x
Badwe et al. (1991)	249	Luteal	145[c]	65[c]	39[c]	119[c]	84[c]	46[c]	x	x	x
Wobbes et al. (1994)	89	No relationship	52	26	11	59	23	7	x	x	x
Badwe et al. (1994)	271	No relationship	x	x	x	x	x	x	x	x	x
Corder et al. (1994)	157	Follicular	x	x	x	x	x	x	x	x	x
Veronesi et al. (1994)	1175	Luteal	926[c]	249[c]	0[c]	905[e]	270[e]	0[e]	x	x	x
Saad et al. (1994)	84	Luteal	36[c]	48[c]	0[c]	48[c]	34[c]	2[c]	x	x	x
Saad et al. (1994)	96	Luteal	36[c]	68[c]	12[c]	48[c]	34[c]	14[c]	x	x	x
Minckwitz et al. (1995)	266	Luteal	120[d]	115[d]	31[d]	126[d]	96[d]	44[d]	x	x	x
Holli et al. (1995)	267	No relationship	126[c]	107[c]	34[c]	172[c]	61[c]	34[c]	x	x	x
Mohr et al. (1996)	289	Luteal	x	x	x	x	x	x	x	x	x
Vanek et al. (1997)	150	Perimenstrual	77	52	21	67	51	32	x	x	x
Milella et al. (1999)	248	Luteal	127 [a]	121 [a]	0[a]	x	x	x	x	x	x
Nomura et al. (1999)	721	No relationship	400	284	37	x	x	x	x	x	x
Holmburg et al. (2001)	774	No relationship	x	x	x	x	x	x	x	x	x
Pujol et al. (2001)	360	No relationship	222[c]	138[c]	0[c]	264[c]	96[c]	0[c]	x	x	x
Takeda et al. (2001)	28	Perimenstrual	4	16	8	x	x	x	x	x	x
Thorpe et al. (2008)	412	No relationship	x	x	x	x	x	x	x	x	x
Grant et al. (2009)	834	No relationship	591	237	6	x	x	x	x	x	x
Kucuk et al. (2012)	90	Luteal	66[a]	24[a]	0[a]	x	x	x	x	x	x
Liu et al. (2015)	554	Follicular	341[b]	213[b]	0[b]	238	256	60	318[b]	168[b]	68[b]

Table 22 Distribution of treatments within studies examining the relationship between menstrual cycle stage and prognosis. Treatment information was unavailable in many studies. Many studies did not include information on hormonal therapies, or treatment regimens for node negative (N0) patients. Studies which included adjuvant therapy as a confounding variable, and adjusted for it in their outcomes are shown. x = the number of patients receiving this treatment was not defined in the methods of the paper. a = adjuvant therapy was not adjusted for, however was noted that treatment distributions did not differ between groups; b= studies adjusted for adjuvant therapy, however was not noted if this included adjusting for hormonal therapies in addition to chemotherapy.

			Treatment					
Author	**Number**	**Favourable outcome**	**Chemo-therapy**	**Hormonal therapy**	**Radiation**	**No therapy**	**Not defined in methods**	**Adjusted for treatment?**
Hrushesky et al. (1989)	44	Follicular	31	x	28	13	x	No
Senie et al. (1991)	283	Luteal	x	x	x	x	283	No
Badwe et al. (1991)	249	Luteal	60	x	1	188	126 (N0 patients)	No[a]
Wobbes et al. (1994)	89	No relationship	46	x	x	x	43 (N0 patients)	No
Badwe et al. (1994)	271	No relationship	54	x	x	66	151 (N0 patients)	No
Corder et al. (1994)	157	Follicular	x	x	x	x	157	No
Veronesi et al. (1994)	1175	Luteal	385	x	x	51	739 (N0 patients)	No
Saad et al. (1994)	84	Luteal	41	x	x	43	x	Yes[b]
Saad et al. (1994)	96	Luteal	43	x	x	53	50 (N0 patients)	No[a]
Minckwitz et al. (1995)	266	Luteal	151	x	x	115	x	No[a]
Holli et al. (1995)	267	No relationship	x	x	x	x	267	No
Mohr et al. (1996)	289	Luteal	35	x	x	99	149 (N0 patients)	No
Vanek et al. (1997)	150	Perimenstrual	x	x	x	x	150	No
Milella et al. (1999)	248	Luteal	248	x	x	x	x	No[a]
Nomura et al. (1999)	721	No relationship	582	429	x	x	x	No[a]
Holmburg et al. (2001)	774	No relationship	x	x	x	x	774	No
Pujol et al. (2001)	360	No relationship	x	x	x	x	360	No[a]
Takeda et al. (2001)	28	Perimenstrual	x	x	x	x	28	No
Thorpe et al. (2008)	412	No relationship	278	278	230	x	x	Yes
Grant et al. (2009)	834	No relationship	624	564	490	x	x	Yes[b]
Kucuk et al. (2012)	90	Luteal	60	x	x	30	x	Yes
Liu et al. (2015)	554	Follicular	x	x	x	x	554	No

Breast cancer hormone receptor expression fluctuates across the menstrual cycle. Breast cancer tissue samples are more likely to be estrogen receptor (ER) positive, and exhibit greater ER positivity when taken during the follicular phase compared to the luteal phase (150, 151). Furthermore, breast cancer samples exhibit greater progesterone receptor (PR) positivity during the ovulatory phase, compared to either follicular or luteal phases (150). The percentage of ER and PR positive cells in a tumour is a predictor of the response to therapy, where increasing hormone receptor expression is associated with an increased benefit to endocrine therapy (157, 158). Changes in hormone receptor expression with menstrual cycle phase might therefore affect the extent to which the tumour responds to treatment.

Similarly, growth factor receptor expression also fluctuates across the course of the menstrual cycle, and could contribute to a phase-specific prognosis. Increased expression of the epidermal growth factor receptor (EGFR) and human epidermal growth factor recpetor-2 (HER2) is observed during the follicular phase of the menstrual cycle (153, 154), and has been associated with increased metastasis and poorer survival outcomes (56, 57). Increased signalling through growth factor receptors during the follicular phase could promote breast cancer cell survival, facilitate metastasis, and contribute to the poorer outcomes observed during the follicular phase.

However, other studies have instead suggested that EGFR and HER2 expression is highest during the luteal phase in the normal breast (155), and that its expression is inversely related to ER expression which peaks during the follicular phase (156). Furthermore, the *in vitro* treatment of breast cancer cells with estrogen and progesterone results in the switching from hormone-driven to growth factor-driven cell growth (43). Together, this suggests that the increasing concentrations of progesterone during the luteal phase may increase growth factor-dependent cancer cell function, and contribute to a poorer prognosis, as opposed to estrogen-dependent cancer cell function during the follicular phase. To date, only one study that examines the relationship between the timing of surgery and patient outcomes has assessed HER2 expression. Liu *et al.* (254) took into account HER2 expression, and found that HER2 expression did not fluctuate across the menstrual cycle, nor was it a prognostic factor for disease free survival. However, the authors did not consider the intensity of HER2 expression.

Several studies (253, 259) have suggested that the effects of menstrual cycle phase are more pronounced in ER positive tumours, however the influence of PR and HER2 positivity on prognosis remains unclear. Expression of ER, PR and HER2 may be influenced by fluctuating concentrations of estrogen and progesterone, affecting cancer cell function and risk of metastasis. Changes in expression of hormone and growth factor receptors may also affect clinical decision-making around use of adjuvant therapies in some premenopausal women (174,

272), which could influence use of adjuvant treatments and explain why one stage of the menstrual cycle is associated with poorer survival outcomes. Therefore, hormone receptor and growth factor receptor expression may be a confounding factor on menstrual cycle phase-specific prognosis, or there may be alterations in tumour cell biology across the menstrual cycle that affect metastatic potential.

Biological mechanisms that link menstrual cycle phase to increased breast cancer cell dissemination

Several studies provide preclinical evidence that the manipulation of breast tumours during surgery or biopsy can increase the number of circulating tumour cells in the blood (273-276). The hormonal environment at the time of surgery may have effects on these circulating tumour cells and their microenvironment, to facilitate the establishment and survival of tumour cell metastases and contribute to phase-specific prognoses (277).

Estrogen and progesterone can modulate angiogenesis, vascular invasion, and the immune system, to promote a proangiogenic and immunosuppressive environment supportive of metastasis. In premenopausal women, breast tumours resected during the follicular phase of the menstrual cycle show increased incidence of vascular invasion (227). Preclinical studies have shown that expression of vascular endothelial growth factor (VEGF), a growth factor that plays key roles in angiogenesis and vascular invasion, is positively associated with estrogen concentration and its expression is blocked by estrogen antagonists *in vivo* (51, 52). VEGF expression is highest during the follicular phase of the menstrual cycle, and expression is reduced with increasing concentrations of progesterone during the luteal phase (53). Any relationship between the timing of surgery and patient outcomes may be influenced by increasing concentrations of estrogen during the follicular phase promoting a proangiogenic environment favourable for breast cancer metastasis.

