Cell-free plasma markers of breast cancer in young women and women at high risk

Thesis submitted for the degree of

Doctoral of Philosophy at the University of Leicester

by

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July 2016

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Abstract

Breast cancer in younger women can have an aggressive course. In pre-menopausal women (<50 years in age), mammography has limited value due to higher breast density. Therefore, there is a need for early and accurate detection to improve patient outcomes. The aim of this thesis was to investigate specific microRNAs (miRNAs) and copy number variations (CNVs) in plasma cell free DNA (cfDNA) as circulating biomarkers in the blood of women who have developed breast cancer at a young age (<50 years) and compare results between breast cancer patients and healthy controls. Candidate miRNAs were selected from TaqMan array card data and quantitative real-time polymerase chain reaction (qPCR) was used to investigate miRNA profiles in patient samples and controls. For plasma cfDNA CNV was compared at key chromosome intervals/genes and results compared with matched normal lymphocytes (as a germline DNA control) using both qPCR and droplet digital PCR (ddPCR) techniques. Both markers were compared in a cohort of women from a family history clinic (97) including 53 women from either *BRCA1* or *BRCA2* family and 44 women with a family and/or personal history of breast cancer as well as 12 healthy controls.

MiRNA profiling showed that five miRNAs (mir-26a, mir-27b, mir-130b, mir-324-3p, and mir-181a) might be useful in monitoring women at high-risk due to *BRCA* mutation, on follow-up after breast cancer surgery. Similarly CNV was identified in cfDNA in five genes (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2*) in women who had had previous surgery for *BRCA1/ BRCA2* breast cancer, but not in *BRCA1/BRCA2* carriers or healthy controls.

The circulating biomarkers (miRNAs and gene specific amplification) identified in the plasma may have use in monitoring women with breast cancer due to inherited *BRCA* mutation after surgery and adjuvant therapy.

Acknowledgements

First and foremost, I give thanks and praise to ALLAH who generously helps me to achieve my dreams and who gave me the ability to successfully complete this project.

I am indebted to my country and my former King, His Royal Highness, the Late King Abdullah bin Abdul-Aziz, and King Salman bin Abdul-Aziz, Custodian of the Two Holy Mosques, the sponsor and generous benefactor of generations of our nation's youth.

In the preparation of this thesis I extend my sincere gratitude to my supervisor, Prof. Jacqueline Shaw, who has been patient; advising and guiding me, as well as providing me with the help I needed. I am eternally grateful for the calmness she gave me to make some sense of the confusion experienced at times throughout my project work. In particular I would also like to thank Dr Shona Elshaw, Dr David Guttery, and Dr Karen Page for their generous support, especially in my miRNAs study. They always responded to my queries and questions instantly and cared about my results during my research. I would also like to extend my greatest thanks to Dr Daniel Fernandez for his support and guidance, as he was always available to answer any question I had during my work with CNV analysis in cfDNA. Special thanks also go to Lindsay Primrose for her help, patience and support she gave me in getting started and for teaching me the principal techniques in the lab. In addition, I am indeed grateful to my colleagues Dr Ottolini Barbara, Dr Abbie Fairs, Dr Patricia Adamo, Dr Howard Pringle, Ms Joan Riley, Miss Vilas Mistry, Dr Basma Rghebi, Ricky Trigg, and Luke Martinson for their assistance, and full support while working in the lab and to all members of staff at Leicester University who offered guidance and support.

Most of all, I cannot find words to express my gratitude to my parents, sisters and brothers for their patience, continual encouragement, valuable assistance and fruitful collaboration as I completed my study, and even more importantly, for taking care of my son, Mohammed. I must also express my appreciation to my husband, Nader Wasfi, for his encouragement and motivation in improving my knowledge and advancing my education throughout my years of study.

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List of Abbreviations

ANOVA	Analysis of variance
ATM	Ataxia telangiectasia mutated gene
BC	Breast Cancer
BCL2	B-cell CLL/lymphoma 2
BMI	Body max index
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
BRIP1	BRCA1 interacting protein C-terminal helicase 1
bp	Base pair
CCND1	Cyclin D1
CDKs	Cyclin dependent kinases
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary deoxyribonucleic acid
cfDNA	Circulating free DNA
CHEK2	Checkpoint kinase 2
CN	Copy number
CNV	Copy number variation
CT	Cycle threshold
CT	Computed tomography
CYP19A1	Cytochrome P450 family 19 subfamily A member 1
DCIS	Ductal carcinoma in situ
ddPCR	Droplet digital PCR
DFS	Disease free survival
DMEM	Dulbecco's Minimal Essential Medium

DMSO	Dimethylsulphoxide
DMXL2	Dmx-Like 2
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
FGFR1	Fibroblast growth factor receptor 1
FISH	Fluorescence in situ hybridisation
FCS	Foetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HER2	Human epidermal growth factor receptor 2
HGDNA	Human Genomic DNA
HIF1A	Hypoxia-inducible factor 1alpha
HRT	Hormone replacement therapy
IHC	Immunohistochemistry
IDC	Invasive ductal carcinoma
kb	Kilo base pairs
kDa	Kilodalton
ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma in situ
LDM	Low-dose metronomic
LOH	Loss of heterozygosity
MCL1	Myeloid Cell Leukemia 1
MDR	Multidrug resistance

$MgCl_2$	Magnesium Chloride
Mg	Milligram
ml	Millilitre
µl	Microliter
mRNA	Messenger Ribonucleic acid
MSI	Microsatellite instability
MTD	Maximum tolerable dose
ng	Nanogram
LKB1	Liver Kinase B1
OS	Overall survival
PALB2	partner and localizer of BRCA2
PBS	Phosphate buffered saline
PBX1	Pre-B-Cell Leukemia Homeobox 1
PI3K	Phosphatidylinositol-3-kinase
PCR	Polymerase chain reaction
PR	Progesterone receptor
P53	Protein 53 or tumour suppressor protein 53
PTEN	Phosphatase and tensin homolog gene
qPCR	Quantitative real-time polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SD	Standard deviation
SEM	Standard error of the mean
SNPs	Single nucleotide polymorphisms

STK11	Serine/threonine kinase 11
TE	Tris EDTA
TN	Triple-negative
TNBC	Triple negative breast cancer
TNC	Tenascin C
TSP-1	Thrombospondin-1
UP	Ultra pure
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Introduction to breast cancer

Breast cancer is one of the most common cancers in women in the UK (CRUK, 2014), accounting for 30% of all cancers. Despite the fact that breast cancer rarely occurs in women under the age of 50, the incidence of breast cancer is quite high (about 2-5%) in young women <35 years of age. A good percentage of patients (15-25%) are in their thirties or forties at the time of diagnosis of breast cancer (Han et al., 2011; DeSantis et al., 2014). In familial breast cancer, an inherited mutation in the *BRCA1* or *BRCA2* gene increases the probability of women getting breast cancer at a younger age (<50 years) (Metcalfe et al., 2008). In England alone, 51,103 new cases were reported in 2015 and around 12,000 women died from breast cancer every year (CRUK, 2014; Breast Cancer Care, 2015; Lawrence et al., 2015). The survival rate has somewhat increased due to the early detection and accurate characterisation combined with improved treatments, newer and more efficient drugs. However, the mortality is still high for patients in certain populations (Tang, 2013). In 2008, about 450,000 women were diagnosed with breast cancer in Europe of which 140,000 deaths were reported, whereas, 68,000 women were diagnosed in Africa corresponding to 37,000 deaths (Ferlay et al., 2013).

Breast cancer manifests in either the ducts that transport milk to the nipple or the lobules (Figure 1.1), which are the milk-producing sections of the breast (Richie and Swanson, 2003). It is a collection of various clinical and pathological features and risk factors that originate in the terminal duct-lobular unit in the breast. It is characterised by the uncontrollable multiplication of abnormal cells in the breast (Alizart et al., 2012).

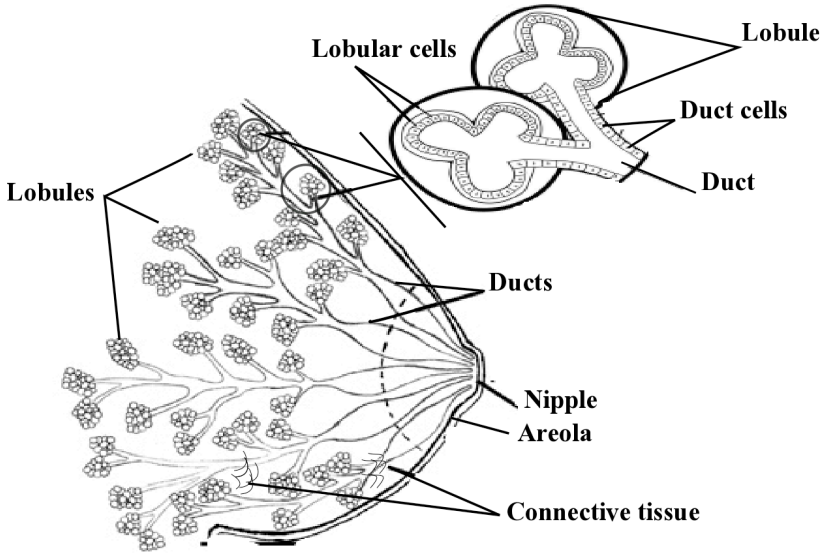


Figure 1.1 Anatomy of the female breast

Adapted from American Cancer Society, 2015.

1.2 Risk factors for breast cancer

In the UK, breast cancer is the most prevalent cancer, with one in eight women likely to receive a breast cancer diagnosis (CRUK, 2014).

With regard to the risk factors involved in breast cancer, age, geographical variation, and diet among others make a significant contribution to the cases of this disease all over the world. Furthermore, family history, diet, body weight, excessive consumption of alcohol, and exposure to ionising radiation also make a contribution to the rise of breast cancer patients (Table 1.1). With regard to the above listed risks, some have high chances of leading one to getting the disease, whereas others have minimal chance. It is with this information in mind that there are main and minor risk factors of breast cancer (Basil, 2008; McPherson et al., 2000).

Table 1.1 Factors increase relative risk of breast cancer in women

The correlation between lifestyle risk factors, breast cancer subtypes and reproductive, Life After Cancer Epidemiology (LACE) study n=(1,821). HRT (hormone replacement therapy); BMI (body max index) (reproduced with permission from Kwan et al., 2009) (see Appendix 3).

Risk Factors	Luminal A (comparison)	Luminal B			Triple Negative			HER2-overexpressing		
	n	n	OR	95% CI	n	OR	95% CI	n	OR	95% CI
Age at diagnosis (years)										
≥65 (Ref)	444	66	Ref	---	43	Ref	---	18	Ref	---
50-64	621	90	0.97	0.69, 1.36	74	1.18	0.79, 1.75	35	1.38	0.77, 2.47
<50	260	69	1.71	1.18, 2.49	82	3.23	2.15, 4.84	15	1.36	0.67, 2.76
Test for trend				P=0.001			P≤0.0001			P=0.35
Race/ethnicity										
White (Ref)	1065	166	Ref	---	149	Ref	---	50	Ref	---
African American	65	11	1.00	0.52, 1.95	27	2.68	1.64, 4.38	3	0.95	0.29, 3.15
Hispanic	89	20	1.35	0.81, 2.27	13	0.86	0.46, 1.60	6	1.39	0.58, 3.33
Asian	84	23	1.60	0.97, 2.62	7	0.52	0.23, 1.15	6	1.48	0.62, 3.56
Other	22	5	1.38	0.51, 3.71	3	0.87	0.25, 2.99	3	2.81	0.81, 9.73
Menopausal status										
Postmenopausal (Ref)	895	129	Ref	---	99	Ref	---	43	Ref	---
Premenopausal	256	70	1.52	0.92, 2.52	66	1.08	0.64, 1.84	10	0.62	0.23, 1.63
Family History										
No (Ref)	1043	189	Ref	---	158	Ref	---	60	Ref	---
Yes	282	36	0.73	0.50, 1.07	41	0.99	0.68, 1.44	8	0.50	0.24, 1.06
Age at first full-term pregnancy (years)										
Nulliparous (Ref)	226	37	Ref	---	38	Ref	---	10	Ref	---
<26	710	128	1.30	0.87, 1.96	115	1.23	0.82, 1.87	45	1.55	0.76, 3.18
≥26	387	60	1.01	0.64, 1.58	46	0.86	0.54, 1.38	13	0.76	0.33, 1.77
Parity										
Nulliparous (Ref)	226	37	Ref	---	38	Ref	---	10	Ref	---
1-2 children	586	109	1.21	0.81, 1.83	93	1.07	0.70, 1.62	27	1.04	0.50, 2.21
≥3 children	513	79	1.13	0.73, 1.75	68	1.11	0.71, 1.75	31	1.53	0.72, 3.25
Lifetime duration of breastfeeding										
Never (Ref)	637	108	Ref	---	110	Ref	---	37	Ref	---
0-3 months	177	36	1.24	0.82, 1.88	27	0.96	0.61, 1.54	8	0.78	0.36, 1.71
≥4 months	489	72	0.81	0.59, 1.12	61	0.67	0.48, 0.95	22	0.75	0.43, 1.29
Alcohol use										
Never (Ref)	481	97	Ref	---	71	Ref	---	29	Ref	---
Ever	624	90	0.72	0.52, 1.00	91	0.97	0.69, 1.38	30	0.83	0.48, 1.42
Smoking history										
Never (Ref)	645	126	Ref	---	107	Ref	---	34	Ref	---
≤10	185	29	0.80	0.52, 1.25	28	0.82	0.52, 1.30	5	0.51	0.19, 1.33
11-19	96	14	0.79	0.44, 1.44	12	0.75	0.39, 1.43	5	1.04	0.39, 2.73
≥20	386	55	0.81	0.57, 1.16	50	0.87	0.60, 1.26	23	1.22	0.69, 2.13
HRT (postmenopausal only) [b]										
Never (Ref)	193	40	Ref	---	27	Ref	---	15	Ref	---
Ever	682	85	0.58	0.38, 0.87	71	0.72	0.45, 1.18	28	0.53	0.27, 1.03
Oral contraceptive use										
Never (Ref)	501	90	Ref	---	61	Ref	---	20	Ref	---
Ever	778	123	0.72	0.52, 0.99	132	0.92	0.65, 1.32	47	1.50	0.84, 2.69
BMI (kg/m^2) [b]										
<25 (Ref)	600	113	Ref	---	77	Ref	---	30	Ref	---
25-29	395	59	0.89	0.63, 1.26	69	1.59	1.10, 2.28	22	1.20	0.67, 2.14
≥30	315	49	0.93	0.64, 1.35	51	1.35	0.90, 2.02	16	1.09	0.58, 2.07

1.2.1 Main risk factors

The main risk factors are simply the key causes of breast cancer. In other words, with the slightest exposure to these factors, one remains at a high-risk of getting the disease. According to Basil (2008), the risks of getting the disease vary with different groups i.e. the exposure of a given group to a given risk may either result in the individual getting the disease or not. Therefore, a risk factor remains relevant to a certain group and not all the other groups.

1.2.1.1 Age at menarche and menopause

Age falls in the category of the main risk factors leading an individual to getting breast cancer. The increase in the age of an individual increases the individual's vulnerability to getting breast cancer (Figure 1.2). The risk of a young person getting breast cancer before menopause remains low compared to the elderly. To the young people, who have not reached menopause, the risk of getting breast cancer doubles after every ten years (CRUK, 2014; Sestak et al., 2012). However, in most countries of the world, after menopause, the cases of one getting the disease remain constant (Chaka, 2015).

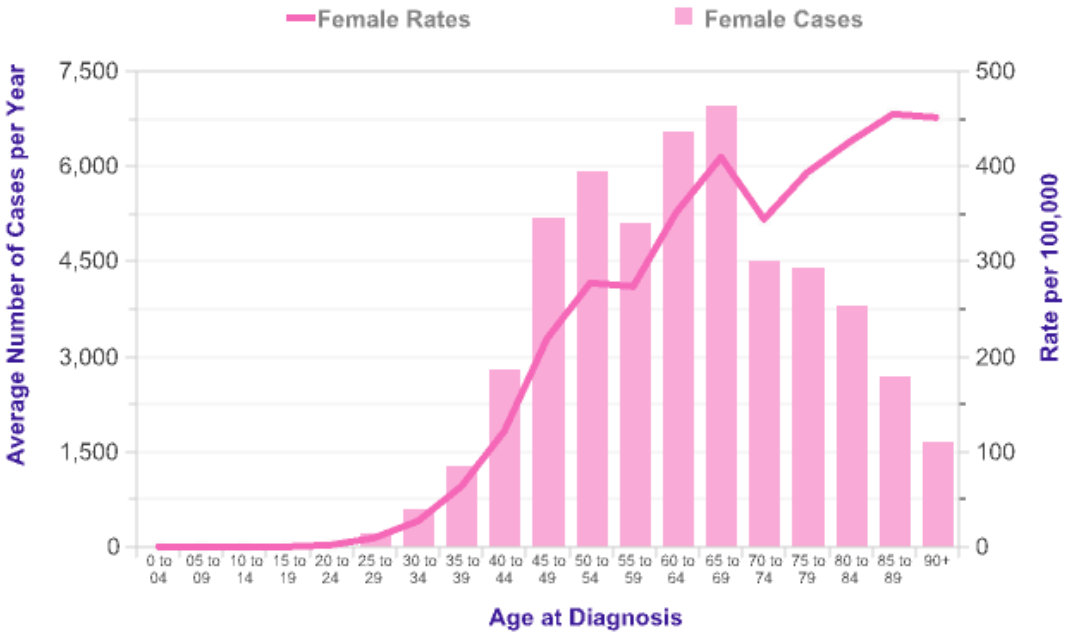


Figure 1.2 Breast cancer incidence by age

Age-Specific Incidence rates per 100,000 females and average number of breast cancer cases per year in UK (from CRUK, 2016).

http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer/incidence-invasive#heading-Zero

1.2.1.2 Weight and diet

Diet plays a chief role in increasing the risks of a person getting the disease. High intake of saturated foods contributes very much to the possibility of an individual getting breast cancer (Bingham, 2003; CRUK, 2014). With regard to the difficulties involved in a changing society and culture in terms of their eating habits, therefore, diet remains a chief risk factor in breast cancer. Furthermore, the gain in weight by more than ten kilograms at the age of eighteen in an individual increases the risks of one getting breast cancer (Sariego, 2010; Wright et al., 2015).