Metastasis involves the migration of cells from the primary tumour in the breast to a distant site at which they must be able to establish. During the follicular phase, unopposed estrogen may facilitate metastasis, by increasing the risk of dissemination of malignant cells during tumour handling during surgery. In addition to stimulating angiogenesis and vascular invasion, estrogen promotes the expression of genes involved in epithelial-to-mesenchymal transition (EMT), and allows for cells to detach and gain access to lymph and blood vessels (278). *In vitro* and *in vivo* stimulation with estrogen promotes proliferation of breast cancer cells and induces protease production. Simultaneously, estrogen downregulates E-cadherin expression, an effect which

can be reversed with anti-estrogenic treatment, consequently increasing the invasive ability of tumour cells (182, 183).

Biological mechanisms that link menstrual cycle phase to suboptimal immune response to breast cancer

The immune system plays a key role in removing cancer cells and preventing metastasis, and therefore an immunosuppressive environment at the time of surgery may increase the metastatic potential of cancer cells. Hormonal fluctuations during the menstrual cycle have direct and indirect effects on the immune system. Circulating estrogen during the follicular phase of the menstrual cycle can reduce immune activity, phagocytic activity, and alter expression of cytokines, which may promote tumour metastasis, establishment and survival. Conversely, progesterone can supress the effects of estrogen.

Macrophages and regulatory T cells (Tregs) play critical roles in the immune evasion abilities of breast cancer cells. The abundance of Tregs correlates with serum concentrations of estrogen; Tregs are most abundant during the follicular phase of the menstrual cycle, and their abundance decreases during the luteal phase (228). Furthermore, treatment with estradiol promotes the proliferation of Tregs and enhances their immunosuppressive functions (229). Similarly, progesterone is known to have immunosuppressive activity, and regulates Treg abundance and phenotype (194). The abundance and function of macrophages also fluctuates across the ovarian cycle of mice, where lowest macrophage abundance is observed in the mouse mammary gland during the estrus phase, when concentrations of estrogen are highest (188, 190).

Reduced natural killer (NK) cell abundance and activity is associated with increased metastatic incidence. Breast cancer patients with low NK activity are at a greater risk of developing metastatic recurrence (279). Furthermore, in mice, the metestrus phase of the estrous cycle shows lowest NK cell activity and interleukin-2 production, and is associated with the highest incidence of pulmonary metastasis (230). The effects of cycle phase on the abundance and activity of NK cells may be mediated by estrogen. Treatment of mice with estrogen results in inhibition of NK cell activity, and is associated with an increased incidence of pulmonary metastasis (231). Similarly, tamoxifen treatment of postmenopausal women resulted in enhanced NK cell activity (280). It is possible that high concentrations of estrogen during the follicular phase reduce NK activity, resulting in an immunosuppressive and pro-metastatic environment; conversely, high progesterone concentrations during the luteal phase promote an environment more resistant to tumour metastasis.

Estrogen also influences the expression of pro-inflammatory cytokines, including CSF1, CSF2, IFNG and TNFA. In mice, expression of pro-inflammatory cytokines is greatest at the estrus phase of the ovarian cycle, when concentrations of estrogen peak, and their increased expression is mitigated by progesterone during different ovarian cycle stages (189). Furthermore, estrogen treatment alone, or in combination with progesterone, can stimulate insulin-like growth factor 1 (IGF1) which can increase breast cancer cell proliferation and inhibit apoptosis (232, 281). Conversely, concentrations of IGF1 in serum are reduced following progesterone treatment alone (232, 282).

A relationship between the gut microbiome and the immune system has been described, where disturbance in diversity and alterations in relative abundance of different bacterial phyla and genera can influence the local and systemic immune environment (283) and increase breast cancer metastasis in mice (284). An association has been suggested between circulating concentrations of estrogen in blood and gut microbiota diversity, whereby increased circulating concentrations of estrogen contribute to a more diverse microbiome (285, 286). If the stage of the menstrual cycle influences gut microbiota diversity, then cross-talk between the altered microbiome and the immune system may result in an environment that favours tumour cell metastasis, and thus the timing of surgery could influence survival outcomes. However, this phenomenon has not yet been explored.

Fluctuations in estrogen and progesterone across the menstrual cycle can influence immune cell abundance and activity, and change the cytokine environment. It is possible that altered immune function at a specific menstrual cycle phase may affect the metastatic ability of breast cancer cells; allowing for tumour cells to evade the immune system, and facilitate the spread, survival, and establishment of metastatic cells following surgery.

Conclusion

The current evidence from clinical studies and animal models supports the possibility that menstrual cycle phase at the time of surgery influences risk of tumour metastasis. However, given the conflicting results, there is insufficient evidence to determine whether there is an optimal time of the month to perform surgery. This issue has dogged breast cancer surgery for decades; knowledge of an optimal time of the month to conduct surgery would be a simple, non-toxic and cost-effective approach to improve patient outcomes. Key considerations for further studies are clear definitions for the different phases of the menstrual cycle based on both last menstrual period and circulating hormone concentrations, stratification by tumour subtype

and nodal status, as well as consideration of confounding factors, including irregular menses, the use of oral contraceptives, and neoadjuvant and adjuvant therapy. The impact of tumour manipulation during both diagnosis and excision on patient prognosis should also be assessed. A significant problem with the current clinical studies is the lack of insight from mechanistic research that would elucidate the variables to control for. While there are a number of plausible biological mechanisms that could collectively lead to altered survival, supporting evidence is limited. Elucidation of the specific confounding factors, as well as biological mechanistic pathways that may explain the potential relationship between timing of surgery and survival will greatly assist in designing robust well-controlled clinical studies to evaluate this paradigm.

APPENDIX B

Hormonal modulation of breast cancer gene expression: implications for intrinsic subtyping in pre-menopausal women

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frontiers in Oncology

REVIEW
published: 14 November 2016
doi: 10.3389/fonc.2016.00241

Hormonal Modulation of Breast Cancer Gene Expression: Implications for Intrinsic Subtyping in Premenopausal Women

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Clinics are increasingly adopting gene-expression profiling to diagnose breast cancer subtype, providing an intrinsic, molecular portrait of the tumor. For example, the PAM50-based Prosigna test quantifies expression of 50 key genes to classify breast cancer subtype, and this method of classification has been demonstrated to be superior over traditional immunohistochemical methods that detect proteins, to predict risk of disease recurrence. However, these tests were largely developed and validated using breast cancer samples from postmenopausal women. Thus, the accuracy of such tests has not been explored in the context of the hormonal fluctuations in estrogen and progesterone that occur during the menstrual cycle in premenopausal women. Concordance between traditional methods of subtyping and the new tests in premenopausal women is likely to depend on the stage of the menstrual cycle at which the tissue sample is taken and the relative effect of hormones on expression of genes versus proteins. The lack of knowledge around the effect of fluctuating estrogen and progesterone on gene expression in breast cancer patients raises serious concerns for intrinsic subtyping in premenopausal women, which comprise about 25% of breast cancer diagnoses. Further research on the impact of the menstrual cycle on intrinsic breast cancer profiling is required if premenopausal women are to benefit from the new technology of intrinsic subtyping.

Keywords: premenopausal breast cancer, intrinsic subtyping, menstrual cycle, gene expression, hormones

OPEN ACCESS

Edited by:
José - Bines, Instituto Nacional de Câncer, Brazil

Reviewed by:
Tomas Reinert, Instituto do Câncer Mãe de Deus e Instituto DeVita, Brazil
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Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *30 August 2016*
Accepted: *27 October 2016*
Published: *14 November 2016*

Citation:
Bernhardt SM, Dasari P, Walsh D, Townsend AR, Price TJ and Ingman WV (2016) Hormonal Modulation of Breast Cancer Gene Expression: Implications for Intrinsic Subtyping in Premenopausal Women. Front. Oncol. 6:241. doi: 10.3389/fonc.2016.00241

INTRODUCTION

Approximately 25% of breast cancers are diagnosed in women under the age of 50 (1). When breast cancer is diagnosed in young women it carries a high burden, with reduced 5-year survival rates compared to breast cancer in older women (2, 3), and a devastating impact on young families. Breast cancer is considered a chronic disease, with increased mortality extending over the next 40 years, even if the breast cancer is diagnosed at an early stage (3).

In premenopausal women, cyclical production of ovarian hormones estrogen and progesterone occur over the course of the menstrual cycle, causing the mammary gland epithelium to undergo cycles of proliferation, differentiation, and apoptosis (4, 5). Estrogen and progesterone play key

roles in the development of breast cancer, with the relative risk of breast cancer related to the breast's cumulative exposure to these hormones (6, 7).

Breast cancer is not a single disease. There are many mutated genes that can drive tumor development, and biomarkers are essential to classify breast cancer into its different subtypes, each of which responds best to different therapies. Currently, immunohistochemical assays that detect abundance of proteins are used to identify expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) and determine the rate of proliferation of the cancer cells (Ki67). These biomarkers are used collectively to diagnose subtype and thus determine the best treatment option for an individual patient.

Now, clinics are increasingly adopting gene-expression profiling to diagnose breast cancer subtype, providing an intrinsic, molecular portrait of the tumor. For example, the PAM50-based Prosigna test quantifies expression of 50 key genes to classify breast cancer subtype. Tests that diagnose intrinsic breast cancer subtype must be robust, and relatively resistant to fluctuations in gene expression. Generally, good concordance is observed in gene expression between pairs of diagnostic and surgical samples and between classification of subtype by gene expression and traditional immunohistochemical techniques.

However, despite their availability for diagnosing breast cancer subtype in premenopausal women, tests that utilize gene-expression profiling were largely developed and validated using breast cancer samples from postmenopausal women. Fluctuations in ovarian hormones estrogen and progesterone across the menstrual cycle may affect expression of genes currently used in intrinsic subtyping tests. Indeed, studies have found poor concordance between classification of subtype by intrinsic tests and traditional immunohistochemistry in premenopausal breast cancer patients (8, 9).

Here, we outline the role of ovarian hormones in regulation of gene expression in the breast and highlight the deficiencies in knowledge around intrinsic subtyping in premenopausal breast cancer. As different therapeutic strategies are required depending on the tumor type, effective subtyping of breast cancers is necessary to help guide treatment decisions and provide accurate prognostic information for each patient. Intrinsic subtyping offers some significant advantages over traditional subtyping methods; however, the current tests have not been sufficiently validated in the context of premenopausal breast cancers, where there are significant fluctuations in estrogen and progesterone. There is a pressing need for more research into hormonal modulation of breast cancer gene expression in order to provide the optimal subtype diagnosis for premenopausal women.

HORMONALLY DRIVEN CHANGES IN THE BREAST DURING THE MENSTRUAL CYCLE

During the follicular phase of the menstrual cycle, increasing levels of FSH produced by the pituitary stimulate maturation of estrogen-secreting ovarian follicles. Estrogen acts on the pituitary to further increase the production of FSH and LH. Eventually, the concentration of estrogen peaks, stimulating a peak in LH secretion that triggers ovulation. Following ovulation, LH promotes differentiation of the ovarian follicle into the progesterone producing corpus luteum. The luteal phase is characterized by a high concentration of progesterone and is accompanied by a smaller second rise of estrogen. Progesterone supresses FSH and LH production, resulting in a decrease in estrogen concentration. Levels of progesterone begin to decrease as the corpus luteum ceases to produce progesterone and collapses. Consequently, the end of the menstrual cycle is characterized by low circulating hormones, which, in turn, relieve the negative inhibition of FSH and LH (10, 11) (**Figure 1**), allowing progression into the next menstrual cycle.

Fluctuations in estrogen and progesterone across the menstrual cycle direct the mammary gland epithelium to undergo sequential waves of proliferation, differentiation, and apoptosis (4, 5, 12, 13). Histological analysis of breast tissue by Vogel et al. identified distinct morphological changes in the mammary gland in accordance with different phases of the cycle (14). Five separate phases of breast morphology have been identified, associated with differing concentration of circulating estrogen and progesterone. Each phase has distinct morphological criteria based on the appearances of the luminal cells, myoepithelial vacuolization, intraluminal secretion, stromal edema, and events of cell turnover (5, 14), as summarized in **Table 1**.

The highest proliferative activity of mammary epithelium is observed in the luteal phase, with rising levels of progesterone (**Figure 1**). As the concentration of progesterone rises, there is an increase in secondary branching, alveoli budding, and stromal development, accompanied by changes to the extracellular matrix (10, 14). Consistent with this, studies in rodents have shown that administration of exogenous progesterone promotes side-branching and normal secretory alveolar development, whereas estrogen stimulates ductal elongation (15). Ferguson and Anderson showed that epithelial apoptosis increases at the end of the cycle, with decreasing circulating concentration of estrogen and progesterone (16); causing an atrophy of the epithelium, closing of the alveolar lumen, condensation of intralobular stroma, and a variable inflammatory infiltrate (14).

Hormonally driven morphological changes are associated with gene expression changes. Estrogen regulates many genes involved in cell cycle progression; such as Cyclin D (17), and c-MYC (18, 19), and is involved in activation of Cyclin E complexes (20). Estrogen also induces cyclin dependant kinases (Cdk) activation and Rb phosphorylation (20, 21) to promote cell cycle progression. In addition, estrogen treatment inhibits genes responsible for the suppression of cell growth, such as p21 (22). Estrogen is also an inhibitor of apoptosis and increases the expression of antiapoptotic proteins, such as Bcl-2 and Bcl-xL (23). Consistent with this, Bcl-2 is expressed almost exclusively in ER-positive breast cancers and is associated with a good prognosis (24).

Progesterone also plays an important role in cell proliferation and differentiation in the breast, specifically acting during the luteal phase of the menstrual cycle. The proliferative role of progesterone is likely mediated by regulation of cell cycle genes,

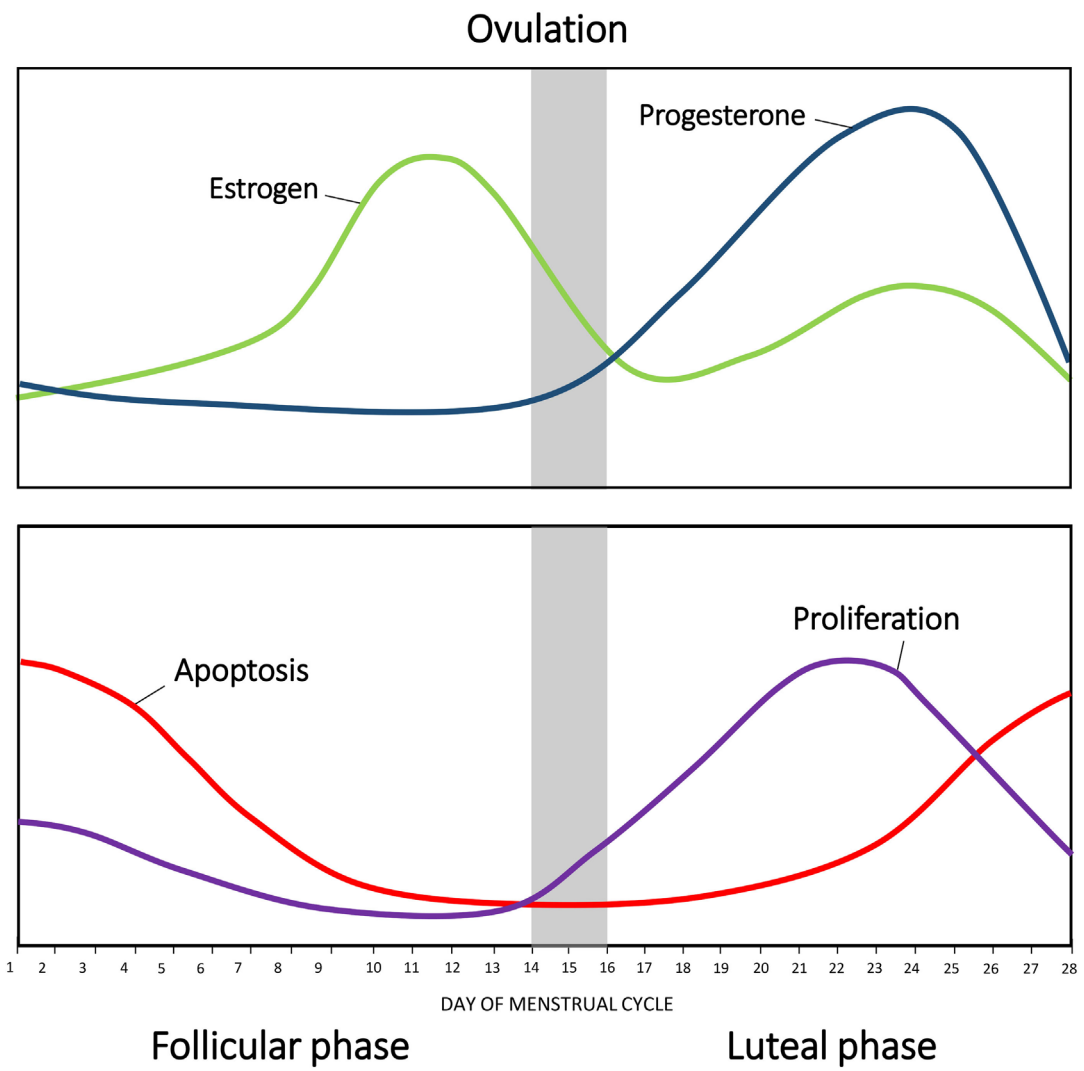

FIGURE 1 | Changes in hormonal levels in accordance with the menstrual cycle. The fluctuations of estrogen (green) and progesterone (blue) during the human menstrual cycle. Net apoptosis (red) and proliferation (purple) in the mammary gland in accordance with the menstrual phase.