1.2.1.3 Radiation

Exposure to ionising radiation remains an important risk factor in breast cancer, more so, on teenagers. People, or rather, teenagers (<20 years) exposed to radiation remain the highest sufferers of breast cancer (Lanfranchi and Brind, 2007; Kennedy and Boughey, 2013; Jardines et al., 2015). According to Bilimoria and Morrow (1995), ionising radiation affects elderly women more than the teenagers. This is whereby; it may remain to be dormant at the times of exposure only to appear at later stages in life. In addition, with the mammographic screening, there is a significant decrease in the mortality cases arising from breast cancer.

1.2.2 Minor risk factors

Minor risk factors of breast cancer are simply the causes of breast cancer, which do not contribute to the high cases of the disease, in the world. In other words, there are few people who get breast cancer because of the minor risk factors (American Cancer Society, 2015; Basil, 2008). In addition, the minor risks at some point can be curbed and weakened thus rendering them incapable in enhancing the spread of the disease. For instance, the geographical factors remain a minor contributor to the cases of breast cancer. Studies of migrants from Japan to Hawaii showed that the rates of breast cancer of migrants assumed those of the host country within the first two generations (Sariego, 2010).

1.2.2.1 Family history

The overall relative risks with breast cancer increase if a woman has a family history of breast cancer. Having one first-degree relative (mother, daughter, or sister) with breast cancer, the risk is about twice as high, whilst having two and three or more first-degree relatives the risk is increase by about three and four times, respectively (American Cancer Society, 2015).

1.2.2.2 Genetic factors

Genetics play a limited but important role as a risk factor in breast cancer. According to Jardines et al., (2015), only about 5% to 10% of breast cancer cases are considered hereditary. Younger women who are diagnosed with advanced breast cancer are more likely to have genetic alterations (see 1.1) in certain genes including *BRCA1* and *BRCA2* (Anders et al., 2009). *BRCA1* and *BRCA2* genes located on the long arms of chromosomes 17 and 13 contribute substantially to high-risk in families to getting breast cancer (Hill et al., 1997; Jardines et al., 2015). Penetrance of germline alterations was discovered in a variety of heritable diseases including breast cancer (Shawky, 2014). Cooper et al., (2013) defined penetrance as "the percentage of individuals having a particular mutation or genotype who exhibit clinical signs or phenotype of the associated disorder or genotype". Women who have a positive chance of developing breast cancer are estimated to have a 50% to 85% lifetime risk, while the risk of developing ovarian cancer is around 15% to 65%, beginning at age twenty-five (Richie and Swanson, 2003). The penetrance of *BRCA1* and *BRCA2* can be modified by a woman's reproductive history, according to some studies (Jernstrom et al., 1999; Rebbeck et al., 2001; Burke and Austin, 2002). Burke and Austin (2002) reported that environmental factors play an additional role in identifying penetrance of *BRCA* genes. Other studies have found that the risk of cancer is relatively higher in younger women who are positive for *BRCA* mutations than in older women (Nelson et al., 1993; de Sanjose et al., 2003; Metcalfe et al., 2008; Pruthi et al., 2010). Table (1.2) shows a list of the hereditary cancer syndromes correlated with breast cancer.

Table 1.2 Breast cancer susceptibility genes

Based on Ripperger et al., 2009; Balmana et al., 2010; Apostolou and Fostira, 2013.

Gene	Cancer syndrome	Associated tumours
	High-penetrance mutations	
BRCA1	Breast/ovarian predisposition	Breast, ovarian, bowel, prostate
BRCA2	Breast/ovarian predisposition	Breast (including male), ovarian, prostate, pancreatic
TP53	Li Fraumeni syndrome	Childhood sarcoma, brain, leukaemia, adrenocortical carcinoma, early-onset breast
PTEN	Cowden's syndrome	Breast, gastrointestinal, thyroid, endometrial (benign and malignant)
STK11/LKB1	Peutz-Jeghers syndrome	Breast, gastrointestinal, pancreatic, ovarian
	Moderate–penetrance mutations	
ATM	Ataxia telangiectasia	Non-Hodgkin lymphoma, ovarian, breast (in heterozygote carriers)
CHEK2	*CHEK2*- related	Breast, colorectal, ovarian, bladder
PALB2	*PALB2*-related	Breast, pancreatic, ovarian, male breast cancers.
BRIP1	Moderate risk breast/ovarian cancer	Breast, ovarian

1.2.2.3 Hormones

Hormonal history is considered a risk factor, since one relative risk of breast cancer results from cumulative exposure to oestrogen and progesterone (Begg et al., 1987; Pike et al., 1979). Li et al., (2003) stated that women using unopposed oestrogen replacement therapy (ERT) had no significant increase in the risk of breast cancer. However, use of combined oestrogen and progesterone HRT had an overall 1.7-fold (95% confidence interval (CI)) increased risk of breast cancer, including a 2.7-fold (95% CI) increased risk of invasive lobular carcinoma (ILC), a 1.5-fold (95%) increased risk of invasive ductal carcinoma (IDC), and a 2-fold (95% CI) increased risk of oestrogen receptor-positive/progesterone receptor-positive (ER+/PR+) breast cancers.

1.2.2.4 Breast density

Though the epidemiology of breast density remains poorly understood, high breast density is a key risk factor for breast cancer (Sestak et al., 2012; Willett, 2009). With

regard to this, nulliparity, late age at first birth, younger age, and lower body mass remain to be the major epidemiological factors influencing breast density.

1.3 Histopathological classification of breast cancer

As highlighted in the introduction, breast cancer manifests in either the breast lobules or ducts. Ductal breast cancer affects the ducts or tubes that transport milk from the tissues to the nipple. The majority of breast cancers are of the ductal type (Gokmen-Polar and Badve, 2012). By contrast, lobular carcinoma affects the lobules, which produce milk. In some instances, scientists term breast cancer as invasive when it affects the ducts, lobules and other breast tissues, as well. In situ or non-invasive breast carcinoma is evident in cases where the cancer concentrates around the ducts or lobules and has not spread to the other breast tissues as shown in Figure 1.3 (Breast Cancer Care, 2012; Richie and Swanson, 2003).

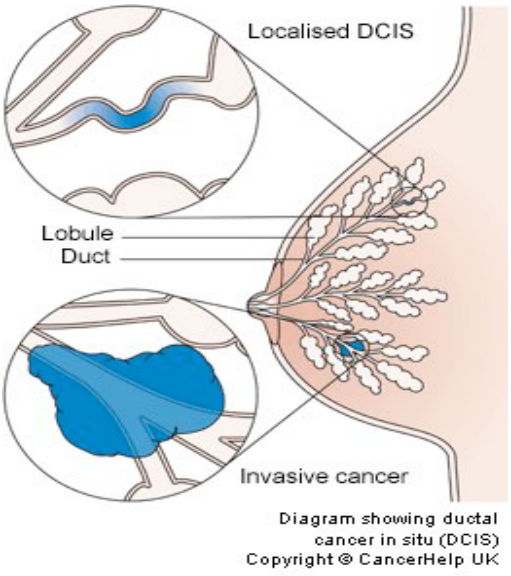


Figure 1.3 Invasive and non-invasive ductal carcinoma (from CRUK, 2014)

http://www.cancerresearchuk.org/about-cancer/type/breast-cancer/about/types/invasive-ductal-breast-cancer

1.3.1 In-situ breast carcinoma

1.3.1.1 Ductal carcinoma in-situ

Intraductal or ductal carcinoma in situ (DCIS) is the term doctors give to breast cancer that manifests in the ductal lining, but not in surrounding tissues. If untreated, however, it could advance into invasive cancer. It is usually discovered accidentally during mammography as a calcification confined to the breast tissue. The malignant cells in DCIS are usually ER-negative (ER−) and Human epidermal growth factor receptor 2 positive (HER-2/neu+ also known as HER2+) (Burstein et al., 2004; Breastcancer.org, 2015).

1.3.1.2 Lobular carcinoma in-situ

Lobular carcinoma in situ (LCIS) is an indicator of a higher risk of invasive cancer affecting one or both breasts (Lakhani et al., 2006). It does not contain calcification, which makes its diagnosis much more difficult on mammogram compared with DCIS. Furthermore, these cells usually express ER and PR and they are almost always negative for HER-2/neu (Li et al., 2006; Lakhani, et al., 2006).

1.3.2 Invasive breast carcinoma

1.3.2.1 Invasive ductal carcinoma

Invasive ductal carcinoma (IDC) is the most common type of breast carcinoma (75%) (Wasif et al., 2010; Breast Cancer Care, 2016). This type of invasive breast cancer is also known as ‘no special type’ (InvC, NST) or ‘invasive carcinoma not otherwise specified’ (InvC, NOS) (CRUK, 2014). These tumour cells have no specific pattern for cell arrangement microscopically like the others and it is regarded as the most aggressive one and usually carries a poor prognosis (Kumar et al., 2014).

1.3.2.2 Invasive lobular carcinoma

Invasive lobular carcinoma (ILC) is responsible for 10% of invasive breast cancers (CRUK, 2014). It is characterised by its specific microscopic features where the cancer cells are arranged in linear patterns called "Indian files". It is further characterised by its bilaterality, therefore some surgeons when finding ILC in one breast prefer to do bilateral prophylactic mastectomy (Fisher et al., 1977; Cha and Weidner, 1996; Kufe et al., 2003). Ellis et al., (1992) have found that the classical, tubulolobular, and lobular mixed types of this tumour had a better overall prognosis than ductal carcinomas.

1.3.2.3 Special types of invasive breast carcinoma

Invasive types of breast carcinomas are characterised by their specific histological criteria that class them as special types, and account for about 25% of invasive breast carcinomas. Special types of breast cancer are classified according to histopathological features into tubular, cribriform, mucinous (colloid), medullary, and papillary carcinomas (Page, 2003; Harris et al., 2010; Malhotra et al., 2010; Kumar et al., 2014). Less than 5% of breast cancer cases involve medullary carcinoma, in which case the affected breast tissue resembles the medulla (brain tissue) in colour, and distinguishes itself from normal breast tissue (Moore and Kinne, 1996). Ductal carcinoma may also advance into metaplastic breast cancer that affects the milk ducts, and then, attacks other breast tissues. Cases of metaplastic breast cancer constitute below 1% of invasive breast carcinomas (Reis-Filho et al., 2005). Other breast cancers such as micropapillary and adenoid cystic cancers are also invasive as they affect breast tissue other than the ducts and lobules (Page, 2003; American Cancer Society, 2016).

In some rare cases, breast cancer affects the blood vessels, fat and muscle tissue of the breast. Scientists term this type of breast cancer sarcoma, as it affects the connective tissue in the breast (Moore and Kinne, 1996). The prevalence of sarcoma is rare, with clinicians recording below 1% of such cases among breast cancer patients, and soft tissue carcinomas below 5%. Instances of inflammatory breast cancer, in which case,

the breasts turn red and swell, are equally less common. Figure (1.4) shows various breast cancer types (Sarkar and Mandal, 2011).

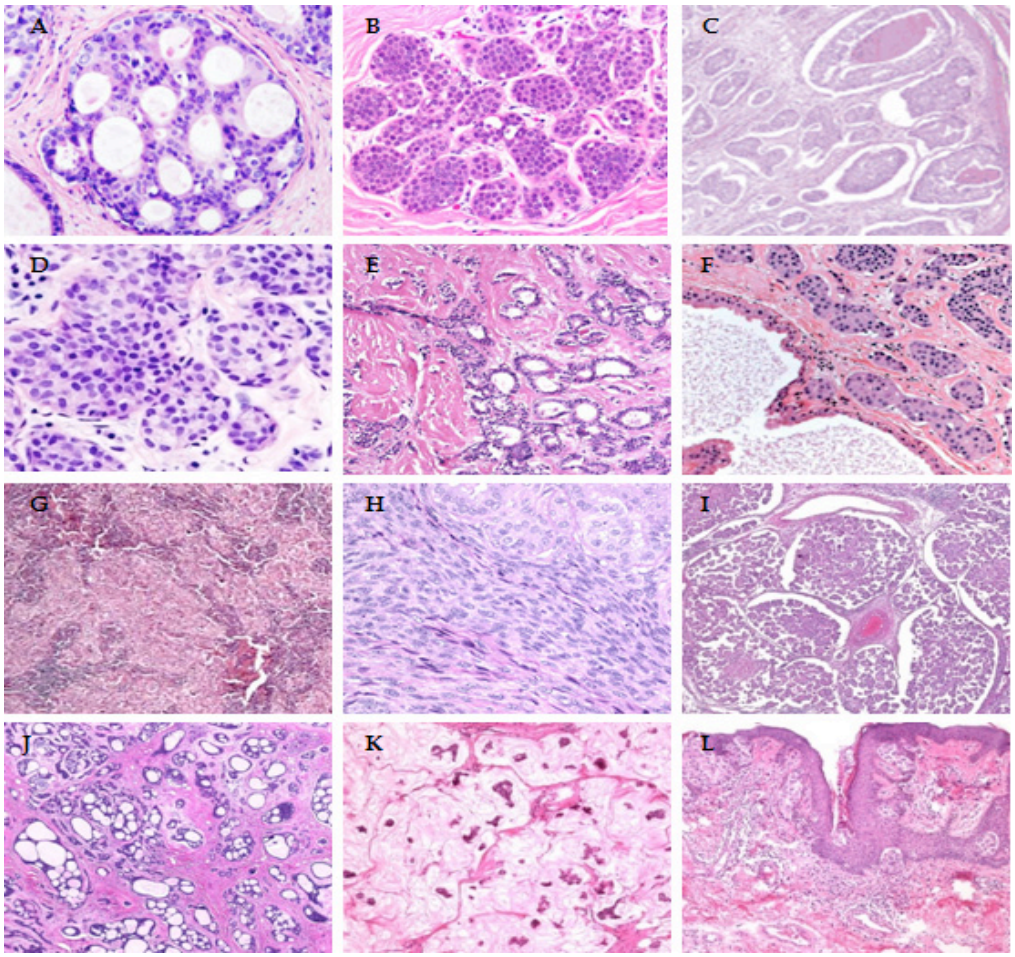


Figure 1.4 Breast cancer histology

Breast cancer can be ductal, as in A, lobular, as in B, and inflammatory, as in C. Sections A-B show in-situ cancer, while parts C-L display invasive carcinoma. Image C is an example of inflammatory invasive cancer, while D illustrates invasive cancer of the lobular type. E displays tubular invasive cancer, F illustrates the apocrine type, G illustrates medullary invasive carcinoma, while H shows metaplastic invasive cancer. I displays micropapillary invasive cancer of the micropapillary type, J illustrates adenoid cystic carcinoma, image K shows mucinous carcinoma, while L illustrates Paget disease (reproduced with permission from Sarkar and Mandal, 2011) (see Appendix 3).

1.4 Molecular classification of breast cancer

Breast cancers were classified according to histopathology (invasive, lobular, grade, ductal, ER and HER-2 status), clinical (node, tumour, age) and molecular (luminal, basal, normal-like, HER-2) values (Sarker and Mandal, 2011).

1.4.1 Immunohistochemistry

The therapeutic and prognostic information can be derived from the immunohistochemistry (IHC) classification. There are four groups on breast cancer that are classified based on IHC profile ER/PR and HER2 (HER2/neu) expression, positive (+) and/or negative (−). The intrinsic gene expression microarray categorisations are well correlated with IHC classification: ER/PR+, HER2− with Luminal A; ER/PR+, HER2+ with Luminal B; ER/PR−, HER2+ and ER/PR−, HER2− with TN/basal-like tumours (Onitilo et al., 2009).

1.4.2 Gene expression microarray studies

There has been a microarray-based platform raised for the introduction of high throughput systems used in the study of thousands of genes depending on a particular experiment. A microarray is a collection of DNA fragments (e.g. cDNA, oligonucleotide) that are spotted in a particular sequence with reference to grids onto a solid surface (a 'chip'). The individual spots contain a unique DNA fragment structure with a high concentration that maps to specific genes as well as genomic sequences (Cummings et al., 2011). The DNA microarrays and patterns of gene expression studies on breast carcinoma have offered evidence to validate that ER+ and ER− breast tumours are basically different at the transcriptomic level (Sotiriou and Pusztai, 2009; Weigelt et al., 2010). This has brought about five different tumour subtypes: normal breast-like, HER2, basal-like, luminal A, and luminal B (Sorlie et al., 2001; Parker et al., 2009). Recently, a new subtype known as "claudin-low" has also been classified (Herschkowitz et al., 2007; Prat et al., 2010).

These molecular subtypes were also identified by hierarchical clustering. Table (1.3) summarises the different breast tumour subtypes associated with the overall survival (OS) and relapse-free survival (RFS).

Table 1.3 Molecular subtypes of breast cancer

Molecular types of breast carcinoma	Frequency (%)	5-year OS (%)	5-year RFS (%)	10-year OS (%)	10-year RFS (%)
Luminal A	50-60	85-95	80-90	75-85	75-85
Luminal B	5-10	70-80	65-75	55-65	54-64
Basal	10-20	63-73	60-70	57-67	45-55
ERBB-2	10-20	55-65	15-20	45-55	15-30
Normal-like	10-15	84-94	80-90	75-85	72-82

Recurrence-free survival (RFS) is the proportion of patients without any other symptoms of breast cancer during the period between the date of breast surgery and the date of second diagnosis, whether it is classified as a breast cancer recurrence or second primary. Overall survival (OS): the percentage of survivors during the period between the date of breast surgery and the date of death of breast cancer (reproduced with permission from Sarker and Mandal, 2011) (see Appendix 3).

1.4.3 Developmental hierarchy of breast cells

In addition to microarray-based gene expression analysis, hierarchical clustering uses statistical tools applied in analysis of gene expression data sets to bring out groups of cases that show signs of similar molecular features (Radmacher et al., 2002). The researchers demonstrated the existence of four molecular subtypes of breast cancer: Basal-like, luminal-like, HER2+, and normal-like (Perou et al., 2000). Resultantly, the gene list was customised and 476 genes were termed as the 'intrinsic gene set'. These were used in a follow-up study that included 78 cancers suggesting three additional subgroups within the luminal class that includes luminal-A, luminal-B, and luminal-C (Andre and Pusztai, 2006). Recent molecular studies have expanded this and now suggest ten distinct molecular subtypes (Dawson et al., 2013).