growth factors, and growth factor receptors. Musgrove et al. illustrated that progesterone treatment of PR-positive breast cancer cells results in an increase in cell cycle progression, which is correlated with an induction of cell cycle genes; including cyclin D1 (25, 26), and c-Myc (25–27). Progesterone also regulates activity of Cdks (28). In addition to stimulating genes associated with cell cycle progression, progesterone has been suggested to inhibit expression of genes responsible for suppression of cell growth, such as tumor suppressor protein p53 (29) and retinoblastoma protein (30). Decline in ovarian hormones also effects gene expression. The fall in estrogen and progesterone at the end of the luteal phase is associated with an increase in apoptotic proteins, such as BAX (10) and FasL (31), and a decrease in antiapoptotic proteins, such as Bcl-2 (10).

In the mammary gland, progesterone elicits its function mainly through a paracrine mechanism. Recently, RANKL has been identified as an important paracrine mediator of progesterone-induced proliferation in the mammary gland (32, 33) and is implicated upstream of Cyclin D (32). Consistent with this, RANKL is required for mammary gland development (34) and was shown to be essential for ductal side branching and alveologenesis in mice (35). In addition, overexpression of its receptor, RANK, in mice resulted in increased proliferation of mammary epithelial cells (36). Wnt-4 has also been identified as a paracrine mediator of progesterone signaling (32, 37), and is important for side-branching of the mammary ductal epithelium (37). To promote optimal proliferation of mammary epithelial cells, estrogen induces expression of the PR. This leads to proliferation of mammary epithelial cells through elevated expression of cell cycle genes, when both estrogen and progesterone are present (38). Conversely, progesterone downregulates its receptor and inhibits synthesis of the ER (39).

TABLE 1 | Morphological changes in the mammary gland in accordance with the menstrual cycle as described by Vogel et al. (14).

Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
Follicular phase (days 3–14)		Luteal phase (days 15–27)		Menstrual phase (days 28–2)
Proliferation	*Follicular phase of differentiation*	*Luteal phase of differentiation*	*Secretory phase*	*Menstrual phase*
Dense cellular stroma	Dense collagenous stroma	Loose broken stroma	Loose fluid-filled stroma	Dense cellular stroma
Tight closed lumen (no stratification)	Defined lumen (radial orientation)	Open lumen (radial orientation)	Open lumen (radial orientation)	Swollen lumen (radial orientation)
No active secretion	No active secretion	No active secretion	Active apocrine secretion from lumen cell	Rare secretion
High levels of apoptotic bodies	Apoptotic bodies rare	Apoptotic bodies rare	Apoptotic bodies rare	Apoptotic bodies rare

Progesterone and estrogen also regulate growth factors and growth factor receptors in the breast, such as epidermal growth factor (EGF) (40) and EGF receptor (EGFR) (41, 42). Many key genes associated with EGFR signaling are upregulated in response to progesterone treatment (41, 43). Furthermore, EGFR signaling has been implicated downstream of estrogen in the mammary gland (44). Estrogen can induce phosphorylation of EGFR, and can directly interact with signal transduction pathways, to activate MAPK, JAK/STAT, SRC, and PI3K signaling pathways downstream of EGFR. In parallel, EGFR can, in turn, phosphorylate and activate ER and PR (45). It has also been shown that EGF family members are induced by estrogen; including EGF (46), TGFα (46, 47), and amphiregulin (Areg) (48, 49), and act as an important mediator of paracrine estrogen-induced proliferation (**Figure 2**). However, estrogen inhibits EGFR expression in ERα positive cells. In ERα negative cells, secreted amphiregulin activates EGFR signaling to promote cell proliferation (50). Recent studies have also indicated that estrogen treatment induces expression of vascular epithelial growth factor receptor (VEGF), a receptor involved in tumor growth, both *in vitro* (51) and *in vivo* (52).

In breast cancer, increased EGFR signaling is associated with a more aggressive phenotype. Overexpression of growth factor receptors has been associated with increased metastasis and poor survival, together with a lack of response to endocrine therapy (53, 54). As estrogen and progesterone play critical roles in regulation of growth factors, it is possible that the fluctuations of these hormones during the menstrual cycle are sufficient to modulate expression of EGFR and affect downstream signaling. In the luteal phase when progesterone is high and estrogen is present, signaling through growth factor pathways may be increased compared to the follicular phase when progesterone concentration is low. Consistent with this, breast tumors in young women often have significantly higher EGFR expression and worse prognosis (55, 56).

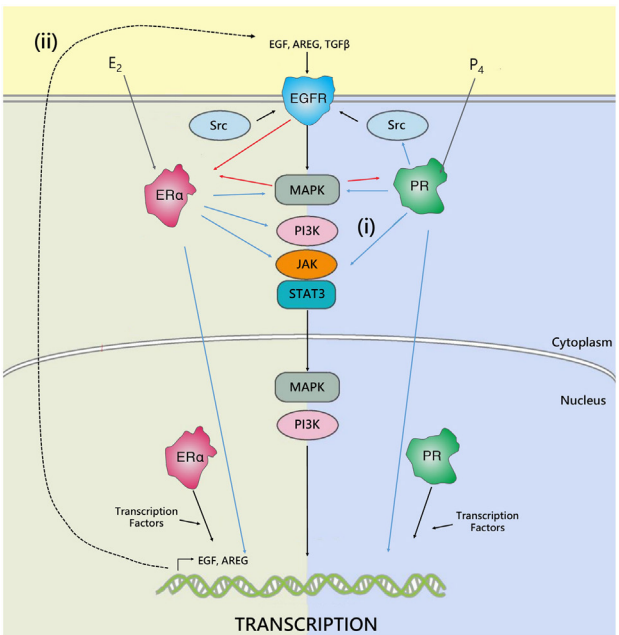

FIGURE 2 | The interplay between ER, PR, and EGFR. Hormone receptors regulate gene transcription either by binding directly to DNA response elements or by recruiting transcription factors and co-regulators. In addition, cross talk occurs between ER, PR, and EGFR to regulate gene expression. The estrogen and progesterone receptor can regulate epidermal growth factor receptor activity by either: (i) directly interfering with their transduction pathways, to activate MAPK, JAK/STAT, SRC, PI3K signaling downstream of EGFR, or (ii) by inducing expression and secretion of paracrine growth factors, such as AREG, TGFβ, or EGF, which act on EGFR to activate pathways involved in cell proliferation, survival, and metastasis. In parallel, EGFR can, in turn, phosphorylate and activate ER and PR. Adapted from Tanos et al. (45).

CLASSIFICATION OF BREAST CANCER SUBTYPES

Breast cancer is a heterogeneous disease, due to its diverse molecular and cellular features, with different therapeutic strategies required depending on the tumor type and stage. The decision to treat patients with adjuvant therapy has been guided by clinical and pathological features of the tumor. With no adjuvant therapy, 12–58% of women will experience a reoccurrence within 5 years (57–59). Of women diagnosed with breast cancer, the majority (approximately 75–92%) receive adjuvant therapy (57, 60, 61), suggesting that many women receive a treatment that may not provide benefit, exposing them to unnecessary side effects. Ideally, the decision to use adjuvant therapy should be based on the prediction of the degree of benefit, to minimize the number

of patients receiving unnecessary treatment. Traditionally, evaluation of ER, PR, HER2, and Ki67 immunoreactivity, together with clinicopathological variables including tumor size, type, and grade, are used to classify breast tumors and guide clinical decisions. Breast cancer can be classified into five major subtypes, i.e., Luminal A, Luminal B, HER2 enriched, Basal-like, and normal breast-like, which show significant differences in incidence, survival, and clinical outcomes (9, 62–64).

Luminal A tumors are the most common, representing 50–60% of all breast cancers (65). Patients with Luminal A breast cancer have a good prognosis; displaying significantly increased overall and disease-free survival compared to other breast cancer subtypes (9, 63, 64). Treatment of early-stage Luminal A breast cancer is based mainly on hormonal therapies, with the addition of adjuvant chemotherapy dependant on the clinical stage. The immunohistochemical profile of Luminal A tumors is characterized by high expression of ER, PR, and luminal cytokeratins 8 and 18, an absence of HER2 expression, and low rate of proliferation measured through Ki67 (65, 66).

Luminal B tumors account for 15–20% of all breast cancers (65). Patients with Luminal B breast cancer have poorer outcomes from endocrine therapy, however, have a better response to chemotherapy, achieving pathological complete response (pCR) to neoadjuvant chemotherapy in 16% of tumors compared to 6% in Luminal A tumors (67). From the immunohistochemical point of view, Luminal B tumors are characterized by a lower expression of ER and PR, and higher Ki67 index, and display a higher histological grade, compared to Luminal A tumors (66). Like Luminal A tumors, they express luminal cytokeratins 8 and 18 (65, 66).

Human epidermal growth factor receptor 2-enriched tumors represent 15–20% of breast cancer subtypes (65). Patients with HER2-enriched tumors have poor prognosis and overall survival (9, 63, 64). The immunohistochemical profile of HER2-enriched tumors is characterized by variable ER or PR expression and overexpression of HER2 (66). Consequently, treatment of HER2-enriched tumors includes monoclonal antibodies which directly target the HER2 receptor given in conjunction with chemotherapy (68).

Basal-like tumors comprise 15–20% of all breast cancers (66), and are associated with an aggressive clinical behavior and a high rate of metastasis (69). Patients with Basal-like tumors have a poor prognosis, displaying lower overall and disease free survival compared to other subtypes (9, 63, 64). Treatment of basal-like tumors involves systemic chemotherapy. The immunohistochemical profile of basal-like tumors is characterized by a triple-negative phenotype; ER, PR, and HER2 negative.

Normal breast-like tumors account for 5–10% of all breast cancers (65). They lack the expression of ER, PR, and HER2, however, are not considered basal-like tumors as expression of basal cytokeratin 5 and EGFR is absent (66). However, normal breast-like tumors are poorly defined, and it is argued that they are an artifact of having a high percentage of normal cells in the tumor specimen (70, 71). It has been suggested that these tumors could be grouped into the recently discovered claudin-low subtype, which also displays basal-like characteristics, while also sharing biomarkers in common with normal-like breast epithelial cells. Similar to the basal-like subtype, claudin-low tumors have been associated with therapeutic resistance and poor survival outcomes (72), due to their highly migratory nature.

In clinical practice, identifying triple-negative and HER-2-positive breast cancers can be achieved with standard pathological testing, and recommendations for appropriate adjuvant therapy in early-stage disease are well defined. However, for patients with ER-positive and HER-2-negative disease, distinguishing between those with Luminal A disease and those with Luminal B disease is more challenging and has implications for treatment recommendations (73). Identifying those patients with good prognosis Luminal A disease who will have a small absolute benefit from adjuvant chemotherapy can avoid unnecessary chemotherapy and its associated side effects, while identifying those with Luminal B disease and a higher risk of relapse can prevent under treatment in this group (73, 74).

GENE-EXPRESSION PROFILING IN BREAST CANCER

In 2000, Perou et al. (62) proposed a new classification system for breast cancer subtypes, separating them into distinct subgroups based on gene-expression profiles, as opposed to protein expression signatures used in traditional subtyping methods. Intrinsic subtyping by gene-expression profiling is predictive of overall and relapse-free survival (8, 9, 63, 64, 75) and can predict the relative risk of relapse and the patient benefit from hormonal therapy and chemotherapy (71). Therefore, gene-expression profiling can be used to inform risk prediction and help guide treatment decisions, to decrease the number of patients receiving unnecessary treatment. The main genes associated with each subtype, together with pathological characteristics and prognosis, are summarized in **Table 2**.

Each subtype displays a distinct gene-expression profile. Luminal A tumors are characterized by a high level of ER, and as such display an increased expression in genes associated with ER function, such as *Bcl2*, *EsR1*, *PgR*, and *FOXA1* (62, 63). Compared to Luminal A, Luminal B tumors display an increase in expression of proliferative genes, and consequently possess a more aggressive phenotype, higher proliferative index, and worse prognosis (9, 63, 64, 67, 79). Like Luminal A tumors, Luminal B tumors also express genes associated with ER activation, including *Bcl2*, *FOXA1*, *CCND1*, and *GATA3* (79); however, increased expression of proliferative genes, such as *CCNB1*, *CCND1*, *CCNE1*, *MYBL2*, and *MKi67*, appears to be the hallmark of luminal B tumors (63, 64, 79).

HER-2-enriched tumors are characterized by a high expression of human epidermal growth factor receptor-2, *ErbB2*, and other genes associated with the HER2 pathway (65, 80). HER2 functions as a receptor tyrosine kinase and signals through PI3K/AKT/mTOR, JAK/STAT, MAPK and Ras/Raf pathways to promote cell survival, proliferation, and migration (66). Consequently, a dysregulation of signaling in HER-2-enriched tumors can lead to sustained proliferative signaling, a hallmark of cancer. HER-2-enriched tumors also overexpress *GRB7*, an adaptor protein involved in receptor tyrosine kinase signaling, which can also promote activation of PI3K/AKT/mTOR, JAK/STAT, and MAPK signaling pathways to allow for sustained

TABLE 2 | Summary of clinical and pathological characteristics, prognosis, and gene-expression changes of breast cancer subtypes.

		Biomarker profile					Prognosis				
Subtype	**Incidence (%)**	**ER**	**PR**	**HER-2**	**Ki67**	**Other**	**OS (%)**	**5 years DFS (%)**	**10 years DFS (%)**	**Gene-expression changes**	**Treatment**
Luminal A	50–60	+	+	–	Low	Luminal epithelial cytokeratins 8 and 18 Low histological grade	89–95	79–85	70–78	Increased expression in genes associated with ER function*: FOXA1*, *PgR*, *BCL2*, *EsR1*, *LIV1*, *ZIP6*, *SLC39A*, *XBP1*, *GATA3*, *ERBB3/4*, and TFF1	Hormonal therapies +/– chemotherapy
Luminal B	15–20	+	+	–	Mod	Luminal epithelial cytokeratins 8 and 18 High histological grade	71–85	60–75	50–60	Increased expression in genes associated with ER function*: FOXA1*, *PgR*, *BCL2*, *EsR1*, *GATA3* Increased expression of proliferative genes *CCNB1*, *CCND1*, *CCNE1*, *MYBL2*, MKI*67*, *v-MYB*	Poorer outcomes from hormone therapy (Low levels of HRs); better pCR to neoadjuvant chemotherapy
HER-2	15–20	–	–	+	High	Luminal cytokeratins	43–78	41–65	45–51	Amplification of *ERBB2* and GRB7 PI3K pathway activation (AKT, pS6, and p4EBP1) correlated with *INPP4B* and *PTEN* loss Increased expression of proliferative genes *BIRC5*, *CCNE1*, *CCND1*, *ORC6L*, *MYBL2*, *MKi67*	HER-2-targeted therapy and chemotherapy
Basal-like	15–20	–	–	–	High	Basal cytokeratins 5, 14, and 17 High EGFR	53–73	48–72	48–65	Increased expression of EGFR Dysregulation of MAPK/AKT/PI3K and Ras/Raf/ and JAK/STAT Increased expression of *FOXM1*, *cMYC*, *CCNE1*, *CCND1*, *CDC20*, *CDC6*, *BIRC5*, *ORC6L*	Chemotherapy Future: EGFR (Gefitinib/Cetuximab), VEGF, or AR inhibition
Claudin-low	12–14	–	–	–	Low	Low luminal markers and high mesenchymal markers	×	~67	×	Loss of tight junction proteins: *claudin 3*,*4*,*7*, *E-cadherin* and *CDH1* (and highest expression of transcript repressors of *CDH1 vimentin*, *SNAI1 and 2*, *TWIST1/2*, *and ZEB1/2*) Enrichment for EMT markers: *SNAI1/2*, *TWIST1/2*, *ZEB2*	Chemotherapy
Normal-like	5–10	–	–	–	High	Negative for CK5 and EGFR	~93	79–87	~85	Loss of tight junction proteins: *claudin 3*,*4*,*7*, *E-cadherin*	Chemotherapy
Reference	(65, 66)	(66)			(66)	(66)	(9, 67, 72, 76, 77)			(65, 66, 71, 72, 78)	(66)

The italics refers gene names.

proliferative signaling (66). HER-2-enriched tumors have increased expression of proliferative genes, including *BIRC5*, *CCND1*, *CCNE1*, *ORC6L*, and *MKi67*, and are often associated with a more aggressive and highly proliferative tumor (80).