1.4.4 Disease outcome

Earlier reports suggest that the biomarkers can be used to assess breast cancers in clinical practice on a routine basis such as ER, PR, and HER2 with reference to the definition by gene expression profiling. Tumours that are ER+ and/or PR+, and HER2− are more likely to be luminal A, whereas those that are ER+ and/or PR+, and HER2+ are in all likelihood luminal B. Those that are ER−, PR−, and HER2+ are likely to be HER2 type. Lastly, those that are PR−, ER−, and HER2− are presumably basal-like (Schnitt, 2010). With respect to outcome, luminal A tumours were shown to have a better prognosis, while HER-2 and basal-like tumours have the worst outcome (Table 1.4) (Hu et al., 2006; Sorlie et al., 2001).

Table 1.4 Breast cancer molecular subtypes associated with clinicopathological characteristics

Adapted from Normanno et al., 2010.

Tumour subtype	Tumour marker	Clinicopathological characteristics
Luminal A	ER+ and/or PR+, HER2−	High levels of luminal epithelial cytokeratins and Other luminal associated markers Low relapse rate Good differentiation Low proliferative index Better prognosis
Luminal B	ER+ and/or PR+, HER2+	Similar to luminal A tumours but: High relapse rate Poor differentiation High proliferative index Poorer prognosis comparing with luminal A
HER2	ER−, PR−, HER2+	Amplification of the *HER2* gene High expression of HER2 and HER2-related gene Poor differentiation Worse prognosis
Basal-like	ER−, PR−, HER2−	High expression of basal myoepithelial markers and epidermal growth Factor receptors (EGFR) Poor differentiation Worse prognosis

ER (Oestrogen receptor); PR (progesterone receptor)

Lately, there have been demonstrations to outperform all of the known clinicopathologic parameters based on a number of studies linking gene expression profiles to clinical outcome in breast cancer. As an outcome to this, some clinical signatures have been developed. For instance, there has been Food and Drug Administration approvals (FDA) to aid in the prognosis of patients with node-negative disease under the receivership of Mammaprint (Agendia, Huntington Beach, CA). Using DNA microarrays, it is possible to measure the expression of 70 genes while calculating a prognostic attainment that classifies the patients into a poor or good prognosis (Eichhorn and Baselga, 2010; Gokmen-Polar and Badve, 2012).

Equally, Oncotype DX (Genomic Health, 2009) has received endorsements on clinical oncology from the American Society for classifying patients with ER+, treating node-negative disease with tamoxifen and quantitative real-time polymerase chain reaction (qPCR) that analyse the expression of 21 known cancer-related genes. These measurements are categorised by the Oncotype DX system into a quantitative recurrence score used to classify patients into high-risk, intermediate-risk, and low-risk groups (Eichhorn and Baselga, 2010; Weigel and Dowsett, 2010).

A 50-gene assay (PAM50) has shown promise to predict risk of relapse as compared to a model utilising only clinical variables (tumour size, node status, and histologic grade) when tested on ER+/node-negative patients (Parker et al., 2009).

1.5 Breast cancer pathology staging

Staging is vital because it reveals the degree to which the cancer has invaded the other body parts. Prognosis and treatment recommendations partly depend on information obtained from breast cancer staging (Breastcancer.org, 2015). The TNM staging system is used by specialists to assess the spread of the cancer (Table 1.5) (CRUK, 2014). Various factors are applicable in breast cancer staging, and these include patient's age,

tumour size, status of the axillary lymph nodes (ALNs), scope of invasion or disease in breast tissue, and status of the hormone receptors (Pinder et al., 1995).

Table 1.5 Breast cancer staging

TNM staging system based on tumour size, status of the axillary lymph nodes, and scope of invasion (from Breastcancer.org, 2015)

Stage	Tumour size	Lymph node Involvement	Metastasis
I	Less than 2 cm	No	No
II	Between 2-5 cm	No or in same side of breast	No
III	More than 5 cm	Yes, on same side of breast	No
IV	Not Applicable	Not applicable	Yes

1.5.1 Lymph node status

Scientists consider the status of the ALN as the sole most significant marker of the survival of breast cancer patients. The 5-year disease-free survival (DFS) is 82.8% for patients with negative nodes, while, for those with positive nodal activity survival rates are 73% for 1–3 positive nodes, 45.7% for 4–12 positive nodes, and 28.4% for ≥13 positive nodes, within this period (Cianfrocca and Goldstein, 2004). Furthermore, the total number of nodes present is vital to breast cancer prognosis, as the presence of four or more active nodes leads to a worse outlook than cases of node-negative disease (Jatoi et al., 1999). Patients in the initial tumour stage are those whose lymph nodes do not reveal any cancer activity. Patients in the second tumour stage have only three or less cancerous lymph nodes, while those in the third stage have four or more affected lymph nodes (Pinder et al., 1995).

1.5.2 Imaging techniques

To facilitate staging, scientists carry out imaging including breast mammograms, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) scans, and the sentinel lymph node biopsy (SLNB). In addition, doctors may perform blood tests to assess the patient's physical well-being, and find out whether the cancer has invaded other vital body organs (American Cancer Society, 2016).

1.5.3 Testing for breast tumour markers

Blood marker tests compliment the use of biopsies in the detection, diagnosis, and management of cancer, in addition to providing accurate results regarding cancer stage. There are different types of breast tumour marker test including; receptor evaluations of ER and PR, and HER2. Further, carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA 15-3) markers are useful in monitoring advanced cancer patients for a relapse (Cheung et al., 2000; Harris et al., 2007; Geng et al., 2014).

1.6 Prognosis and predicting breast cancer

The therapeutic decision for breast cancer is highly correlated with prognostic and predictive markers that are derived from either the tumour type or characteristics of the patient (Weigel and Dowsett, 2010). Prognostic factors related to the natural progression of the condition in the patient, and predictive factors involve the patient's reaction to therapy (Bradley, 2007). Indeed, many predictive factors for the selection of optimum treatment are increasingly being recognised as prognostic factors for clinical outcome such as ER/PR status and *HER2* gene amplification and/or over-expression (Clark, 1995; Sweep et al., 2006). For instance, there has been a particular emphasis on predictive biomarkers for ER− and triple-negative (TN) disease, because these subtypes have a poor prognosis if untreated. However, there is high-risk of recurrence and disease progression in TN disease, which is sensitive to chemotherapy, and therefore more targeted, less toxic therapies are needed (Perou, 2011). ER− patients revealed a

significantly lower survival after relapse than ER+ patients (Howell et al., 1984). In a 2004 study, women with ER+ breast cancer had a 5-year DFS and OS of 74% and 92%, respectively, while the women with ER− breast cancers had a five-year DFS of 66% and OS of 82% (Cianfrocca and Goldstein, 2004).

1.6.1 Prognostic markers

Any measurement available that correlates with DFS or OS of breast cancer patients and relates to the natural history of the disease is considered as a prognostic factor. Numerous clincopathological features of potential prognostic value have been extensively examined in multicentre clinical trials, including tumour size, lymph node metastasis, lymphovascular invasion, histological subtype, and tumour grade (Galea et al., 1992; Harries et al., 2003; Cianfrocca and Goldstein, 2004). Other molecular markers have previously been described as important in breast cancer prognosis, including *HER2*, *EGFR*, *MYC*, *CCND1*, and *MDM2* (Al-Kuraya et al., 2004).

1.6.1.1 Lymph node involvement

Patients with negative nodal involvement have a ten-year survival of around 80%. The number of involved lymph nodes is also of prognostic significance; the ten-year free survival falls to half of the above percentage when there is less than four lymph nodes involved and may reach 10% when more than ten nodes are positive, therefore this prognostic parameter is regarded as the most significant one among all other parameters (Chae et al., 2009; Kumar, 2014).

1.6.1.2 Tumour size

There is a relationship between the size of the tumour and lymph node involvement, for example, if the breast cancer patients have tumours less than one centimeter in diameter without lymph node involvement, they had a five-year OS close to 99% in comparison

to those that have between one and three, and three and five centimeter tumour size where the percentage decreased to 89% and 86%, respectively (Carter et al., 1989; Rosen et al., 1993).

1.6.1.3 Histological subtype

The prognosis of breast cancer differs according to the histopathological type, for example, women have much better prognosis if their breast lesion is of tubular, mucinous, colloid or papillary type than if they have NOS breast cancer, which accounts over 70% of cases and such women with NOS breast cancer usually have a bad prognosis (Simpson and Page, 1992).

1.6.1.4 Tumour grade

Women with grade I breast cancer have better prognosis than those who have grade III breast cancer and those with grade II are usually in between (Le Doussal et al., 1989).

The current grading system of breast cancer, with the most widely accepted being the Scarff-Bloom-Richardson (SBR) classification also called the Nottingham grading system (NGS) (Bloom and Richardson, 1957; Elston and Ellis, 1991; Rakha et al., 2008), measures three features of the tumour: the tubule structure (degree of differentiation), mitotic count/index (proliferation) and nuclear pleomorphism/grade (Cummings et al., 2011). Histological grading for breast cancer is now considered essential as part of the minimum data set for pathological reporting, and is becoming widely recommended by the United Kingdom Royal College of Pathologists (UK RCPath) (Ellis et al., 2005), the College of American Pathologists (CAP) (Fitzgibbons et al., 2000), the European Commission (van Engen et al., 2010), and by the World Health Organization (WHO) (Travis and Harris, 2004).

1.6.2 Predictive markers

Predictive markers can predict the outcome of a specific therapy. For example, ER/PR expression is used as a predictive indicator of endocrine therapy (Byar et al, 1979; Cianfrocca and Goldstein, 2004). In ER+ patients, the main growth stimulus is the steroid hormone, estradiol. Thus, this impact was associated with response to endocrine therapy (Howell et al., 1984; Weigel and Dowsett, 2010). ER− patients achieve a greater response from neoadjuvant chemotherapy than the ER+ patients (Colleoni et al., 2004; Ring et al., 2004). At present, HER2 is probably the most powerful individual predictive marker introduced for treatment, selecting patients with advanced breast cancer with trastuzumab therapy (Duffy, 2005; Mass et al., 2005; Romond et al., 2005; Smith et al., 2007).

1.7 Diagnosis of breast cancer

The most critical concern of pathologists is the diagnosis of invasive breast cancer. A diagnosis of invasive breast cancer is mainly based on the microscopic rating of tissue specimens, which often begins with an assessment of image-guided core biopsies obtained by radiologists (Allred, 2008).

1.7.1 Breast cancer diagnostic tests

Fluoro-2-deoxyglucose positron emission tomography (FDG-PET) is an accepted tool that covers disease diagnosis in most malignancies, including breast cancer. It is employed for monitoring response to treatment in local tissues in advanced and metastatic breast cancer, when a change in therapy is expected (Kelloff et al., 2005). FDG-PET evaluation is based on increased glycolytic level and glucose avidity in cancer cells in comparison to normal cells. Medical research continues to enhance PET tracers to facilitate more specific cellular procedures than glucose metabolism. In breast cancer diagnosis, these tracers contain thymidine such as fluoro-L-thymidine to examine DNA replication and multiplication of cancerous cells (Quon and Gambhir, 2005).

Currently, various schemes of diagnostic surgical techniques and different adjuvant therapies are employed for longer survival for breast cancer patients, including: mammogram, breast ultrasound, chest radiography, CT, SLNB, complete ALN dissection, MRI, and isotope bone scanning (Chae et al., 2009). There are many blood tests and diagnostic imaging procedures, in addition to physical examination, that help detect asymptomatic distant metastases (Perrone et al., 2004).

1.7.2 Molecular techniques

Some of the molecular methods most suitable for breast carcinoma diagnosis include real-time RT-PCR, IHC, the use of DNA microarray, and fluorescence in situ hybridization (FISH) (Schnitt, 2010). IHC is used for classification of breast carcinoma to ER positive and negative tumours, whereas FISH is used to classify breast cancer disease to human epidermal growth factor-2 (HER-2) amplified or non-amplified classes (Schnitt, 2010). Recently, flow cytometry has been used for breast cancer diagnosis, in the detection of PR, ER, E-cadherin, and HER-2 markers (Lostumbo et al., 2004).

1.8 Breast cancer treatment

1.8.1 Surgery

Breast cancer treatments depend on age, stage, and hormonal and receptor status. Surgery is the main method for treating localised and invasive ductal breast carcinoma, and often the initial choice. Often, surgery is combined with further treatments, such as radiation therapy, hormone therapy, chemotherapy, and targeted therapy (American Cancer Society, 2015). The amount of tissue removed with the tumour differs according to the kind of breast cancer surgery, the characteristics of the tumour, and the patient's wishes. If the patient undergoes timely diagnosis, doctors merely excise the tumour and conserve the breast (lumpectomy), rather than remove the entire breast, as in a mastectomy (Veronesi et al., 2002).

1.8.2 Radiotherapy

In the early stage, breast cancer patients need radiotherapy to the preserved breast after breast conserving surgery as an appropriate method of primary therapy to lower the risk of cancer and to kill any residual cancer cells after the surgery (Fisher et al., 2002; CRUK, 2016). Radiation adjuvant therapy is a cancer treatment that employs high-energy X-rays or other types of radiation to destroy cancer cells left in the breast, underarm area, or chest wall after surgery, or that reduces tumour size before surgery (Jassem, 2000; VanderWalde and Hurria, 2012). Two types of radiation therapy are given. The first type is external radiation therapy, which uses a machine outside the body to send radiation across the cancer. The second type is internal radiotherapy also called a radiotherapy implant, which uses radioactive substances that are placed directly into or near the cancer (Rozen and Ashton, 2012).

1.8.3 Chemotherapy

Chemotherapy is made up of drugs that are applied to reduce the tumour size that has metastasised in a neoadjuvant (before surgery) setting and to lower the risk of breast cancer relapse in an adjuvant (after surgery) setting (Rastogi et al., 2008; VanderWalde and Hurria, 2012). In fact, various drugs are recommended for use with other drug types in early breast cancer, including: Methotrexate, Cyclophosphamide, Fluorouracil (5-FU), Doxorubicin (Adriamycin), Docetaxel (Taxotere), Paclitaxel (Taxol), and Epirubicin (Sarkar and Mandal, 2011). The decision to offer chemotherapy is dependent on the patient characteristics, as well as on clinical and histopathological parameters (Goldhirsch et al., 2003; Paik et al., 2004; Gonzalez-Angulo et al., 2007).

1.8.4 Endocrine therapy

The human body naturally produces several hormones for regulating the growth and development of the normal cells. Oestrogen is one such hormone that can prompt the growth of certain breast cancer cells. Thus, hormone therapy can prove to be effective only if the breast cancer cells contain ERs. This treatment can shrink the breast cancer

that has spread to other parts of the body either by preventing oestrogen from attachment to their receptors or through preventing their production (Jin and Mu, 2015). Numerous hormonal drugs are used during breast cancer treatment. These include aromatase inhibitors, tamoxifen, as well as ovarian suppression (Chia and Wolff, 2011; Early Breast Cancer Trialists' Collaborative Group, 2011; CRUK, 2014).

1.8.5 Biological therapy

Another approach is the biological therapy, which includes the use of drugs to modify cancer cell interactions so as to prevent the cancerous cells from passing signals to multiply. There are numerous approaches to biological therapy in the treatment of breast cancer (Mayo Clinic, 2014). They include sunitinib, trastuzumab (Herceptin), everolimus, and lapatinib (Craft et al., 2007).

1.9 Cancer subtypes in the middle - east area Saudi Arabia

In the Kingdom of Saudi Arabia (KSA), breast cancers account for about 19.8% of all cancers among Saudi females (Al Diab et al., 2013). It is the second most common malignancy among Saudi females (Al Qahthani, 2007). A report of the Saudi Cancer Registry (2014) showed an increasing number of breast cancer cases among females from 429 (1994) to 1473 (2010). Locally advanced breast malignancy disease is uncommon among women in the Western countries. In other parts of the globe, including the KSA, locally advanced breast cancer accounts more than 40% of all (non-metastatic) breast cancer (Ezzat et al., 1999).

1.9.1 Racial/Ethnic variation in biological features in younger women

Breast cancer frequency rates have varied considerably, and have shown a remarkable geographical variation, with the uppermost rates in western states, and lowest in Asian and African states. The breast cancer prevalence is increasing in nearly all regions; the

changes have peaked in areas where rates were formerly low. Environmental features might be the cause for such global variations (Sait et al., 2010).

Roughly, about 3.7%–7.5% of the entire number of breast cancer victims diagnosed each year in Western Europe and US are younger than 40 years. In Saudi Arabia, the fraction of breast cancer victims less than 40 years at diagnosis is spectacularly larger with 25.1% (Rudat et al., 2013). Numerous retrospective progressions and subset analysis of larger unsystematic trials have depicted that youthful patients with breast cancer have a poorer prognosis as compared to an older age at diagnosis. Breast cancer patients ≤40 years are likely to have more TN and fewer luminal A and B breast cancers (Al Tamimi et al., 2010), tumours of higher grade, more extensive intraductal component, more lymphovascular invasion, more likely ER− tumours, and more often *BRCA1* or *2* germline mutations (Rudat et al., 2012; Shannon and Smith, 2003).

Breast cancer in young women has been shown in several studies to be an independent interpreter of aggressive biological behaviour (Cancello et al., 2010; Nixon et al., 1994; Albain et al., 1994). Age 35 years and below is taken to be an absolute indication for adjuvant systemic chemotherapy irrespective of other tumour characteristics, these results were as a result of several consensus guidelines regarding the age limit. In Saudi Arabia and the Middle East, analysis based on prognostic data and clinico-patholical details are scarce. Breast cancer in young women is perhaps the result of a multifarious interaction between genetic, non-genetic and environmental patient related factors (Choi et al., 2004; Loman et al., 2001; de Sanjosé et al., 2003; Anders et al., 2008; Althuis et al., 2003). However, no major difference of the family history was found between breast cancer patients less or equal to 40 years and greater than 40 years in the study (Rudat et al., 2012).

1.9.2 *BRCA1* and *BRCA2* mutations

Diverse histological phenotypes occurring in *BRCA1* and *BRCA2* mutation carriers as a result of subsequent somatic mutations and the germline mutation occurring in breast tissue (Table 1.6) (Armes et al., 1999). In familial breast cancer, mutations in the tumour suppressor genes (*BRCA1* and *BRCA2*) lead to a high-risk of developing breast cancer (O'Donovan and Livingston, 2010). At the cellular level, *BRCA1* and *BRCA2* genes code as large proteins that appear to operate as tumour suppressor genes, and play a role in the protection of genome integrity. Expression and action of *BRCA1* and *BRCA2* in human breast cancer cells can result from DNA-damaging agents such as topoisomerase inhibitors, ultraviolet radiation and ionising radiation (O'Donovan and Livingston, 2010). The malignancies are characterised by low onset age and a tendency of diagnosis at late stages of malignancy as proved from an analysis of recent data on breast/ovarian cancers among the female population of the KSA. *BRCA1* and *BRCA2* transformations are likely to throw in the pathogenesis of familial breast cancer in feminine patients from KSA (El-Harith et al., 2002).

Table 1.6 Different molecular pathogeneses in breast tumours from *BRCA1/2* mutation carriers

***BRCA1* phenotype**	***BRCA2* phenotype**
Features specific to way of tumour progression and lead to *BRCA* genes defect (adapted from Turner et al., 2004; Welcsh and King, 2001)	
Triple negative	No specific subtype
ER−/ PR−	ER+ in similar proportion to sporadic cancer
EGFR expression	-
Lymphocytic infiltration	-
Ductal carcinoma in situ	Ductal carcinoma in situ
c-MYC amplification	? *c-MYC* amplification
TP53 mutation	*TP53* mutation
Features that reflect loss of function of BRCA genes (adapted from Petrucelli et al., 2010; Welcsh and King, 2001)	
Defects in DNA repair	Defects in DNA repair
Defects in transcription	Defects in transcription
Abnormal centrosome duplication	Abnormal centrosome duplication
Defective G2/M cell cycle checkpoint regulation	Defective G2/M cell cycle checkpoint regulation
Chromosomal abnormalities	Chromosomal abnormalities

1.9.3 Comparison of biomarkers among younger women in the Middle East and Europe

The importance of knowledge of ethnic molecular features due to the increase in use for predicting cancer prognosis and therapy response. To detect potential molecular variances between breast cancers in the Middle East and Europe, consecutive breast cancer groups from Switzerland (n=2197) and Saudi Arabia (n=204) were analysed. An analysis was carried out by FISH for *HER2*, *CCND1*, *MYC*, and *EGFR* amplification based on the tissue microarrays. The statistics revealed marked differences linking Saudi and Swiss patients. Saudi breast cancers had a distinctly higher frequency of *HER2* (31 vs 17%; P<0.0001) and *MYC* (16 vs 5%; P<0.0001) amplifications as compared to Swiss breast cancers. Remarkably, this was influenced by the high incidence in grade 3 cancers in the Saudi than in the Swiss population (65 vs 32%; P<0.0001). Nonetheless, differences in amplification frequency hold some element of truth within grade 3 cancers (*HER2*: 40 vs 30%, P<0.05; *MYC*: 22 vs 11%, P=0.002) (Al-Kuraya et al., 2005; Al Diab et al., 2013).

It was attested that, the frequency of ER, PR, and HER2 amplification among Saudi Arabia breast cancer patients is comparable to the rest of the globe. Besides, there is a negative correlation among hormone receptor expression and HER2 amplification. The hormone receptor expression and the lower grade tumour are correlated accordaning to Arafah et al., (2012) study; nonetheless not all the high-grade tumours were HER2 positive.

1.10 MicroRNAs

MicroRNAs (miRNAs) are naturally occurring ~22-nucleotide non-coding RNAs which interact with mRNA and play a role in gene expression at post-transcription. They have evolved as a new class of regulatory molecules that play an important role in the biological development, differentiation, apoptosis, and proliferation (Figure 1.5) (Krol et al., 2010) and control about one-third of all human genes (Mendell and Olson, 2012).

In fact, they have been recognised as tumour suppressors and oncogenes in breast cancer (Figure 1.5) (Ahmed et al., 2013) and are included in the development of novel cancer treatments (Garofalo and Croce, 2011). According to a recent study, nearly 50 percent of the annotated miRNAs in the human reside within the fragile sites of the genome. These fragile sites are known to be linked with cancer. This indicates the possible role of miRNA in the progression of cancer (Esquela-Kerscher and Slack, 2006). MiRNA genes are typically located in genomic areas in the form as individual transcriptional units; they also exist in polycistronic unit clusters, which transmit several miRNAs' information (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee et al., 2002; Reinhart et al., 2000). Lately, the potential of circulating miRNAs as novel markers of several diseases have attracted attention in several studies. Research has established their most potent use as minimally invasive breast cancer markers in blood plasma at an early stage (Cuk et al., 2012).

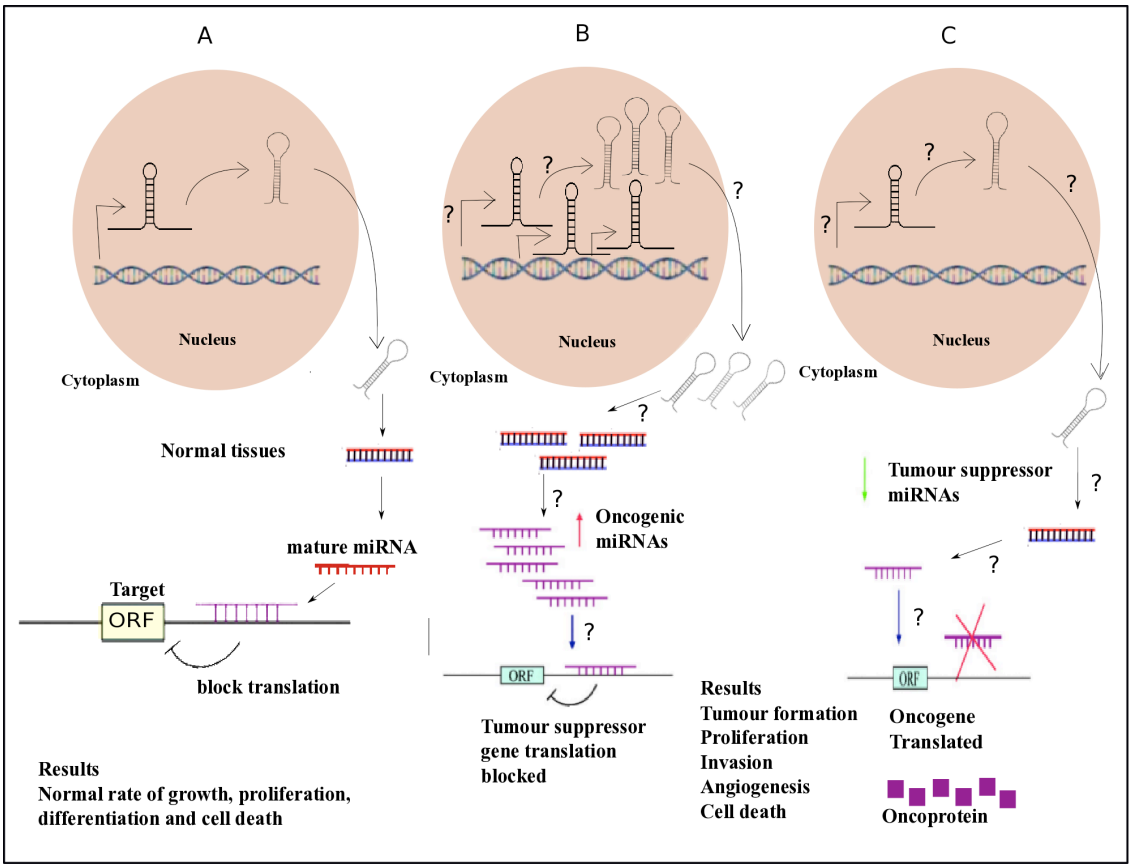


Figure 1.5 MiRNAs can serve as suppressors for tumours as well as oncogenes

A | In the case of normal tissues, proper miRNA processing, transcription, as well as binding with complementary sequences on target mRNA leads to the repression of target gene expression due to suppressing protein translation or altered mRNA stability (not shown). The final outcome is a normal degree of cell growth, differentiation, proliferation, as well as cell death. **B** | Moreover, an overexpression of miRNA with an oncogenic function is also a causative factor in tumour development. Cancer growth may occur as a result of the elimination of the miRNA-target tumour suppressor gene expression due to the production of an excess quantity of miRNA in other tissues or during inappropriate times. Causes for the increase in the levels of mature miRNA can be amplification of the miRNA gene, which is a significantly active promoter, increased miRNA stability, and increased efficiency in the processing of miRNA (indicated by question marks). **C** | A deletion or reduction of a miRNA, which plays a critical role in tumour suppression, can initiate tumour development. Mature miRNA levels may decline or get destroyed due to defects occurring at any miRNA biogenesis (indicated by question marks) stage, eventually causing an inappropriate miRNA-target oncoprotein (purple squares) expression. The final result involves enhanced proliferation, angiogenesis or invasiveness, reduced apoptosis levels, or de-differentiated tissue or undifferentiated, ultimately leading to tumour formation. Open reading frame (ORF) (adapted from Esquela-Kerscher and Slack, 2006).

1.10.1 Biogenesis of microRNAs

RNA polymerase II is responsible for transcribing the miRNA genes, producing long primary transcripts (pri-miRNAs) that are about 1000 nucleotides (Ambros and Lee, 2004). This is followed by the production of mature miRNAs that involves two stages. These two steps involve use of RNase-III enzymes and companion double-stranded RNA-binding domain (dsRBD) proteins (Figure 1.6).

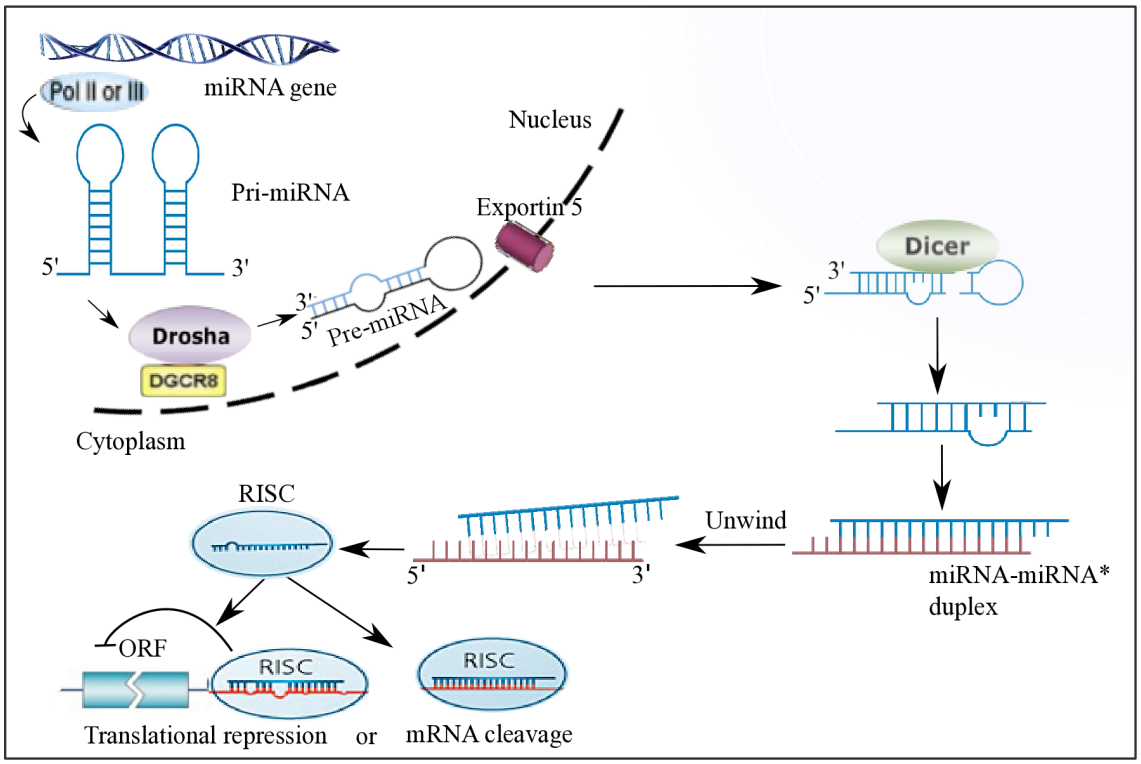


Figure 1.6 The biogenesis of microRNAs

The biogenesis of miRNA initiates within the nucleus, and then it is processed and becomes mature in the cytoplasm of a eukaryotic cell. RNA polymerase II is responsible for transcribing the miRNAs as a long primary transcript (pri-miRNAs) characterised by a hairpin-like structure. They are then cleaved into smaller molecules of around 70-nucleotides (pre-miRNAs) by the Drosha enzyme. The molecules are then transferred by the Exportin 5/Ran-GTP complex to the cytoplasm where there are acted upon by the RNAse III Dicer. This process results in the formation of double-stranded RNAs known as duplex miRNA/miRNA* of 22-24 nucleotides. The strand relevant to the mature miRNA is integrated into a sizeable protein complex known as RISC (RNA-induced silencing complex), which interacts with the 3′ UTR of the targeted messenger RNA. If the miRNA and the 3′ UTR are entirely compatible, the 3′ UTR is cleaved by the RISC. However, translational repression occurs if the matching is imperfect (adapted from Achard et al., 2004; Gregory et al., 2006).

The RNase III–type enzyme Drosha acts upon the long primary transcripts (pri-miRNA) within the nucleus. This produces a hairpin like arrangement (pre-miRNA), comprising of nearly 70 nucleotides. These pre-miRNAs are then exported into the cytoplasm. Within the cytoplasm, they are processed by the RNase III protein Dicer to yield unstable, 19-25 nucleotides miRNA duplex structures (Sontheimer, 2005).

1.10.2 Mechanism of miRNA secretion and incorporation

The inclusion of plasma RNA in protein or lipid vesicles ensures that it is protected from degrading. This was demonstrated by El-Hefnawy et al., (2004); Arroyo et al., (2011); Turchinovich et al., (2011). These particles are called exosomes, apoptotic bodies, or microvesicles (MVs) depending on their size and method of expulsion from cells (Figure 1.7) (Kosaka et al., 2010).

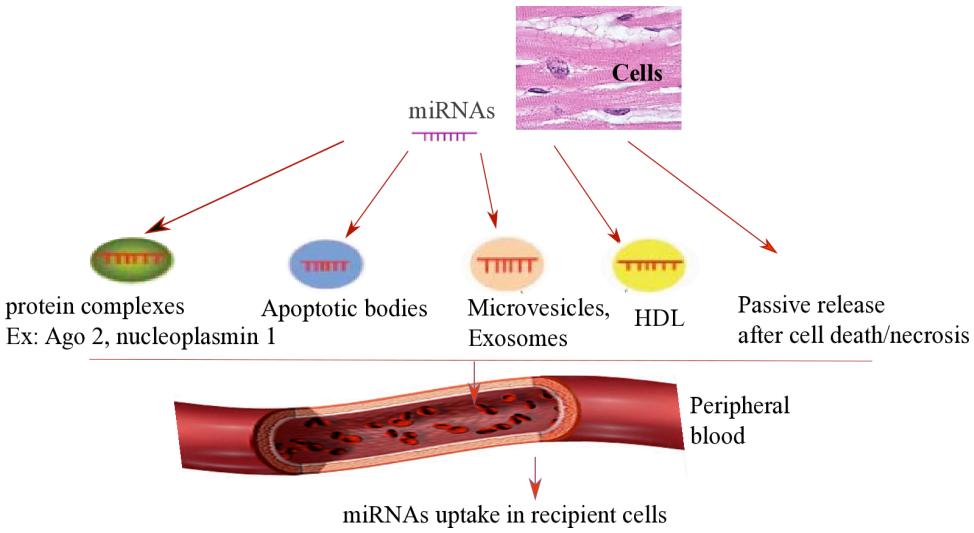


Figure 1.7 Cellular expulsion of miRNAs in the peripheral blood circulation and their uptake by recipient cells

The extracellular miRNAs can reside within vesicles, including microvesicles, exosomes, and apoptotic bodies, and also within proteins like Ago2 (Argonaute 2) and HDL (High density Lipoprotein) (adapted from Metzinger-Le Meuth et al., 2012).

1.10.3 The role of microRNA in breast cancer

Over the years, the miRNAs have been reported to play a significant role in the progression and initiation of breast cancer. MiRNAs, mir-10b, mir-125b, mir-145, mir-21, and mir-155 appear to be most consistently deregulated in breast cancer amongst the differently expressed miRNAs. Amongst these, mir-10b, mir-125b, and mir-145 are down-regulated whereas mir-21 and mir-155 are up regulated. This implies that these down-regulated miRNAs as well as up regulated ones might play a role as potential tumour suppressor genes or oncogenes respectively (Enright et al., 2004; Krek et al., 2005).

1.10.3.1 MicroRNA as oncomirs

The miRNAs that play a role in cancer are referred to as oncogenic miRNAs (oncomirs), and they usually stimulate cancer development by negatively inhibiting tumour suppressor genes that regulate apoptosis or cell differentiation (Lal and O'Day, 2010; Zhang et al., 2006; Sochor et al., 2014). For instance, mir-15 and mir-16 have been found to induce apoptosis by targeting B-cell CLL/lymphoma 2 (*BCL2*). Also, the miRNAs in the mir-17-92 cluster work as modulators of tumour formation while functioning as oncogenes by influencing the translation of E2F transcription factor 1 (E2F1) mRNA (Cho, 2007; He et al., 2005). Aditionally, the gemcitabine-induced apoptosis is modulated by oncogene mir-21, which regulates the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) that is a central player in the signaling of Phosphatidylinositol-3-kinase (PI3K) (Cho, 2007).

Wang et al., (2010) reported that mir-21 is associated with advanced stages of breast cancer, poor prognosis, and lymph node metastasis. The oncogene modulates the downstream pathway and regulates the target genes, thereby promoting tumour invasion and metastasis (Wang et al., 2010). Jiang et al., (2010) reported that mir-155 is up-regulated in breast cancer tissues, where it negatively regulates Suppressor of cytokine signaling 1(*SOCS1*) gene. According to Guttilla and White (2009) mir-27a is another

oncogene that is highly expressed in breast tumour cells and promotes cell growth by targeting Forkhead box O1 (*FOXO1*). In addition, Tang et al., (2012) have documented that mir-27a negatively regulate Zinc finger and BTB domain containing 10 (*ZBTB10*) which result in the progression of angiogenesis.

1.10.3.2 MicroRNA as tumour suppressors

MiRNAs have also been reported to act as tumour suppressors that usually inhibit cancer progression by negatively preventing oncogenes that control apoptosis or cell differentiation (Johnson et al., 2007; Zhang et al., 2006). Nimmo and Slack (2009) reported that let-7 family suppresses breast tumour cells through the heterochronic pathway. The let-7 family facilitates the correct initiation, differentiation, and metastasis of tumour cells at a suitable time. Sun et al., (2012) have argued that let-7 loses the ability to suppress Ras mRNA when it is down-regulated in breast cancer cells, and this leads to activation of p-ERK and p-Ras.