Basal-like tumors express high levels of basal cytokeratins 5, 14, and 17, and do not express ER, PR, and HER2. Consequently, basal-like tumors cannot be treated with many conventional therapies, however, have a better response to chemotherapy compared to other subtypes. The EGFR is often overexpressed in basal-like tumors, where increased EGFR expression correlates with poor patient survival. Basal-like tumors display a dysregulation in PI3K/AKT, JAK/STAT, and ERK/MAP signaling pathways and a high expression of proliferative genes, such as *FOXM1*, *c-MYC*, *CCNE1 BIRC5*, and *CCND1* (66). In addition, basal-like tumors overexpress genes involved in the progression through the cell cycle (*CDC20*, *CDC6*) (66) and genes associated with the EGFR pathway (43). Absence of ER expression results in low expression of estrogen-related genes, *EsR1*, *Bcl2*, and *PgR* (66).

Claudin-low tumors are enriched for epithelial-to-mesenchymal transition markers, such as *SNAI1/2*, *TWIST1/2*, and *ZEB2* (72), with low expression of tight junction proteins, such as claudin 3, 4, and 7, E-cadherin, and *CDH1* (72). The claudin-low subtype is highly migratory and therefore has a poor prognosis (76).

PAM50

PAM50 is a list of 50 genes that classify breast cancers into one of five intrinsic subtypes from formalin-fixed, paraffin-embedded tissues by real time polymerase chain reaction (RT-PCR) (62). These 50 genes identified were refined from a list of 1,906 genes, which were found in four previous microarray studies. The list was minimized to genes that have passed previously established formalin-fixed paraffin-embedded (FFPE) performance criteria, and were further refined through statistical analyzes, allowing for identification of genes which showed a highest correlation to each intrinsic subtype. Differential gene expression between subtypes is shown by microarray in **Figure 3**. Subsequent studies have shown that classification of intrinsic subtypes using the PAM50 test retains the prognostic and predictive significance characteristic to breast cancer subtypes (9, 70, 74, 81, 82). Furthermore, several studies have shown that the PAM50 classification method provides better information on prognosis than immunohistochemistry-based surrogates (8, 9, 64). This suggests that subtyping by immunohistochemistry is inferior to genomic profiling, identifying a requirement for gene-expression profiling in a clinical setting.

Accurate testing of predictive biomarkers is important, as discrepancies between IHC and intrinsic classification of breast tumors may lead to differences in treatment decisions and patient outcomes. To address this, ER, PR, and HER2 concordance between immunohistochemistry and gene profiling has been investigated. A number of studies have reported a discordance of below 10% for ER and PR status (8, 9, 70, 83, 84). Cheang et al. evaluated concordance in subtype classification between PAM50 and immunohistochemistry, finding that of Luminal tumors as defined by PAM50, 8% did not stain positive for ER through immunohistochemistry (8). Consistent with this, Chia et al. identified 8% of Luminal tumors instead being classified as either HER-2-positive, or triple-negative through immunohistochemistry (9). As patients with ER negative or HER-2-positive tumors will receive chemotherapy, any discordances in classification of luminal tumors can have critical implications on treatment options, where women may receive unnecessary chemotherapy with no benefit.

Although several studies have reported high overall concordance in HER-2 expression between gene profiling and immunohistochemistry (85–88), others report low concordance (70, 81). Chia et al. (9) found that of HER-2-enriched tumors identified by PAM50, only 66% of these stained positive for HER2 expression by immunohistochemistry. Instead, 31% of HER2-enriched tumors were classified as Luminal A or B tumors, and 4% classified as basal-like through immunohistochemistry. Cheang et al. (8) also identified a low concordance of 69% in HER2 status between PAM50 and immunohistochemistry, with 6% of tumors instead classified as Luminal A tumors, and 16% as triple-negative.

As different subtypes each respond best to different treatments and the absolute benefit of adjuvant therapies depends on the risk of relapse, this poses the question; in discordant cases which result should be used to guide treatment decisions, and is it appropriate to deny a patient treatment that would otherwise be indicated by a different subtyping method? Studies by Chia et al. (9) and Cheang et al. (8) included only premenopausal women and described low concordance between immunohistochemistry and gene profiling. This low concordance may be due to the fluctuations in circulating hormones during the menstrual cycle and the relative effect of hormonal stimulation on gene versus protein expression. While it is believed that PAM50 is more reflective of the true biology of the tumor than protein-based immunohistochemistry, the paucity of data on premenopausal women makes it difficult to determine the efficacy of the PAM50 test compared to the traditional gold standard for tumor subtyping.

Prosigna is an *in vitro* diagnostic assay which is based on the PAM50 gene signature assay. The Prosigna test is performed on FFPE tissue and identifies the patient's risk of distant reoccurrences of disease; it aims to aid clinicians and patients in treatment decisions. The development of Prosigna from PAM50 is summarized in **Table 3**. Of note, the validation of the Prosigna test – necessary for FDA approval – was based on two clinical trials (the TransATAC and ABCSG-8 clinical trials) incorporating data from over 2,400 postmenopausal women enrolled in adjuvant aromatase inhibitor trials. Although studies have examined the use of PAM50 in managing adjuvant therapy in premenopausal breast cancer (8, 9), there have been no large-scale clinical trials into the efficacy of the Prosigna test for premenopausal women. Thus, the accuracy of the Prosigna test has never been properly validated in the context of the hormonal fluctuations that occur during the menstrual cycle in premenopausal women.

Oncotype DX

Oncotype DX evaluates the expression of 21 genes associated with tumor proliferation, invasion, and estrogen signaling (94) (**Table 4**). In 2004, Paik et al. selected 250 candidate genes from

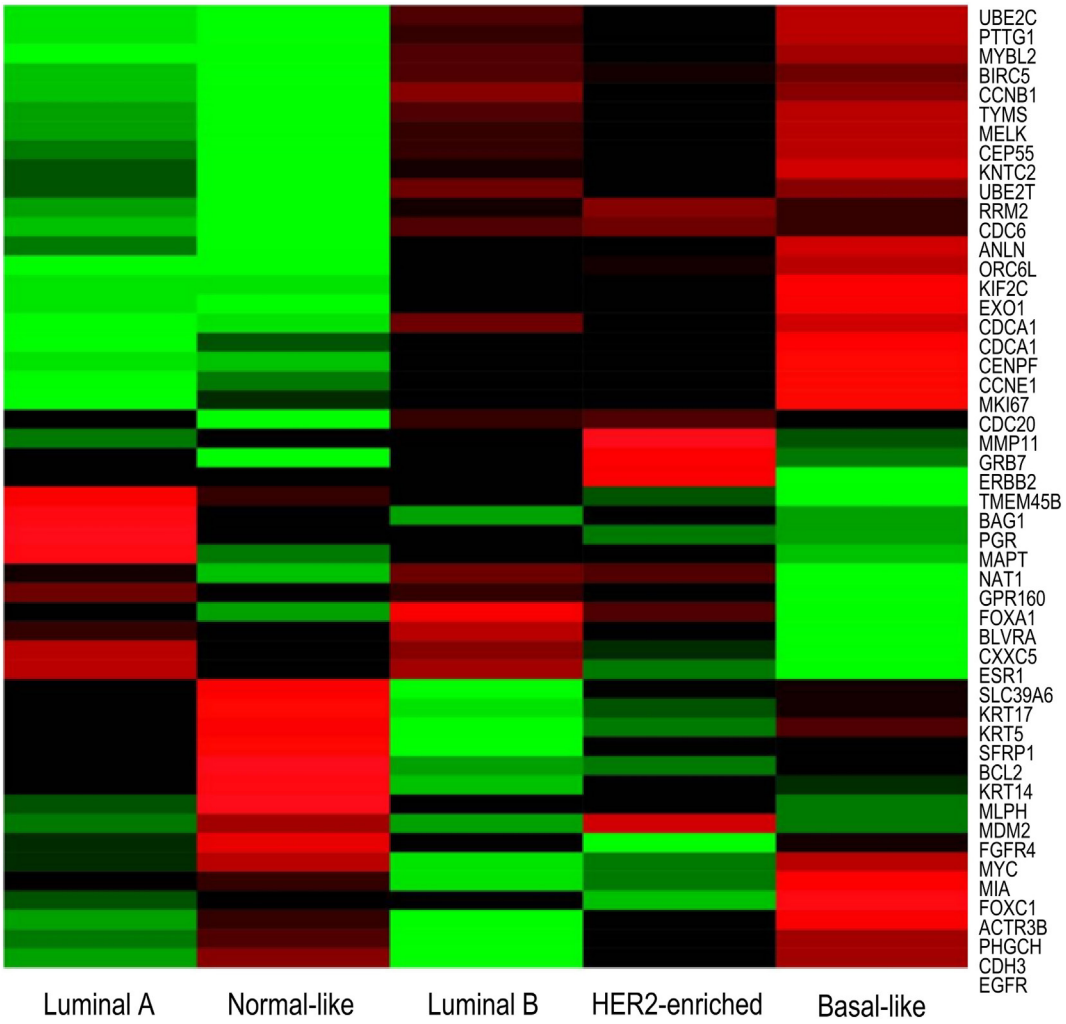

FIGURE 3 | Microarray heatmap of PAM50 genes expression in "intrinsic" breast cancer subtypes. Molecular profiles have distinct gene expression. Expression values of genes included in the PAM50 signature are shown as red/green according to their relative expression level for each subtype. Highest gene expression (red), lowest (green), and average (black) (71).