Spizzo et al., (2010) have shown that mir-145 negatively regulates the ER- α and, as a result, mediates the growth of breast cancer cells. Hu et al., (2012) have also shown that mir-145 inhibits the tumour cells by blocking the octamer-binding transcription factor 4 (*OCT4*). The mir-200 family also has a significant impact on regulating the transition between non-stem like and cancer cell-like phenotypes (Park et al., 2008). Li et al., (2013) have found that mir-200b regulates tumour cell metastasis and plasticity in a moesin-dependent manner. Radojicic et al., (2011) have also pointed out mir-205 to be down-regulated in breast tumour cells, and this negatively regulates the Epithelial-mesenchymal transition (EMT) when it targets zinc finger E-box binding homeobox 1 and 2 (*ZEB1*) and (*ZEB2*). Other miRNAs like mir-335 are also common in the metastasis of breast cancer. Png et al., (2011) have shown that mir-335 represses the invasion and migration of breast cancer by targeting a set of genes associated with metastasis, including the transcription factor SRY-box 4 (*SOX4*) and the extracellular matrix protein tenascin C (*TNC*).

1.10.4 MicroRNAs as biomarkers in breast cancer

MiRNAs have been shown to be associated with specific tumour stages, poor survival, lymph node metastasis, response to specific therapies, and disease outcomes (Kodahl et al., 2014). As such, miRNAs can be beneficial as diagnostic, prognostic, and predictive biomarkers.

1.10.4.1 Diagnostic biomarkers

MiRNAs are present in the circulation of breast cancer patients and was capable to distinguish between early stage breast cancer and controls (van Schooneveld et al., 2015; Igaz, 2016). In a study by Kodahl et al., (2014), mir-15a, mir-18a, mir-107, and mir-425 have been identified at higher levels in serum during early breast cancer. However, other diagnostic biomarkers have been found at lower levels, including mir-139-5p, mir-145, mir-143, mir-133a, and mir-365. Those miRNAs were able to distinguish between ER+ early stage breast cancer and controls, accordingly could be serve as diagnostic markers (Kodahl et al., 2014). Additional study by Cuk et al., (2013) have used plasma and found mir-148b to be upregulated, in addition to mir-376c, mir-409-3p, and mir-801. These biomarkers can detect stage I and stage II of early breast cancer. A further study by Ng et al., (2013) has found mir-16, mir-21, and mir-451 to be up-regulated in plasma and tissue, while mir-145 is down-regulated in plasma and tissue samples. A cohort study by Godfrey et al., (2013) has reported that mir-18a, mir-181a, and mir-222 can also be used as potential diagnostic markers given their overexpression in serum samples compared to the control samples.

1.10.4.2 Prognostic biomarkers

A number of gene-expression studies have reported several miRNA prognostic markers associated with outcome of breast cancer patients. There are both positive and negative prognostic miRNA signatures in breast tumours. Some positive prognostic miRNA signatures for breast cancer have been reported by a number of studies in the recent past. Sempere et al., (2007) reported let-7b from a case-cohort reporting the detection of

Locked Nucleic Acid-based in situ hybridization (LNA-ISH) in early invasive breast cancer. Another study by van Schooneveld et al., (2015) has reported that let-7b with luminal breast cancer was positively correlated with survival. Quesne et al., (2012) have demonstrated mir-205 to be a positive prognostic signature in ductal breast cancer.

The miRNAs that are associated with a negative prognosis include mir-122, mir-27b-3p, mir-21, mir-210, mir-9, mir-187, and mir-155. Guttilla and White (2009) have reported the mir-27 family to regulate the progression of the cell cycle and promote survival of the tumour suppressive *FOXO1*. Shen et al., (2014) have identified and validated a prognostic biomarker for patients with lymph node status in distant metastasis survival. Mulrane et al., (2012) have identified mir-187 to be involved in the progression of breast cancer. However, mir-187 is linked with poor breast cancer outcome, and mainly the lymph node-positive serum samples, when it comes to patient survival.

1.10.4.3 Predictive biomarkers

MiRNA predictors can help individualise diagnosis of and therapy for breast cancer in respect of correlating with the given treatment and its benefits. MiRNAs can be associated with targeted therapies such as mir-210 that have been reported by Jung et al., (2012). For example, in HER2+ patients, higher level of mir-210 expression was found in trastuzumab resistant cells. Ward et al., (2013) found that the expression of mir-375 associates with sensitivity to tamoxifen by targeting metadherin (*MTDH*). Cittelly et al., (2010) have also reported that the expression of mir-342 associates with response to tamoxifen in ER+ cancer cells. Zhou et al., (2010) have demonstrated the role of mir-125b in Taxol resistant breast cancer cells inducing BCL2 antagonist/killer 1 (*BAK1*) suppression. Wang et al., (2012) also reported high mir-125b expression in patients showing non-responsive to 5-FU. MiRNAs have also been associated with radiotherapy (van Schooneveld et al., 2015). Stankevicins et al., (2013) have reported that mir-34a is up-regulated via p53 in response to low-dose X-radiation in breast cancer cell lines.

1.10.5 Methods of microRNA profiling

A number of techniques have been used in miRNA profiling, including MiRNA arrays, RNA-seq, multiplex miRNA profiling, and qPCR techniques. MiRNA qRT-PCR is the most commercially available technique that uses proprietary and pre-designed miRNA-specific primers for reverse transcription (Lu et al., 2005). This method has been used by several studies (Markou et al., 2014; Lee et al., 2011; Ono et al., 2015). The technique is also sensitive, flexible, and inexpensive, which makes it a preferred choice to validate new miRNAs in small-scale experiments. Nonetheless, some scholars like Wang et al., (2014) have noted the limitations of qPCR like large experiments becoming very labour intensive. Besides, different from conventional qPCR, it is only a single flanking primer that can be specific to the miRNA.

Additionally, miRNA arrays are used to cover multiple targets of miRNA. Thousands of miRNA probes can be easily spotted on a slide or in photolithography, and this makes it possible to parallel track the known miRNAs. The probing is done by hybridising fluorescently marked RNA or DNA samples (Wang et al., 2007). Individual brightness spots can be observed to infer relative alterations in sample expression (Git et al., 2010). However, similar to the qPCR, it is difficult to distinguish similar sequences. Even so, a careful selection of the controls, stringent cleaning, and analysis can prevent these problems (Git et al., 2010; Giovannetti et al., 2012).

RNA-seq of miRNA uses a high throughput capacity of next-generation RNA sequencing. Although the method cannot quantify the levels of miRNA using a molar resolution of qPCR, researchers have noted that the deep sequencing of miRNA shows to have a greater advantage of being in a position of the entire miRNAs available in a sample, and this makes the technique an ideal tool for miRNA discovery. Importantly, the said miRNA sequences are commonly read directly, as the RNA-sequencing can distinguish even the closely related isoforms and miRNAs (Baker, 2010).

The Multiplex miRNA profiling is a recent technique that is used to validate multiple miRNA across several samples, without the challenges of large sample requirement of other techniques or intense labour workflow. The technique relies on hydrogel particles that have custom selected probes targeted against miRNAs. The targeted miRNA binds to the selected probes and are ligated to the sequence adaptors for pre-detection or detection of miRNA amplification (Tackett et al., 2015).

1.11 Circulating free DNA

This is defined as extracellular DNA in the blood (Figure 1.8) and was first described in 1948 by Mandel and Metais. Between 1966 and 1975 the presence of higher levels of DNA were detected in the plasma/serum of patients with systemic lupus erythematosus (Tan et al., 1966*)*, rheumatoid arthritis, leukemia, and other diseases (Perlin and Moquin, 1972; Koffler et al., 1973; Steinman, 1975). The development of sophisticated techniques resulted in the detection of cfDNA in healthy people (Kamm and Smith, 1972; Steinman, 1975). Several years later, Leon et al., (1977) described the existence of cfDNA in the plasma and serum of patients with malignant tumours. However, it was only during the late eighties when it was verified that the circulating free DNA (cfDNA) found in the plasma of cancer patients were the DNA originating from the tumours themselves (Stroun et al., 1989). This research demonstrated that normal tissues and malignant tumours had distinct physical and biological properties of their cfDNA. Beginning in the year 2000, the role of cfDNA as a marker for diagnosis and prognosis in cancer has sparked great interest among researchers (Rainer et al., 2003; Chang et al., 2003; Hu et al., 2010; Cabral et al., 2010; Arnalich et al., 2010; Liggett et al., 2010; Kamat et al., 2010).

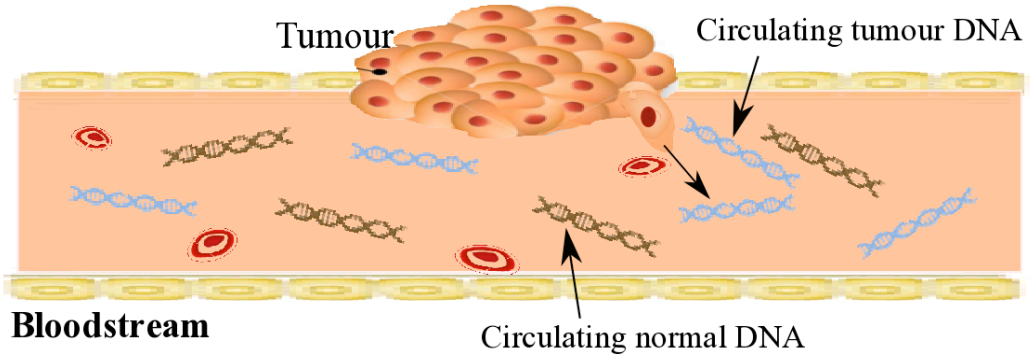


Figure 1.8 The presence of cfDNA in the blood as a biomarker

Adapted from Roschewski et al., 2015.

1.11.1 Characteristics of cfDNA

cfDNA comprised double-stranded DNA molecules with size ranging from 0.18 kB (Jahr et al., 2001) to 21 kB (Stroun et al., 1987; Carpentier et al., 1981). These DNA fragments have conspicuously lower molecular weights (Wang et al., 2003) compared to genomic DNA (Jung et al., 2010). The majority of the DNA in the human body circulates in the form of complexes such as nucleoprotein (Fournié et al., 1992; Skvortsova et al., 2006); or is attached to the surface of the cells through proteins with specific nucleic acid binding properties (Rieber and Bacalao, 1974; Juckett and Rosenberg, 1982; Chelobanov et al., 2004; Laktionov et al., 2004). The major significance of cfDNA as a biomarker is its ease of isolation, its permanence (cfDNA is stable in stored samples of serum or plasma), and its availability, offering potential to access through less-invasive methods (a "liquid biopsy'' by taking a simple blood sample) (Schulz, 2005; Page et al., 2013). Patients with advanced cancers have higher levels of cfDNA than healthy individuals (Zhong and Holzgreve, 2009). A mechanism that could account for this difference is the lower level of DNase activity. In some advanced states of disease, a little more than 100 ng/mL of DNA is detected (Leon et al., 1977). Still, it is important to note that benign breast diseases may also present with increased levels of cfDNA therefore, levels of cfDNA are not specific tumour markers (Guttery et al., 2012).

1.11.2 Origin of cfDNA

The origin of cfDNA in blood under normal and pathological circumstances remains uncertain (Koffler et al., 1973). Apoptotic cells serve as the origin of cfDNA in healthy individuals (Suzuki et al., 2008; Stroun et al., 2001). In cancer patients, cfDNA can have several origins: active release of DNA fraction from all living normal and diseased cells (Stroun et al., 2001; Stroun et al., 2001), and from necrotic and apoptotic processes, feature cancer cells with high cellular turnover (Stroun et al., 2001; Li et al., 2003). Moreover, they have proposed that cfDNA originates from phagocytic action on released necrotic cells from a tumour tissue, and is highly fragmented (Figure 1.9) (Benesova et al., 2012; Jung et al., 2010). Additionally, intact cells have been retrieved from the bloodstream of patients with breast, prostate or liver cancers (Racila et al., 1998; Komeda et al., 1995; Kar and Carr, 1995; Gormally et al., 2007). Both lysis of circulating tumour cells (CTCs) and turnover of disseminated tumour cells (DTCs) in micrometastatic deposits may also release cfDNA into the blood (Guttery et al., 2012; Alix-Panabieres et al., 2012).

1.11.3 Quantitative analysis of cfDNA

There are several approaches to the quantitative analysis of DNA. These include radioimmunoassays (Leon et al., 1977; Shapiro et al., 1983), a procedure utilising nick translation (Fournié et al., 1995), competitive PCR (Jahr et al., 2001), qPCR (Thijssen et al., 2002), fluorimetric quantitation (Thijssen et al., 2002), spectrophotometric determination (Shao et al., 2001), and visual comparison with known standards (Sozzi et al., 2001). So far, all studies, whether or not serum or plasma was used as a sample, demonstrated that patients with cancer have significantly higher levels of DNA than healthy individuals. However, there are variations in the level of DNA found across different studies. This is due to the differences in the type of DNA, the type of cancer investigated and the methodologies used (Chen et al., 1999; Lee et al., 2001; Lui et al., 2002).

1.11.4 Qualitative, tumour specific alterations of cfDNA

Several studies have debated the usefulness of qualitative and quantitative analysis of cfDNA with respect to; DNA strand integrity, microsatellite alterations, mutation of genes, and methylation alterations, as non-invasive prognostic, diagnostic, and monitoring markers in patients with cancer so as to get a better understanding (Anker et al., 1999; Stroun et al., 1987; Stroun et al., 2000) (Figure 1.9).

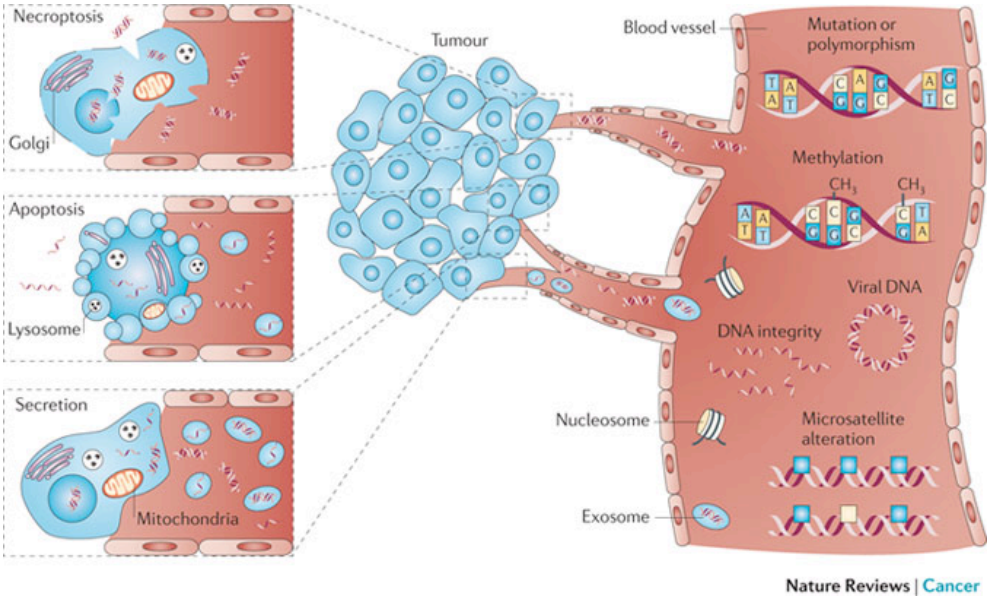


Figure 1.9 Cell-free nucleic acids in the blood

Methylation, mutations, microsatellite alterations, viral DNA, and DNA integrity might be revealed in cfDNA in the blood. The tumour cells release tumour-related cfDNA in the blood of cancer patients. This tumour-related cfDNA circulates in their bodies in various forms and at different levels. The tumour cells may discharge the DNA owing to numerous cell physiological events like apoptosis, secretion, and necrosis. Single as well as double-stranded DNA can be obtained as a result of DNA shedding (reproduced with permission from Schwarzenbach et al., 2011) (see Appendix 3).

1.11.4.1 cfDNA strand integrity

A DNA integrity index defined as the ratio of longer (>200 bp) to shorter (<200 bp) DNA fragments was suggested for characterising the source of cfDNA (Wang et al., 2003). It has been postulated that tumour necrosis causes release of DNA of varying sizes, which contrasts apoptosis in normal tissue that releases smaller and more uniform DNA fragments (Schwarzenbach et al., 2011; Alix-Panabieres et al., 2012). To test the hypothesis that increased DNA integrity, i.e., a longer DNA strand, is a tumour-associated marker in plasma, the genomic DNA integrity index in plasma DNA was determined using qPCR assays targeting different genes with amplicon sizes from 201 to 618 bp for the longer fragments and from 100 to 200 bp for the shorter fragments (Ellinger et al., 2008; Umetani et al., 2006; Schmidt et al., 2008; Sunami et al., 2008; Ellinger et al., 2009; Deligezer et al., 2008; Wang et al., 2003; Gang et al., 2010; Holdenrieder et al., 2008; Chan et al., 2008; Ellinger et al., 2008; Umetani et al., 2006; Jiang et al., 2006; Hanley et al., 2006). In other studies, they determined the apoptosis index, which was the inverse index of the ratio of the shorter to the longer fragments of DNA (Ellinger et al., 2008; Ellinger, 2008).

1.11.4.2 Mutations of tumour suppressor genes and oncogenes

The first tumour-associated variations discovered in circulating free plasma DNA were mutated ras oncogenes (Sorenson et al., 1994; Vasioukhin et al., 1994). *K-ras* ranks second to *p53* in terms of the most frequently analysed gene (Silva et al., 1999; Silva et al., 2002). Alterations in the *K-ras* gene are an early event in malignant transformation. There are 3 mutation hotspots in the codons 12, 13, and 61, which allow for the utilisation of specific and sensitive tests (Lecomte et al., 2002). *K-ras* is a frequent target gene of many research studies because *K-ras* mutations are usually absent from plasma/serum of healthy individuals. However, *K-ras* mutations in the plasma DNA of patients do not necessarily have similar alterations in the matched tumour samples (Ziegler et al., 2002; Kopreski et al., 2000; Kopreski et al., 1997). These mutations can be also found in the plasma DNA of patients with chronic pancreatitis with no evidence of tumour. Colorectal cancer studies revealed the highest consistent results (83–86%) between matched tumour and plasma samples in over 10 patients (Kopreski et al., 2000;

Anker et al., 1997; Mulcahy et al., 2000). In terms of clinical significance, two studies showed that circulating *K-ras* mutations did not usually correlate with clinical and pathological outcomes. However, in pancreatic cancer patients, it was found that *K-ras* mutations may be relevant in terms of tumour size, stage, and relapse risk (Castells et la., 1999; Yamada et al., 1998; Kopreski et al., 1997).

Plasma *p53* mutations were investigated as a single marker in breast and colorectal cancer patients (Mayall et al., 1998). This gene was found to have the most frequent mutations in patients with malignant tumours (Silva et al., 1999; Silva et al., 2002). Breast cancer studies were faced with the lack of control subjects, which disrupted the researchers to calculate the specificity in the said research. But still, the larger patients number employed in the studies and the presence of matched tumour and plasma samples strengthened the quality of the data obtained from these studies. With a sensitivity of 65%, *p53* mutations were detected in the plasma at about 24%. There were statistically significant findings that were relevant to the clinical outcome. Whether or not nodes were involved, patients with mutations in both the solid tumour and the plasma had the worst outcome in terms of survival. Compared to the breast cancer study, the specificity of the colorectal study was higher and the sensitivity was 75%, with overall detection of 17% in the plasma (Mayall et al., 1998). Further mutations measured in plasma cfDNA fragments of cancer patients are those of genes *APC* (Diehl et al., 2005), *BRAF* (Daniotti et al., 2007; Chuang et al., 2010), *EGFR* (Mack et al., 2009; Bai et al., 2009; Kuang et al., 2009), and *MYC* (Park et al., 2009; Gotoh et al., 2005). In principle, their limited diagnostic benefit concurs with the data obtained for *K-ras* and *p53*. Mutations of *EGFR*, *K-ras*, *p53*, and other genes were also used as prognostic indicators and in monitoring treatment effects (Downward, 2003; Levine and Oren, 2009).

1.11.4.3 Methylation alterations

Alterations of the DNA methylation pattern such as hypo- and hypermethylation of DNA are part of the epigenetic changes frequently found in cancer. The term

“epigenetic” refers to altered patterns of gene expression of a number of genes involved in cell cycle control and apoptosis (Dumitrescu, 2012). In the mammalian genome methylation takes place primarily at cytosine bases. Methylation is catalysed by DNA methyltransferase enzymes that use s- adenosyl-methionine as a methyl donor to replace a hydrogen atom with a methyl group at the carbon 5 position of the cytosine pyrimidine ring (Ramsahoye et al., 2000) This only occurs at cytosine bases located 5′ to a guanosine in a CpG dinucleotide (Herman and Baylin, 2003). Methylation of CpG sites within promoter regions is an important mechanism for controlling gene expression in normal cells (Klose and Bird, 2006).

Numerous genes have been demonstrated to serve as methylation markers in different cancers, and analysed in cfDNA extracts: *APC*, *DAPK*, *GSTP1*, *MGMT*, *p16*, *RASSF1A*, and *RARβ2* (Jung et al., 2010). Methylation-specific PCR (MSP) has enabled the detection of methylated DNA sequences in plasma and serum (Herman et al., 1996). Researches have focused on the methylation status of genes that have been associated with cancer in the plasma or serum. Adenomatous polyposis coli (*APC*) gene methylation was prevalent in malignant tumours in lung cancer (96%), making it a valuable disease marker (Usadel et al., 2002). With high-levels of plasma or serum, *APC* gene methylation proved to be an independent factor that predicted for shorter survival. However no correlation was found with common clinico-pathological parameters.

The *p16* tumour suppressor gene has been studied in various types of malignancies. In breast cancer, its methylation state has also been assessed either as a single biomarker (Silva et al., 1999) or in combination with additional variations (Silva et al., 2002; silva 1999). However, these studies did not demonstrate any clinical outcome with *p16* methylation. This might be due to the low frequency of *p16* methylation in breast cancer tissue (22%–23%), which led to overall detection rates in plasma of about 10–14%. The *p16* alterations were more prevalent in liver cancer, where two studies found methylation of *p16* between 67% and 73% of tumours (Wong et al., 2000; Wong et al., 1999).

1.11.4.4 Microsatellite alterations

A microsatellite is defined as a region in a chromosome that contains DNA sequences with lengths of 1–6 nucleotides repeated 1–60 times in tandem. Microsatellites are dispersed throughout the entire genome. The number of units of repeat motifs varies between different alleles (Garbus et al., 2015). Microsatellite alterations, detected by PCR and subsequent analyses of the PCR products, for example, by electrophoresis, are frequently found in tumours as a consequence of defective DNA mismatch repair genes. These alterations appear as loss of heterozygosity (LOH) or as microsatellite instability (MSI). Historically, these alterations were only used in clinical analysis as markers of genome instability, but they are currently considered to directly affect neoplastic processes (Shah et al., 2010). Tumours in their early stages can exhibit microsatellite alterations, as shown in various studies. These finds were confirmed in stage I lung cancer individuals (Sozzi et al., 2001; Sozzi et al., 1999), in plasma DNA of breast cancer individuals who have a carcinoma in situ tumour (Chen et al., 1999), and in serum DNA from early stage ovarian cancer individuals (Hickey et al., 1999).

1.11.5 Copy number variation

Chromosomal aberrations occur frequently during cancer development. These abnormalities can exist in the form of numerical and structural alterations (Dos Santos and Van Kessel, 1999). Numerical aberrations, including aneusomy which is the loss (monosomy) and/or gain (trisomy) of a whole chromosome. When there is an extra copy of every chromosome, the phenomenon is called triploidy (Sudoyo and Hardi, 2011). Structural chromosomal abnormalities such as deletions (loss of chromosomal material), translocations (occurs when multiple chromosomes exchange material), insertions (a chromosomal material moves to a new, interstitial position in the same or another chromosome), inversions (a 180 rotation of a chromosome fragment), and amplifications (multiple copies of DNA segments) are present in a significant number of tumours (Dos Santos and Van Kessel, 1999).

Copy number variations (CNVs), present in around 12% of the genome in human populations, (Zhao and Zhao, 2013; Kuiper et al., 2010) stand as the most frequently occurring type of structural alteration in the human genome (Krepischi et al., 2012). CNVs are amplified or deleted regions of the genome, of different size, which account for physiological normal human genome variability (lafrate et al., 2004; Sebat et al., 2004) and contribute significantly to phenotypic variation (Redon et al., 2006). The DNA segment has lengths ranging from one kilobase to five megabases compared with the reference genome (Kuusisto et al., 2013; Freeman et al., 2006), and at least 11,700 CNV regions larger than 443 bp have been observed (Sebat et al., 2004; Suehiro et al., 2012).

CNVs have been implicated in various diseases such as cancer and neuropsychiatric disorders (Beckmann et al., 2007; Beroukhim et al., 2010; Jia et al., 2011). The two classifications of disease-associated CNVs are rare and common CNVs (Alkan et al., 2011). In diseases such as Autism, ADHD, and schizophrenia, rare or *de novo* CNVs occur in greater frequency unlike in normal controls (Sebat et al., 2007; Stone et al., 2008; Stefansson et al., 2008; Walsh et al., 2008; Xu et al., 2008; Elia et al., 2009). Common diseases that have shown involvement of CNV are HIV (Gonzalez et al., 2005), malaria (Hedrick, 2011), chronic obstructive pulmonary disease, and Crohn's disease (Bentley et al., 2010). CNVs can be used in both the diagnosis and treatment of diseases (Rodriguez-Revenga et al., 2007).

Rare constitutional CNVs may affect important cancer-associated genes or pathways, providing an explanation for high-risk cancer families. Common CNVs with an allele frequency of more than 5% account for about 80% of the observed copy number changes between pairs of individuals (Kuiper et al., 2010).

In a study on breast cancer patients during routine follow-up, tumourigenic CNVs were found within cfDNA isolated from plasma and distinguish breast cancer from healthy

controls. This finding shows that genomic analysis of cfDNA from plasma can be utilised in screening and monitoring breast cancer (Shaw et al., 2012). High-throughput technologies such as next-generation sequencing often detect thousands of CNVs involved in the pathophysiology of diseases (Zhao and Zhao, 2013).

Several unique CNVs are associated with breast cancer. These CNVs may be feasible markers for assessment of the risk of breast cancer (Suehiro et al., 2012). In a study by Stephens et al., (2012) on the 100 primary breast cancers, copy number changes or somatic point mutations were found in at least 40 of the cancer genes. From the forty cancer genes, only seven of them were mutated in more than 10 % of the cases. These are *TP53*, *PIK3CA*, *ERBB*, *MYC, FGFR/ZNF703*, *GATA*, and *CCDN1* (Hicks et al., 2006). This project will cover *HER2*, *CCND1*, *FGFR1*, *MYC*, *CDKN2A*, *PBX1*, *CYP19A1*, and *DMXL2* as the genes of interest in order to shed light on CNV as it relates to breast cancer.

1.11.5.1 Genes of interest in our study

1.11.5.1.1 Human epidermal growth factor receptor 2

The Human epidermal growth factor receptor 2 (*HER2*) or *ERBB2* is a proto-oncogene also known as *HER-2*/*neu* (C-*erb*B-2) localised to chromosome 17q12 (Slamon and Clark, 1988; De Potter, 1994). The HER2 protein is a member of the epidermal growth factor family (EGFR or HER 1-4). This gene was confirmed as a well-established marker that is overexpressed in a wide range of cancer types including breast, ovarian, colorectal, gastric, endometrial, and pancreatic cancers (Lupu et al., 1995). The *HER2* gene encodes a cell-surface glycoprotein of 185 kDa (p185 (HER2)), with intrinsic tyrosine kinase activity, that plays a fundamental role in regulating cell differentiation and development (Ullrich and schlessinger, 1990; Menard et al., 2000).

In a study by Page et al., (2011), *HER2* gene was found to be highly amplified (mean

RQ=14.3) in human breast cancer cell line (SK-BR-3). Moreover, *HER2* gene has been amplified and overexpressed in 20-30% of invasive breast cancers and DCIS and has been correlated with positive lymph node status, high-grade (Burstein, 2005) and poor clinical outcome (Slamon et al., 1987; Slamon et al., 1989). Additionally, the amplification and/or overexpression of *HER2* gene could predict the outcome of targeted therapy for HER2+ breast cancer in the adjuvant and neoadjuvant settings (Slamon et al., 2001; Piccart-Gebhart et al., 2005; Romond et al., 2005). Monoclonal antibodies (trastuzumab) are more effective in *HER2*-amplified breast tumours (Akin et al., 2014).

Currently, HER2+ patients are selected for adjuvant and/or neoadjuvant targeted therapy based on IHC tests to measure HER2 protein expression on the cell surface and and FISH test to measure *HER2* gene amplification (Pauletti et al., 2000; Bartlett et al., 2001; Lebeau et al., 2001; Wolff et al., 2012). Furthermore, the quantitative estimation of the tissue *HER2* status is determined based on qPCR technology (Bergqvist et al., 2007; Egervari et al., 2009; Schlemmer et al., 2004; Tse et al., 2005).

However, because the requirment of these tests for tumour biopsy, they are not appropriate for repeated measurement for treatment monitoring. This fact led to innovation of new minimally invasive method for plasma or serum analyses through a “liquid biopsy” (Page et al., 2011; Gal et al., 2004), as the entire amount of cfDNA in breast cancer patients could have potential as a marker (Weigel and Dowsett, 2010). Detection of *HER2* amplification in cfDNA in plasma measured by qPCR may well be of both predictive and prognostic importance (Bechmann et al., 2013).

1.11.5.1.2 Cyclin D1

The cyclin D1 gene (*CCND1*) localised to chromosome 11q13 is involved in the pathogenesis of breast cancer and other human cancers (Arnold and Papanikolaou, 2005). *CCND1* acts as a vital cell cycle regulator, through transforming into a complex

with cyclin-dependent kinases (CDK) 4/6, and phosphorylation of retinoblastoma protein (pRb), which leads to promoting progression through the G1-S phase (Lundgren et al., 2012). Biochemically, the *CCND1* oncogene encodes the key cell cycle G1 regulatory protein cyclin D1, which stimulates the CDK4 and CDK6, but which also has CDK-independent function and may trigger oestrogen receptor (ER)-mediated transcription in a ligand-independent manner (Holm et al., 2012). When *CCND1* is overexpressed, the G1 phase of the cell cycle is shortened (Arnold and Papanikolaou, 2005). In breast cancer, *CCND1* amplification has been found in 15–20% but is overexpressed in 45–50% of cases, suggesting that other mechanisms rather than amplification may also dysregulate *CCND1* expression (Holm et al., 2012), including transcriptional and post-transcriptional dysregulation (Arnold and Papanikolaou, 2005).

In terms of breast cancer subtypes, the 11q13 amplification was strongly correlated with the luminal A subtype and negatively correlated with the basal subtype (Letessier et al., 2006). There have been conflicting evidences on the real impact of *CCND1* overexpression on prognosis. Several reports have shown that *CCND1* overexpression in invasive breast cancer is associated with better DFS and OS, specifically for ER+ patients (Bilalovic et al., 2005; Gillett et al., 1996), while contrasting studies have demonstrated that *CCND1* amplification relates to a poorer prognosis in ER+ individuals (Michalides et al., 1996; Seshadri et al., 1996).

1.11.5.1.3 Fibroblast growth factor receptor 1

The fibroblast growth factor receptor 1 (*FGFR1*) gene was reported to be amplified in about 10-15% of breast carcinomas, the amplicon located on 8p11.2-p12. This amplicon has a complex structure, consisting of four separate cores. The *FGFR1* gene is an important oncogene and is found on the second core (Courjal et al., 1997; Theillet et al., 1993). It encodes a member of family of receptor tyrosine kinases made up of four kinases (FGFR1-4), located on chromosomes 8p12, 10q26, 4p16.3, and 5q35.1-qter respectively, that regulate cell differentiation, proliferation, migration, and inhibition of apoptosis, and angiogenesis of various cancers, when dysregulated (Turner and Grose,

2010; Eswarakumar et al., 2005). Activation by amplification, mutation, or translocation leads to tumour cell proliferation and survival in many cancers (Kim et al., 2013; Kim et al., 2013).

FGFR1 amplification accounts for a significant independent risk factor for poor DFS and OS in ER+ but not in ER− cases (Elsheikh et al., 2007). In breast cancer patients wherein this key gene is amplified or overexpressed, brivanib treatment may improve outcomes because of its anti-proliferative, and anti-angiogenic action (Shiang et al., 2010).

1.11.5.1.4 *C-MYC*

About three decades ago, *c-MYC* (*MYC*) was known as *v-MYC* avian myelocytomatosis viral oncogene homolog. The *MYC* gene is located on chromosome 8q24, and is encoding a nuclear protein that regulates cell cycle progression. *MYC* amplification occurs in about 20% of breast cancers and is correlated with a poor clinical outcome (Bonilla et al., 1988; Aulmann et al., 2006).

The transition from G1 to S phase was controlled by the *MYC* gene through activating downstream targets like cyclin E/CDK2 and inhibiting CDK inhibitor $p21^{Cip1}$. *MYC* also promotes cell growth via repression of the $p27^{Kip1}$ CDK inhibitor gene transcription. Up to 53% of patients with *BRCA1* germline mutation carriers showed *MYC* gain, compared to 20% of patients with sporadic breast cancers. Unfortunately, there are contradictory studies between the frequency of *MYC* amplification and its prognostic relevance in human breast cancer. In node-negative breast cancer patients, *MYC* amplification acts as a strong prognostic biomarker of early recurrence. However, other studies confirmed that there was no correlation between *MYC* amplification and established risk factors. Amplification of *MYC* gene was associated with high-grade carcinomas with a high proliferation index, in agreement with some studies (Deming et al., 2000).

In hormone-responsive breast tumours, *MYC* expression is stimulated by oestrogen and down-regulated by tamoxifen (Dubik and Shiu, 1988; Le Roy et al., 1991). Amplification of *MYC* gene was associated with the absence of oestrogen receptor, which is in agreement with some studies (Aulmann et al., 2006; Naidu et al., 2002) but contradictory to others (Varley et al., 1987; Berns et al., 1992). The main treatment of tumours expressing *MYC* is chemotherapy with doxorubicin and cyclophosphamide (Pereira et al., 2013).

1.11.5.1.5 Cyclin-dependent kinase inhibitor 2A

The cyclin-dependent kinase inhibitor 2A (*CDKN2A*), is a tumour suppressor gene, located on choromosome 9p21 and encodes two unrelated cell cycle inhibitor proteins, $p16^{INK4a}$ and $p14^{ARF}$ (Foulkes et al., 1997). The p16 protein (encode exons 1α, 2, and 3) is a CDK inhibitor and is extensively expressed in different tissues including breast cancers (Aveyard and Knowles, 2004; Debniak et al., 2005), where it usually prevents abnormal cell growth and proliferation by binding to complexes of CDK 4 and 6, and cyclin D; thereby inactivating these kinases, which arrests the cell cycle in the Gl phase (Foulkes et al., 1997). $p14^{ARF}$ protein (encode exons 1β, 2, and 3) inhibits p53 degradation through binding to MDM2 (Aveyard and Knowles, 2004). Pathway alterations including cyclin, CDK, CDK inhibitor, and Rb protein are present almost across malignancies, including breast cancer (Sutherland and Musgrove, 2004; Gonzalez-Angulo et al., 2006).

Gorgoulis et al., (1998) reported that analyses based on deletion mapping, PCR-SSCP, and a PCR-based methylation assay were able to show that deletion and silencing of *CDKN2A* through methylation might account for the main mechanisms of inactivating this tumour suppressor gene in breast cancers (Nielsen et al., 2001). Furthermore, genetic abnormalities such as deletion or LOH at the *CDKN2A* locus (9p21) is relatively usual in breast cancer (Gorgoulis et al., 1998). This gene is frequently deleted in a homozygous pattern or mutated in about 50-60% of the breast cancer cell lines and in cell lines of various tumours (Berns et al., 1995). Around 20% of melanoma-prone

families possess a point mutation at *CDKN2A* locus, which (Lesueur et al., 2008) also has been implicated in pancreatic cancer families (Monzon et al., 1998; Whelan et al., 1995). Borg and colleagues (2000) found that protein truncating *CDKN2A* mutations predispose females to breast cancer.