published literature and genomic databases that have been shown to be correlated with disease outcome. The list of genes was reduced to 16 genes, which showed the highest correlation to distant recurrence after 10 years. Relative expression of these genes, in relation to expression of five reference genes, provide a reoccurrence score which is significantly correlated with likelihood of breast cancer reoccurrence in 10 years (94–98). Oncotype DX, therefore, impacts adjuvant treatment decisions and influences treatment recommendations. The development of Oncotype DX is summarized in **Table 5**. Like the PAM50 gene set, the genes used in the Oncotype DX test rely heavily on genes related to ER and growth factor signaling and proliferation, which are differentially expressed by normal breast epithelial across the menstrual cycle, as discussed above.

EndoPredict

EndoPredict is an RT-PCR-based diagnostic test which evaluates the expression of eight proliferative and hormone receptor-associated genes. In conjunction with the tumors clinicopathological factors, it identifies the risk of distant metastasis within 10 years (105). EndoPredict is used to guide treatment decisions for both chemotherapy and hormonal therapy in ER-positive, HER-2-negative breast cancer.

The EndoPredict gene signature was identified from gene expression profiles of breast cancer samples taken predominantly from postmenopausal women (105). Similar to Prosigna, initial clinical validation of EndoPredict was based on two clinical trials (ABCSG-6 and ABCSG-8 clinical trials), which incorporated data exclusively from postmenopausal women, who were enrolled in aromatase inhibitor trials (105). In 2014, results of EndoPredict prognostic validity from a third clinical study were published. This study included both pre- and postmenopausal women, 54 and 46% of patients, respectively, and suggested that EndoPredict is prognostic in both pre- and postmenopausal women with breast cancer (106). The development of EndoPredict is summarized in **Table 6**. There are a lack of studies which validate the

TABLE 3 | Development of Prosigna, a PAM50-based subtype classifier.

Reference	Total	Menopausal status			Receptor status	
		Premenopausal	Postmenopausal	Unknown	ER +	ER−
Development of prosigna						
Parker et al. (71)	761	–	–	761	544	195
Neilsen et al. (81)	786	20	752	14	768	9
Bastien et al. (70)	154	49	101	4	100	49
Chia et al. (9)	398	398	0	–	291	107
Cheung et al. (8)	476	476	0	–	300	168
Martin et al. (89)	820	443	377	–	645	172
Liu et al. (90)	1094	757	337	–	638	456
Nielsen et al. (91)	43	–	–	15	43	0
Sestak et al. (92)	2137	0	2137	–	213	0
Wallden et al. (82)	746	91	433	222	547	177
Clinical validation of prosigna						
TransATAC (93)	1007	0	1007		1007	0
ABCSG-8 (74)	1478	0	1478		1464	17

Following the development of a 50-gene subtype classifier by Parker et al. in 2009, subsequent studies by the same group clinically and analytically validated the prognostic value of the 50-gene signature. TransATAC and ABCSG-8 trials provided evidence of the clinical validity of Prosigna. Currently in recruitment, is a study evaluating the treatment impact of Prosigna. The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

TABLE 4 | Panel of 21 genes used in the Oncotype DX assay to determine the risk of distant recurrence.

Proliferation	Invasion	HER-2	Estrogen	Other	Reference
Ki67	*MMP11*	*GRB7*	*ER*	*GSTM1*	*ACTB*
STK15	*CTSL2*	*HER-2*	*PgR*	*CD68*	*GADPH*
Survivin			*BCL2*	*BAG1*	*RPLPO*
CCNB1			*SCUBE2*		*GUS*
MYBL2					*TRFC*

Genes are grouped on the basis of function, correlated expression, or both. The recurrence score is derived from gene expression normalized to reference genes.

efficacy of EndoPredict in premenopausal women, and whether EndoPredict is an appropriate tool for guiding treatment decisions in premenopausal women has not yet been sufficiently investigated.

MammaPrint

MammaPrint is a diagnostic test which evaluates the expression of 70 genes associated with metastasis, proliferation, invasion, survival, and angiogenesis (113). The list of 70 genes was identified from whole-genome expression arrays, and selected for on the basis of those which were significantly correlated with disease outcome (113). Interestingly, MammaPrint does not measure expression of commonly used diagnostic markers ER, PR, or HER2.

Through the relative expression of these 70 genes, MammaPrint classifies tumors into high or low risk groups, which corresponds with patient's clinical outcome. Studies have shown that risk groups identified by MammaPrint correspond with patients overall survival (114), disease-free metastasis (75, 115), and the benefit from adjuvant chemotherapy (116, 117). Most studies validating the diagnostic capabilities of MammaPrint were small-scale retrospective studies, which included both pre and postmenopausal women, as summarized in **Table 7**. MammaPrint had initially been developed and validated in patients under the age of 55, suggesting that MammaPrint is targeted toward the younger population. Furthermore, the first prospective study which evaluated the impact of MammaPrint in assisting with treatment decisions included patients under the age of 55 (118, 119). As MammaPrint was developed in a younger population, and does not measure the gene expression of ER or PR, it is likely that MammaPrint is appropriate for diagnosing breast cancer in premenopausal women.

In 2016, results from a second prospective study were published, comparing MammaPrint to clinicopathological tools for selecting patients for adjuvant chemotherapy (124). The median age of the patients was 55 years. The study found that approximately 46% of patients, who were classified as high risk by clinicopathological features, were also classified as a low risk of metastasis by MammaPrint. Although these tumors presented with a high clinical risk, the results from the study suggested that these patients received no significant benefit from chemotherapy. Therefore, the authors concluded that using MammaPrint to guide treatment can reduce the number of patients receiving unnecessary chemotherapy.

Breast Cancer Index

The Breast Cancer Index is an RT-PCR based assay which classifies patients into risk groups to predict the likelihood of benefit from endocrine therapy, and the risk of early or late recurrence (125–128). The Breast Cancer Index evaluates two independent biomarkers; the HOXB13:IL17BR gene ratio, which is associated with endocrine therapy response (129), and the molecular grade index, which is determined by the expression of five proliferative-related genes (130). Classification of breast cancer through the expression of these seven genes aims to identify patients which are most likely to benefit from adjuvant therapy. The development of the Breast Cancer Index is summarized in **Table 8**. Similar to Prosigna, the clinical validation of the Breast Cancer Index was based on retrospective studies which used samples exclusively

TABLE 5 | The development of Oncotype DX, a 21-gene assay which identifies patient benefit from chemotherapy.

		Menopausal status		Receptor status	
Reference	**Total**	**Premenopausal**	**Postmenopausal**	**ER +**	**ER−**
Development of oncotype DX					
Paik et al. (94)	668	194	474	668	0
Esteva et al. (99)	149	122	27	103	46
Gianni et al. (100)	89	–	–	52	31
Habel et al. (101)	790	209	581	682	108
Albain et al. (102)	367	0	367	367	0
Clinical validation of oncotype DX					
NSABP B20 (97)	651	289	362	651	0
E2197 (103)	465	193	272	465	0
NSABP B14 (96)	1023	298	725	1023	0
TransATAC (93)	1231	0	1231	1231	0
Tailorx (104)	1623	480	1143	1621	5

Following the identification of 21-genes which showed high correlation to distant reoccurrence of breast cancer at 10 years, subsequent studies verified its predictive and prognostic value. The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

TABLE 6 | The development and clinical validation of EndoPredict.