A study by Ehab and Elbaz, (2016) found the significant role of direct screening of tumours for RB expression as the primary marker of palbociclib response in breast cancer cells. Further studies were conducted to demonstrate the efficacy of palbociclib in combination with doxorubicin in TNBC cell lines. For advanced ER+ breast cancer, palbociclib could be a promising therapeutic agent (Finn et al., 2009).

1.11.5.1.6 Cytochrome P450 family 19 subfamily A member 1

The gene belongs to cytochrome P450 family and is located on chromosome 15q21.1 encoding the aromatase enzyme necessary for the conversion of androgens to oestrogen (Shao et al., 2015; Zhao et al., 1995; Santen et al., 2009). The size of the cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*) gene has been estimated to be 123 kb and is comprised of a minimum 10 coding exons and 5′-end noncoding exons (Sebastian et al., 2002; Bulun et al., 2004). The gene can be determined through high-throughput technologies such as, next generation sequencing, comparative genome hybridization, and single nucleotide polymorphism (Shozu et al., 2014).

Both deletion and amplification of the gene may contribute to the breast cancer risk; however, the information concerning the contribution of the variant of *CYP19A1* in breast cancers is inconsistent (Olson et al., 2007). The association of *CYP19A1* and oestrogen makes the gene an important prognostic biomarker of breast cancer (Shao et al., 2015; Miyoshi et al., 2003). The gene is often overexpressed due to polymorphism and is suggestive of increased risk to breast cancer in some women but not in others. Genetic polymorphism identified in *CYP19A1* gene that is involved in oestrogen biosynthesis may influence oestrogen concentration (Coughlin and Piper, 1999;

Dunning et al., 1999; Kristensen and Borresen-Dale, 2000). Tworoger et al., (2004) suggests that the tetra-nucleotide repeat and/or the deletion polymorphisms are associated with oestrogen concentrations in postmenopausal women not taking hormone therapy. The common approach of treatment is endocrine therapy with aromatase inhibitors like letrozole and anastrozole after the completion of the usual tamoxifen treatment. Resistance to treatment is related to expression of cycline D1 (Artigalás et al., 2015).

1.11.5.1.7 Pre-B-Cell Leukemia Homeobox 1

The Pre-B-Cell Leukemia Homeobox 1 (*PBX1*) is mapped to 1q23.3 locus that is usually overexpressed or duplicated together with ERα in breast tumours (Bertolino et al., 1995; Woodcroft and Lebrun, 2010; Magnani et al., 2011). Magnani et al., (2011) established that 70 % of the oestrogen response is controlled by *PBX1* thus making it an important determinant of therapy and prognosis. In fact, *PBX1* can be used alone to determine the outcome of breast cancer a priori. Normally, it regulates the ERα transcriptional response to the EGF. It is considered a pioneer gene occupying the chromatin before the recruitment of ERα. The amplification of *PBX1* significantly influences the progression of breast cancers. Usually, patients in whom cfDNA shows overexpression of *PBX1* in the presence of ERα have a very poor prognosis making *PBX1* a significant prognostic biomarker. These results were revealed through ChIP-Seq and microarray genome wide analysis (Magnani et al., 2011). ER targeted adjuvant endocrine therapy combined with tamoxifen is used in treating cancers expressing *PBX1* genes (Zhao and Ramaswamy, 2014). The *PBX1* can be down-regulated using GSI, MRK003, and PF03084014 (gamma secretase inhibitors) (Magnani et al., 2015). The resistance to treatment is related to overexpression of cycline D1, which affects both tamoxifen and aromatase inhibitors, which are also often used as endocrine inhibitors.

1.11.5.1.8 Dmx-Like 2

The Dmx-Like 2 (*DMXL2*) gene is located on chromosome 15q21.2 and usually encodes for transmembrane proteins (NCBI, 2016). There is an association between the *DMXL2* gene and the ERα gene hence ERα positive breast cancers show over expressed *DMXL2* gene making it a novel biomarker for ERα positive breast tumours. In about 20 % of cases where both the *DMXL2* and ER genes are overexpressed, the prognosis of the breast cancer is very poor (Faronato et al., 2015). Resistance to endocrine therapy is associated with overexpression of the *DMXL2*. Furthermore, LOH was found at chromosome 15q21.2 in 12% of patients with breast cancer according to Mao et al., (2005). *DMXL2* regulates Notch receptors cleavage and chromatin recruitment, development of endocrine therapy resistant cells in vivo, and epithelial to mesenchymal transition. Defective Notch signaling has been associated with breast cancer development and formation (Bolos et al., 2013). Since Notch demonstrates resistance in endocrine therapy, patients with this type of mutation may benefit from targeted therapies. The *DMXL2* gene has been found to play a unique role in Notch signaling (Yan et al., 2009; Sethi et al., 2010). Treatment with bafilomycin A1 may under regulate *DMXL2*, which then leads to a decrease in Notch signaling hence lowering the progress of the ERα positive breast cancers (Faronato et al., 2015).

1.12 Background to this thesis

This project follows on from a study by Shaw et al., (2012) where they compared matched normal leucocyte DNA, tumour DNA, and cfDNA samples from 15 primary breast cancers taken before any surgery or treatment, 50 patients on follow-up following surgical removal of the primary tumour, and 8 healthy female controls. Subsequently, the research group, compared plasma results with mammograms and MRI scans. 50 ng DNA were subjected to whole genome amplification (WGA) in order to generate sufficient DNA template for hybridization to Affymetrix® GeneChip Human Mapping SNP 6.0 arrays and data analysis was performed using the Genotyping Console™ (GTC) 4.0.

In the plasma of the 50 patients on follow-up 38,560 copy number (CN) segments were identified. By filtering for amplification in more than 10% of patients led to identification of 23 chromosomal intervals with the majority of more than 50 kb in size with more than 50 markers. These genes/markers within CNVs were then validated by qPCR using both assays developed in house and inventoried CN assay (Applied Biosystems). Their findings revealed that cfDNA of healthy women who have inherited a *BRCA* gene mutation have similar genomic instability to that seen in patients with breast cancer, before detection of cancer by either mammograms or MRI scans. Moreover, results of both principal component analysis (PCA) and hierarchical clustering of cfDNA data suggested the presence of circulating tumour DNA. Analyses of candidate intervals identified in their SNP 6.0 study were therefore investigated in a validation study as part of this thesis.

MiRNAs were also investigated in the Shaw lab. A study by Palmieri et al., (2014) showed that candidate circulating miRNAs were able to discriminate breast cancers from healthy controls and may have value for monitoring response to treatment. Therefore miRNAs wre also investigated in this thesis.

1.13 Hypothesis and aims

The hypothesis of this thesis is that circulating nucleic acids have utility as screening biomarker in women with a family history of breast cancer. The aim of this thesis is to investigate circulating nucleic acids (miRNA and cfDNA) in plasma of women with a family history of breast cancer compared to healthy female controls.

My focus will be in breast cancers in young women with a view to addressing the following 3 key research questions:

i) Are circulating free plasma nucleic acids helpful for breast cancer screening and monitoring in women with a family history?
ii) Can we use miRNA profiling in clinical assessment making?
iii) Are CNVs at specific loci in cfDNA useful as non-invasive markers and needed for more effective detection in breast screening and monitoring?

These questions will be addressed by analysing plasma samples from a cohort of 97 women at high-risk of breast cancer due to family history.

1.13.1 Objectives

A) MiRNA profiling:

1) Analyse TaqMan Microfludic card data generated by Dr. Shona Elshaw to identify candidate miRNAs for investigation in breast cancer and healthy controls.
2) Examination and validation of individual miRNA assays selected for analysis in cancer and controls using cell line models. Six breast cell lines were investigated (HBL-100, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D) by qPCR.

3) Evaluate and compare the levels of circulating miRNAs by qPCR (TaqMan MiRNA Assay) to plasma samples of breast cancer patients and healthy controls.

B) CNV profiling:

1) Examination of individual CNV assays (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) in six cell lines (SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D) using qPCR and ddPCR.
2) Evaluation and comparison of copy number-altered genes in cfDNA of breast cancer patients in paired plasma and healthy lymphocyte DNA by qPCR.
3) Assessment of 5 selected CNV assays (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2*) with clear evidence of CN alteration by qPCR in plasma cfDNA to be compared to the matched lymphocyte DNA by ddPCR.
4) Sequencing of DNA for CNV and mutation detection of 9 plasma cfDNA samples and matched lymphocyte DNA (as a control) using the Ion Torrent Personal Genome Machine (PGM).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Breast cancer cell lines

All cell line models listed in Table (2.1) were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD, US).

Table 2.1 Breast cell lines used in this study and their characteristics

Cell line	ER status	PR status	INV	Cell type	Tissue origin	References
HBL-100	Neg	Neg	22.75	Epithelial	Milk of 27 years old healthy female	Gaffney, 1982; de Fromentel et al., 1985
MDA-MB-231	Neg	Neg	42.1%	Mesenchymal	51 years old Caucasian women with adenocarcinoma	Cailleau et al., 1978; Engel and young, 1978
MCF-7	Pos	Pos	0.9%	Epithelial	69 years adult Caucasian women with adenocarcinoma	Brooks et al., 1973; Engel and young, 1978
ZR-75-1	Pos	Pos	?	Epithelial	63 years old Caucasian women with ductal carcinoma	Engel et al., 1978; Engel and young, 1978
T47-D	Pos	Pos	2.2%	Epithelial	54 years adult women with ductal carcinoma	Keydar et al., 1979; Engel and young, 1978
MDA-MB-468	Neg	Neg	16.2%	Epithelial	From 51 year old black women with adenocarcinoma	Cailleau et al., 1978

Neg: negative Pos: positive ER: Oestrogen receptor PR: progesterone receptor
INV: mean invasion index (data from Gordon et al., 2003).

2.1.2 Cell culture

Table 2.2 Equipment and materials required for cell culture experiments

Equipment and Material	Supplier
Medium appropriate to cells: Dulbecco's minimal essential medium (DMEM) and Phenol red free medium (RBM1)	Gibco or Sigma
Foetal calf serum (FCS)	Sera-Lab
Trypsin/EDTA 1x solution	Gibco
Balanced salt solution (BSS) without Calcium or Magnesium	Gibco
Dulbeccos Phosphate Buffered Saline (DPBS)	Gibco
Dimethylsulphoxide (DMSO)	Sigma
70% IMS	Sigma
L-Glutamine	Gibco
Pen-Sterp (penicillin- streptomycin)	Gibco
Protease (Dispase)	Gibco

2.1.3 Isolation of DNA from cell lines

Materials used for DNA isolation from cell lines are listed in Table (2.3).

Table 2.3 Materials used for DNA isolation from cell lines

Material	Supplier
Chloroform/IAA	Sigma
Phenol/Chloroform/IAA	Sigma
Sodium chloride	Sigma
1M Tris PH 8.0	Sigma
10% SDS	Sigma
Proteinase K	Roche
Ethanol	Hayman
TE buffer	Made in-house

2.1.4 Total RNA extraction using Tri-reagent combined with Qiagen RNeasy Kit

Materials used for RNA extraction from cell lines are listed in Table (2.4).

Table 2.4 Materials used for total RNA extraction using Tri-reagent combined with Qiagen RNeasy Kit

Material	Supplier
Tri-reagent	Sigma
RNeasy Kit	Qiagen
Absolute ethanol	Hayman

2.1.5 Tri-reagent separation followed by Ambion mirVana miRNA Isolation Kit

Materials used for RNA extraction from 1 ml plasma are listed in Table (2.5).

Table 2.5 Materials required for RNA extraction from 1 ml plasma

Reagent	Supplier
5N Acetic acid (1 ml Glacial acetic acid + 2.48 ml sterile UP water)	
Tri-Reagent	Sigma
Ethanol	Hayman
mirVana miRNA isolation Kit	Ambion

2.1.6 Isolation of Genomic DNA from Lymphocytes using QIAamp® DNA Blood Mini Kit

Materials used for DNA isolation from Lymphocytes are listed in Table (2.6).

Table 2.6 Materials used for DNA isolation from Lymphocytes using QIAamp® DNA Blood Mini Kit

Material	Supplier
QIAamp DNA Blood mini Kit	Qiagen
PBS	Sterile (nuclease-free)
Absolute Ethanol	Fisher
1.5 Lo-Bind tubes	Eppendorf (Fisher)

2.1.7 DNA extraction from plasma using QIAamp Circulating Nucleic Acid Kit

Materials used for DNA isolation from plasma are listed in Table (2.7).

Table 2.7 Materials used for DNA isolation from plasma using QIAamp Circulating Nucleic Acid Kit

Material	Supplier
QIAamp Circulating Nucleic Acid Kit	Qiagen
Propan-2-ol	Fisher
Absolute Ethanol	Fisher

2.1.8 Quantification of DNA/RNA using the Nanodrop spectrophotometer

Equipment and materials used in DNA/RNA quantification are listed in Table (2.8).

Table 2.8 Equipment and materials used in DNA/RNA quantification using Nanodrop

Equipment and Material	Supplier
Nanodrop Spectrophotometer (ND-1000)	Fisher
Sterile water	NA
1xTE	Fisher

NA: not applicable.

2.1.9 TaqMan RT individual microRNA expression analysis in cell lines/plasma

All materials used for RT individual miRNA expression in cell lines and plasma are listed in Table (2.9).

Table 2.9 Materials used for TaqMan RT individual miRNA expression in cell lines and Megaplex with pre-amplification for miRNA expression analysis in plasma

Material	Supplier
TaqMan microRNA RT Kit	Applied Biosystems
Megaplex RT primers	Applied Biosystems
TaqMan Pre-amp Mastermix	Applied Biosystems
Megaplex Pre-amp primers	Applied Biosystems
TaqMan MicroRNA Assay card	Applied Biosystems
TaqMan Universal PCR Mastermix	Applied Biosystems

2.1.10 Pre-Amplification of cfDNA

All materials used for cfDNA Pre-Amplification are listed in Table (2.10).

Table 2.10 Materials required for cfDNA Pre-Amplification

Equipment and Material	Supplier
TaqMan PreAmp Mastermix	Applied biosystems
10x PreAmp Primermix (200 pmol/µl)	Sigma
Sterile ultra-pure water	NA
GeneAmp® PCR System 9700	Applied biosystems
MicroAmp ™ Fast Reaction Tubes (8 Tubes/Strip)	Applied biosystems
MicroAmp® Optical 8-Cap Strip (8 Caps/Strip)	Applied biosystems

NA: not applicable.

2.1.11 Quantitative PCR

2.1.11.1 Assay Design for qPCR

The primers and probes sequences used in this study were previously designed by our research group (Table 2.11).

Table 2.11 Primers and probes sequences used in this study

Gene Primers and Probes	Primer and Probe Sequence 5'-3'	Fluorophore/Quencher	Supplier
GAPDH-100-F	GGCTAGCTGGCCCGATTT	-	Sigma
GAPDH-100-R	GGACACAAGAGGACCTCCATAAA		
GAPDH probe	ATGCTTTTCCTAGATTATTC	FAM-MGB qPCR VIC ddPCR	Applied Biosystems
CNTNAP1 F	ACCTGGATGCGCTATAACCTACA	-	Sigma
CNTNAP1 R	GGCTCAGCATGTGGGAGAAC		
CNTNAP1 probe	TGCGCTCTGCAGCCA	FAM-MGB qPCR VIC ddPCR	Applied Biosystems
RPPH1 F	CGGAGGGAAGCTCATCAGTG	-	Sigma
RPPH1 R	GACATGGGAGTGGAGTGACA		
RPPH1 probe	CACGAGCTGAGTGCGT	FAM-MGB qPCR VIC ddPCR	Applied Biosystems
KDELC2 F	GATGCTGGGAAAGGAAGCAA	-	Sigma
KDELC2 R	CCTCAGGCCTGGCAGAAGA		
KDELC2 probe	CAATCTGTGGTTCCTTGGT	FAM-MGB qPCR VIC ddPCR	Applied Biosystems
CCND1-1 (EX) F	GAGCCCCAACAACTTCCTG	-	Sigma
CCND1-1(EX) R	TCACACTTGATCACTCTGGAGA		
CCND1 probe	CTACCGCCTCACACGCTTCC	FAM-MGB	Applied Biosystems
CDKN2A-2 F	AAGTCGTGGCCTTTCAACAC	-	Sigma
CDKN2A-2 R	AGTGCGCTTCTGAGACTCTT		
CDKN2A-2 (Intronic) probe	TCTCCATCTGGCTTGGAAGG	FAM-MGB	Applied Biosystems
CYP19A1-F	TCCTACCTCCAGCATTGCAA	-	Sigma

CYP19A1-R	ACAGACGAAAGGAGGCTTCA		
CYP19A1 probe	TCAAGGCCCAGAACAAGTCCCA	FAM-MGB	Applied Biosystems
DMXL2-F	AGGGCTGAGGTTTCTTCATCT	-	Sigma
DMXL2-R	GGCAGCCTTACACGTAACAG		
DMXL2 probe	CAGCATCCCATGATTCTGGTGTGCA	FAM-MGB	Applied Biosystems
FGFR1-2 F	TCTGCGCTGAGTTGCTTGCT	-	Sigma
FGFR1-2 R	CGTGCCTGGCCTGTATTGCT		
FGFR1-2 (Intronic) probe	TCCTAGGAGAGACTTGGACA	FAM-MGB	Applied Biosystems
HER2 F	ATAACACCCACCTCTGCTTCGT	-	Sigma
HER2 R	GGTGCGGGTTCCGAAAG		
HER2 probe	CACACGGTGCCCTGG	FAM-MGB	Applied Biosystems
MYC-1 F	GGTGCCACGTCTCCACACAT	-	Sigma
MYC-1 R	CCCTCTTGGCAGCAGGATAGTC		
MYC-1 (Exonic) probe	CAGCACAACTACGCAGCG	FAM-MGB	Applied Biosystems
PBX1-F	AGCCCACTCATCTTACGTGAC	-	Sigma
PBX1-R	ACGAAATTCCACTCCAACTCCA		
PBX1 probe	GCTCAGGCCTATCTTCTGGA	FAM-MGB	Applied Biosystems

F: Forward primer; R: reverse primer.

2.1.11.2 Reagents

All materials and equipment used for qPCR are listed in Table (2.12).