		Menopausal status		
Reference	**Total**	**Premenopausal**	**Postmenopausal**	**Unknown**
Development of EndoPredict				
Filipits et al. (105)	964	245	589	
Muller et al. (107)	80	–	–	80
Dubsky et al. (108, 109)	1702	0	1702	
Muller et al. (110)	167	–	–	167
Martin et al. (106)	566	300	255	
Buus et al. (111)	928	0	928	
Clinical validation of EndoPredict				
ABCSG-6 (105)	1324	0	1324	
ABCSG-8 (105)	378	0	378	
GEICAM-9906 (112)	566	300	255	

The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

TABLE 7 | The development of MammaPrint.

		Menopausal status		
Reference	**Total**	**Premenopausal**	**Postmenopausal**	**Unknown**
Development and validation of MammaPrint				
van't Veer et al. (113)	97	66	31	
Van de Vijver et al. (75)	295	246	49	
Buyse et al. (115)	302	203	99	
Bueno-de-Mesquita et al. (118)	427	292	135	
Wittner et al. (120)	100	24	76	
Bueno-de-Mesquita et al. (114)	123	83	40	
Mook et al. (121)	241	125	116	
Mook et al. (122)	148	0	148	
Knauer et al. (117)	541	231	310	
Straver et al. (116)	167	119	39	9
Drukker et al. (119)	427	292	135	
Drukker et al. (123)	295	246	49	
Cardoso et al. (124)	6693	2226	4467	

The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

TABLE 8 | The development of the Breast Cancer Index.

Reference	Total	Menopausal status		
		Premenopausal	Postmenopausal	Unknown
Development and validation of the Breast Cancer Index				
Ma et al. (131)	80	2	78	
Ma et al. (130)	836	81	327	428
Jankowitz et al. (132)	265	80	185	
Jerevall et al. (128)	588	0	588	
Mathieu et al. (133)	150	66	84	
Sgroi et al. (126)	665	0	665	
Zhang et al. (125)	958	0	958	
Habel et al. (134)	608	162	446	
Sanft et al. (135)	96	13	76	
Sgroi et al. (127)	292	0	292	

The numbers of pre- and postmenopausal women included in the studies are indicated. In studies where menopausal status was not given, women under the age of 50 were defined as premenopausal and women over the age of 50 as postmenopausal.

from postmenopausal women and, therefore, results cannot be generalized to premenopausal women.

HORMONAL MODULATION OF BREAST CANCER BIOMARKERS

The abundance of ER fluctuates in normal breast tissue during the menstrual cycle, with ER positivity higher during the follicular phase, compared to the luteal phase (136, 137). Similarly, ER positivity in breast cancers is significantly higher in the follicular phase, when progesterone is absent, compared to the luteal phase (138, 139). This suggests that hormonal fluctuations during the menstrual cycle alter expression of hormone receptors and are highly likely to affect expression of genes associated with hormone signaling.

If menstrual cycle stage affects hormonal receptor signaling and the expression of genes and proteins used in subtype diagnosis, it is expected that there would be discrepancies between diagnostic and surgical breast cancer samples, which would be taken from the woman at different times and, therefore, different stages of the menstrual cycle. However, studies on concordance between biopsy and surgical samples have not specifically investigated premenopausal women. In 2013, Dekker et al. assessed the concordance of ER and HER2 expression between core needle biopsy and surgical resections (140). A concordance of ER was found in 99.1% of patients, and in 96.4% of cases for HER2. The menopausal status of women was not specified in the study; however, the mean age of women enrolled was 63 years old, which suggests that a majority of patients were postmenopausal. In a pooled study of 2507 invasive breast tumors, predominantly from postmenopausal women, concordance of ER was found for 93.4% of patients, and 97.8% for HER2 (140). This high concordance in receptor status led the authors to conclude that ER and HER2 status can be reliably determined from the core needle biopsy. However, these studies focused primarily on postmenopausal women, and the effect of premenopausal factors was not investigated.

In addition to studies investigating concordance in traditional biomarkers, several studies have evaluated the gene expression changes between core biopsies and surgical excisions (141–143). In 2012, Riis et al. compared whole gene-expression profiles of 13 women (1/13 premenopausal; 12/13 postmenopausal) and identified 228 genes differentially expressed, a majority of which are immunoregulatory or stress related (142). Two genes from the PAM50 signature gene list had differential expression between samples; *GRB7* and *ACTR3B*. From the Oncotype DX test, only one gene showed differential expression, *GRB7*. Additionally, Jeselsohn et al. compared gene expression between core biopsies and surgical excisions in postmenopausal women with ER-positive breast cancer (141). The authors identified significant changes in the expression level of 14 genes, a majority of which are immunoregulatory. Two genes involved in the Oncotype DX test and PAM50 intrinsic classification, *MYC* and *CCNB1*, showed differential expression between core biopsies and surgical excisions. A recent study utilized a genome-wide approach to determine gene expression changes between core biopsies and surgical excisions. The authors collected 56 paired core-cuts from postmenopausal breast cancer patients, and classified tumors into one of the five intrinsic subtypes based on the PAM50 gene signature (143). No systematic differences in categorization of the tumors into intrinsic subtypes were identified; however, discordances were identified between the Luminal A versus Luminal B subtype. While these studies have generally found good concordance between diagnostic and surgical samples, concordance in premenopausal women has not been specifically investigated.

A change in biomarker status can have important clinical consequences for adjuvant treatment. Studies evaluating the concordance of hormone receptors and gene expression profiles between core biopsies and surgical excisions suggest that biopsies taken at diagnosis are representative of the whole tumor. However, these studies were performed predominantly again in postmenopausal women, and it is possible that hormonal fluctuations in premenopausal women may alter the expression of these biomarkers. As such, the biopsy taken at diagnosis or during surgery may be influenced by fluctuating concentrations of estrogen and progesterone and, therefore, not represent the true tumor profile. This can lead to an incorrect

diagnosis and risk prediction and sub-optimal treatment of premenopausal women.

A recent study compared the expression of estrogen-related genes between pre- and postmenopausal women with ER-positive breast cancer (144). It was suggested that the different hormonal environments of pre- and postmenopausal women may affect the biological characteristics of the breast tumor. The authors found that expression of estrogen-related genes PgR, TFF1, and GATA3, were significantly higher in premenopausal women compared to in postmenopausal women. Consistent with this, studies have also shown that expression of estrogen-regulated genes is significantly associated with the level of estrogen in the blood (145, 146). It is likely that the fluctuating concentration of estrogen during the menstrual cycle affects the expression of these estrogen-related genes. In 2013, Haynes et al. compared the expression of estrogen-related genes between women at different stages of the menstrual cycle (145). They found that the expression of key estrogen-related genes was highest during the follicular phase of the menstrual cycle when estrogen concentration peaks. However, it remains unknown how menstrual cycling can affect the expression of these genes within the same tumor. As Oncotype DX and Prosigna rely heavily on the expression of estrogen-related genes for diagnosing breast cancer, changes in expression of these genes across the menstrual cycle may affect the diagnosis of breast cancer by these tests.

Despite the known role of estrogen and progesterone on the function of the breast and on breast cancer risk, the effect of menstrual cycling on breast tumors remains unknown. In support of the possibility that menstrual cycle critically affects the gene-expression profile of breast cancers, a recent *in vitro* study has suggested that the combination of estrogen and progesterone results in the switching from a Luminal A to Basal-like intrinsic subtype in breast cancer cells, and increases the Oncotype DX Recurrence Score (43) compared to estrogen treatment alone. Tests that utilize gene expression profiling in breast cancer classification were developed and validated from studies predominantly in postmenopausal women, and there is a scarcity of research on how applicable these biomarkers are to premenopausal women, and the extent to which this impacts on treatment response. It is important to understand how hormonal fluctuations affect predictive and prognostic biomarkers, to provide premenopausal women with the optimal treatment for their individual cancer.

CONCLUSION

Breast cancer clinics are increasingly adopting gene expression profiling to subtype tumors and identify the best therapies. However, despite their availability to young women, such tests were largely developed and validated in postmenopausal women – patients in whom fluctuations in estrogen and progesterone associated with the menstrual cycle are absent. Yet, these hormones are highly likely to affect breast cancer gene expression in premenopausal women – and the diagnosis and treatment trajectories that stem from its measurement – could fundamentally depend on a patient's menstrual cycle stage at the time of tissue sampling. Leading diagnostic tests harness intrinsic subtyping of breast cancers, but whether these tests are accurate for premenopausal women remains a startlingly open question. Quite simply, young women may be at risk of receiving inaccurate subtype diagnoses; with ramifications spanning inaccurate prognoses, suboptimal and unnecessary treatments, and reduced survival.

AUTHOR CONTRIBUTIONS

All authors contributed intellectually to the development and writing of the manuscript.

FUNDING

This work was supported by the Hospital Research Foundation (THRF), the Australian National Breast Cancer Foundation (NBCF), and TQEH Haem/Onc Scheme A. WI is an NBCF/THRF Fellow.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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