Table 2.12 Materials used for DNA Quantification/ CNV Analysis using qPCR

Equipment and Material	Supplier
Human Genomic DNA (HGDNA) (200 ng/µl)	Roche
TaqMan Universal Fast PCR mastermix 2x	Applied Biosystems
Forward and Reverse Primers, 200 pmol/µl	Sigma Genosys
Taqman FAM-MGB Probe	Applied Biosystems
MicroAMP^{TM} Fast Optical 96-Well Reaction Plate	Applied Biosystems
MicroAmp Optical Adhesive Film	Applied Biosystems
MicroAmp Splash-Free 96-well Base	Applied Biosystems
MicroAmp Adhesive Film Applicator	Applied Biosystems
StepOnePlus machine	Applied biosystems

2.1.12 Droplet digital PCR

Same primers and probes sequences were used with qPCR (Table 2.11). Materials and equipment used for ddPCR are listed in Table (2.13).

Table 2.13 Equipment and materials required for the ddPCR experiment

Equipment and Material	Supplier
2x digital PCR supermix for probes	Bio-Rad
ddPCR Buffer control Kit	Bio-Rad
Rainin LR tips 20 µl-200 µl	Anachem
0.2 ml qPCR tubes	e.g. Starlab
0.2 ml qPCR 8-well tube strips/caps	e.g. Starlab
DG8 Cartridges	Bio-Rad
DG8 Gaskets	Bio-Rad
Droplet Generation oil for probes	Bio-Rad
Eppendorf semi-skirted PCR plates	Fisher Scientific
Rainin Low Retention PCR filter tips	Amersham
Pierceable foil heat seals	Bio-Rad
QX200 droplet generator	Bio-Rad
PX1™ PCR plate sealer	Bio-Rad
C1000 Touch™ Thermal Cycler	Bio-Rad
QX200 droplet reader	Bio-Rad

2.1.13 Sequencing of DNA with the Ion Torrent Personal Genome Machine

All materials and equipment used for Sequencing are listed in Table (2.14).

Table 2.14 Equipment and materials used for DNA sequencing with the Ion Torrent PGM

Equipment and Material	Supplier
Ion AmliSeq™ CNV Panel	Life Technology
Ion AmliSeq™ Library Kit 2.0	Life Technology
Ion PGM™ Template OT2 200 Kit	Life Technology
Ion PGM Enrichment beads	Life Technology
Ion Sphere Quality Control Kit	Life Technology
Ion PGM™ Sequencing 200 v2 Kit	Life Technology
1M NaOH	Fisher
Ion Xpress™ Barcode Adapters 1-16	Life Technology
Qubit dsDNA HS Assay Kit	Life Technology
Nuclease-free water	NA
Absolute Ethanol	Fisher
AMPure®XP beads	Beckman Coulter

NA: not applicable.

2.2 Methods

2.2.1 Sample information

The study protocol was approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee 2 (NHS Trust; REC reference number: 11/EM0023). Blood sample collection was conducted in accordance with the Declaration of Helsinki. All individuals gave written informed consent prior to participation.

In total, 97 patients were recruited by Dr. Julian Barwell and the cancer genetics team for the study prior to the commencement of this PhD project. 53 women were recruited who had either a *BRCA1* or *BRCA2* mutation present in their family and 44 women with a family and/or personal history of breast cancer.

2.2.2 Cell culture

2.2.2.1 Resuscitation of cells from liquid nitrogen

The cryotubes were taken out of the liquid nitrogen bank. To prevent explosion, the caps of each were loosened slightly and then retightened so as to release pressure. Again, the details were recorded in the liquid nitrogen bank record book. The cells were then thawed out quickly. After removal from the liquid nitrogen bank, the cryotube was placed in a dewar flask of liquid nitrogen. Then, the cryotube was held upright with forceps in a 37°C water bath for about 3 minutes. Care was taken not to immerse the cryotube completely. Once the cryotube was fully thawed, it was transferred to the tissue culture cabinet. 70% alcohol was used to wipe the hood and all objects placed inside it. The medium was prepared by adding 50 ml FCS, 5 ml L-Glutamine, and 5 ml Pen-Sterp to the medium bottle after removing 60 ml to make a total of 500 ml. After that, a centrifuge tube with 10 ml of pre-warmed full culture medium was prepared. Once this was done, the cryotube was opened and its contents were pipetted into the centrifuge tube. This was then placed in the centrifuge for 5 minutes at 100 g. Afterwards, all cell pellets were again suspended in full culture medium before being transferred to a clean flask. The flask was then placed in the tissue culture incubator

overnight. An inverted microscope was used to observe the cells the following day and observations were recorded.

Cell debris was removed from the adherent cell cultures by replacing the culture medium. As for cell debris in suspension cells, the flask was held upright for 5 minutes to allow the cells to settle before the medium was replaced. The cells were monitored daily using a microscope. The manufacturer's protocol was then consulted as to appropriate cell cultures once confluency or sufficient density was observed.

2.2.2.2 Release of adherent cells from a tissue culture surface

Firstly, cell confluency was monitored using phase contrast inverted microscope until 70-100% was reached. Then, growth medium (DMEM for ER− cell lines and RPMI for ER+ cell lines) and balanced salt solution were warmed to 37^{o}C, while the trypsin/EDTA solution was thawed to the same temperature. To avoid contamination, the hood and all objects placed inside it were wiped with 70% ethanol both before and after usage. The growth medium was poured into a dry container. Then, to remove traces of the medium, the adherent cell monolayer was lightly washed with 5 ml of BSS. Afterwards, the thawed trypsin/EDTA was added (using enough to cover the cells). After some minutes, the cells were observed to dislodge, round up, and become phase bright and so the container was tapped gently to help the process. Once no changes were observed, the suspension was collected in centrifuge tubes. To deactivate the trypsin, an equal amount of complete growth medium was added. The cells were then placed in the centrifuge for 5 minutes at 100 g. Afterwards, all cell pellets were suspended in 5 ml of growth medium, wherein a cell count was executed. Then the cells with medium were removed into a new T25 flask containing 5 ml of medium (T75 flask containing 14 ml of medium), which was shaken lightly before being incubated at 37°C.

2.2.2.3 Cryopreservation of cell lines

A suspension of cells was prepared as mentioned in section (2.2.2.2). The freezing medium was then prepared using 8 ml of complete culture medium with 10% FCS and 1 ml each of FCS and DMSO. Cells were then centrifuged at 100 g for 5 minutes in full culture media. Afterwards, the cell pellets were resuspended in the freezing medium in order to obtain a final cell concentration of 2 to 5 x 10^6 cells/ml. This was then divided into 1 ml portions for each cryotube. Each tube was labeled with the cell name, number of cells, date, and initials before being placed in an Eppendorf container. Subsequently, the container was placed in the freezer, which was set at −80°C, and left overnight. The next day, the cryotubes were transferred to the vapour phase of a liquid nitrogen bank and the details of the experiment were recorded in the liquid nitrogen bank record book.

2.2.3 Isolation of DNA from cell lines

Isolation of DNA was started by adding 350 µl of 0.05 M Tris pH8/0.1% SDS to resuspend the cell pellet (2 to 5x10^6 cells/ml) and then mixed gently by pipetting up and down to ensure complete cell lysis. Then, 17.5 µl of Proteinase K (10 mg/µl) reagent was added to the mixture to give a final concentration of 0.5 mg/ml and vortexed vigorously for 10-15 seconds. The sample was then incubated for an hour in 37°C to digest proteins. The DNA was then purified by adding an equal volume (350 µl) of Phenol/ chloroform/IAA (25:24:1), vortexed for 10-15 seconds and centrifuged at 13100 g for 2 minutes. The top layer (aqueous phase) was transferred carefully into a clean 1.5 ml tube and the aqueous layer was re-extracted with phenol to ensure removal of proteins. The following step was chloroform extraction to remove traces of phenol by adding an equal amount (350 µl) of chloroform/IAA (24:1). Afterwards, the mixture was vortexed at max speed for 10 seconds then centrifuged at 13100 g for 2 minutes and the supernatant was transferred into a new tube. The volume was then measured.

Ethanol precipitation was carried out by adding 1/10 volume of 1 M sodium chloride. 1 ml (volume×3) of ice-cold 100% ethanol was added to the solution and inverted until

the thread-like strands of DNA formed a visible mass. The tube was then placed at −20°C overnight to precipitate DNA. The solution was centrifuged for 10 minutes at 11300 g at 4°C to pellet DNA. Subsequently, ethanol was discarded carefully. The DNA pellet was then washed using 70% of ice-cold ethanol. The DNA was afterwards centrifuged for 10 minutes at 11300 g at 4°C. All of the ethanol was aspirated carefully; the pellet was then air-dryed for 10-15 minutes. DNA was re-suspended in 100 µl of TE buffer. Then, tubes were inverted several times to mix the solution and then stored (4°C) overnight. Finally, DNA concentration was measured using a spectrophotometer.

2.2.4 Total RNA extraction using Tri-reagent combined with Qiagen RNeasy Kit

Firstly, the cell line was thawed in Tri-reagent (1 ml), vortexed, and then allowed to stand at RT for 5 minutes. Then, 200 µl of Q20D chloroform was added. This was again vortexed and allowed to stand at RT for 3 minutes. After 3 minutes, the sample was placed in a microfuge for 15 minutes, at 11300 g and at 4°C. The clean aqueous phase was transferred to a labeled sterile bijou, wherein its volume was measured. Subsequently, absolute ethanol was added to the mixture, the volume of which was 1.25x of the measured aqueous phase volume. The solution was then mixed thoroughly before being placed on ice. Then, 700 µl of the solution was applied to RNeasy column (comes with RNeasy Kit), where it was placed in the microfuge for 15 seconds at 11300 g, and the flow-through was discarded. This was repeated until the entire sample was applied to the column. Once this was done, it was washed with 700 µl buffer RW1, then microfuged again for 15 seconds at 11300 g. The flow-through was discarded. Then, it was washed with 500 µl buffer RPE, then microfuged again for 15 seconds at 11300 g. The flow-through was discarded. Lastly, it was again washed with 500 µl buffer RPE, but microfuged for 2 minutes at 11300 g. Afterwards, the column was transferred to a clean Eppendorf container. This was microfuged for 1 minute at 11300 g. Subsequently, the column was moved to a clean-labelled Eppendorf container, wherein the appropriate volume of Rnase free water was added (100 µl for cell line RNA). This was microfuged for 1 minute at 11300 g. Finally, the column was discarded. RNA was thus stored at −20°C. All used extraction reagents were disposed of in the ‘waste phenol’ container.

2.2.5 Tri-reagent separation followed by Ambion mirVana miRNA isolation Kit

The mirVana Kit was used to extract RNA from 1 ml of plasma. Briefly, 100 µl 5N Acetic acid was added to 1 ml of plasma, after vortexing the mixture was transferred into a 15 ml tube. 3750 µl of Tri-reagent was added and vortexed, then left at room temperature for 5 minutes. 1 ml chloroform was added (Vortexed) and stood at room temperature for 3 minutes. The mixture was centrifuged at 1100 g for 15 minutes at 40°C. The aqueous phase was transferred into a clean 15 ml tube. RNase free water was pre-heated to 95°C. The volume of the aqueous phase was measured and 1.25x the volume of 100% ethanol was added and Vortexed. 700 µl was applied to a mirVana column and microfuged at 2800 g for 30 seconds. The flow-through was discarded. This step was repeated until the entire sample had been put through the column and 700 µl of wash solution 1 was applied into the spin column and microfuged at 2800 g for 30 seconds. The flow-through was again discarded and 500 µl of wash solution 2/3 was added and centrifuged at 2800 g for 30 seconds (this step was repeated twice with centrifugation). After discarding the flow-through from the second wash, the column was transferred to a clean Eppendorf and microfuged at 2800 g for 1 minute to dry the column. The spin column was placed to a clean labelled Eppendorf and 50 µl of pre-heated (95°C) RNase free water was added and centrifuged at 11300 g for 30 seconds. The RNA was stored at −20°C.

2.2.6 Isolation of Genomic DNA from Lymphocytes using QIAamp® DNA Blood Mini Kit

This protocol was designed for the extraction of DNA from the lymphocyte (buffy coat) using the QIAamp DNA Blood Mini Kit. Briefly, 20 µl of protease was placed into a 1.5 ml Eppendorf. Thereafter, 200 µl of the buffy coat was added to the protease (PBS was added to the samples with volume less than 200 µl to increase the final volume to 200 µl). After 15 seconds of vortexing, 200 µl of buffer AL was added and vortexed again for 15 seconds. The mixture was then incubated in 56°C water bath for 10 minutes then briefly centrifuged. 200 µl of absolute ethanol (room temperature) was added and vortexed for 15 seconds followed by a brief centrifugation. The mixture was then applied to the spin column and centrifuged at 4300 g for 1 minute. The spin

column was transferred to a new collection tube and 500 µl of buffer AW1 was added and centrifuged at 4300 g for 1 minute. The spin column was placed in a new collection tube and 500 µl of buffer AW2 was applied then microfuged at 13100 g for 3 minutes. Next, the collection tube was emptied and the column was replaced and centrifuged at 13100 g for 1 minute to dry the column completely. The spin column was transferred to a clean 1.5 ml Lo-Bind Eppendorf and 200 µl of buffer AE was added and incubated at room temperature for 5 minutes, then microfuged at 4300 g for 1 minute. The DNA was then stored at 4°C.

2.2.7 DNA Extraction from Plasma using QIAamp Circulating Nucleic Acid Kit

QIAamp Circulating Nucleic Acid Kit was used to isolate cfDNA from 1 ml plasma samples. Briefly, plasma samples were thawed at room temperature, and then centrifuged at 1000 g for 5 minutes. Meanwhile, 100 ml of QIAGEN proteinase K was added to a clean 50 ml Falcon tube. 1 ml of the plasma supernatant was added to the Falcon tubes. Thereafter, 0.8 ml of ACL buffer was added to the Falcon tubes and vortexed for 30 seconds. The tubes were then incubated at 60°C in a water bath for 30 minutes. 1.8 ml of Buffer ACB was added, then vortexed for 30 seconds. The lysate-Buffer ACB mix was then incubated on ice for 5 minutes. An appropriate number of luer plugs were removed from vacuum manifold and replaced with VacConnectors. QIAamp Mini columns were then removed from the collection tubes and inserted into VacConnectors. 20 ml extenders were inserted into the open columns. Next, samples were loaded into column extenders using a Gilson. Vacuum was then applied. When the entire sample had passed through the column, the vacuum was turned off. After the extenders were removed and discarded, 600 µl Buffer ACW1 was applied to the mini columns. Lids were left open, and the vacuum was turned on until all the buffer had passed through, then the pump was switched off. After that, 750 µl Buffer ACW2 was applied to the QIAamp Mini columns, the lid was left open, and the pump was switched on. When the entire buffer had passed through, the pump was switched off. 750 µl of ethanol was applied to the mini columns, the lid was left open, and the pump was switched on. After the entire buffer had passed through, the pump was switched off. Next, lids were closed, removed from manifold, and the VacConnector were then

discarded. The columns were afterwards placed in retained collection tubes and centrifuged at 13100 g for 3 minutes. The spin columns were transferred to new 2 ml collection tubes, and the lids were opened and incubated assembly at 56°C for 10 minutes to dry membrane completely. The spin columns were placed in 1.5 ml Lo-Bind tubes, and 50 µl buffer AVE was applied. The tubes were incubated at room temperature for 3 minutes, and centrifuged at 13100 g for 1 minute to elute nucleic acids, then eluted DNAs were stored at −20°C.

2.2.8 Nanodrop spectrophotometer

RNA and DNA concentration were measured using this method. First of all, the equipment was turned on. The program was opened by clicking on the ND-1000 icon, and then clicking on Nucleic Acid icon. The lid was opened and a clean tissue was used to wipe the pedestal and top. Once cleaned, 1.5 µl UP H_2O or RNase-free water was dropped on the pedestal. The lid was then closed, allowing the system to initialise. Because the samples should be read against a blank, the pedestal and top were again cleaned with a tissue. The appropriate sample type was chosen, such as DNA, RNA etc. Then, 1.5 µl of UP H_2O or TE was added to the pedestal as blank, depending on which was used earlier as a sample diluent. Once the lid was closed again, the Blank icon was clicked. Thus, the blank was measured, achieving a reading of 0.0 ng/µl. Once blank was achieved, the pedestal and top were again cleaned with a dry tissue. The sample was then mixed well before 1.5 µl of it was added to the pedestal. Then, the sample ID was typed in the box for each sample. Once the lid was closed again, the Measure icon was clicked. The concentration in ng/µl, print on the graph, and the 260/280 values were all recorded accordingly. This was repeated between each sample of which the pedestal and top were wiped clean with a dry tissue. Once all samples were taken, the pedestal and top were wiped with UP H_2O and then again with a dry tissue.

2.2.9 Quantification of DNA using the Qubit® 2.0 Fluorometer

This fluorescence-based method was used for DNA quantification. The basis of each Qubit assay is a fluorescent dyes that only fluoresce when bound to DNA. All assay reagents should be at room temperature before beginning. For cfDNA plasma and cell line DNA quantification dsDNA HS (0.2-100 ng) and dsDNA BR (2-1000 ng) assay Kits were used respectively. First, the Qubit® working solution was prepared for (n+1) tubes (including the two standards) by diluting the Qubit® dye reagent 1:200

199 µl assay dilution buffer + 1 µl dye reagent per tube

The standard (2 Qubit® assay tubes) and sample tubes were prepared according to the table below using the working solution prepared earlier.

Table 2.15 Preparing standards and sample tubes for DNA quantification using Qubit®2.0 Fluorometer

	Vol. of sample	Working Solution	Total volume
Standards	10 µl	190 µl	200 µl
User Samples	1-20 µl	To 200 µl	200 µl

Thereafter, all tubes were vortexed for 2-3 seconds then incubated for 2 minutes at room temperature. The Qubit® 2.0 Fluorometer was switched on (DNA was selected as assay type). The appropriate icon e.g. dsDNA Hi Sens for the dsDNA HS Kit was selected. Then, Yes was selected to Read new standard. When prompted, the Qubit® assay tube containing Standard 1 was placed and Read icon was then pressed. When instructed to do so, Standard 1 was replaced with Standard 2; the lid was closed and measured as before. When instructed to do so, Standard 2 was replaced with the Sample tube; the lid was closed and measured as before. The DNA concentration of the original samples was obtained by selecting Stock conc and then inputting volume of sample used for each reading.

2.2.10 Quantification of DNA using qPCR

This protocol used HGDNA standard curve for the quantification of DNA and testing the primers involved in this study. Generally, GAPDH was used as a reference gene. The standard curve consisted of 7 points, with a dilution factor of 1:2, allowing 3.6 µl per well. The unknowns (lymphocyte DNA and cell lines) were diluted to 1:10 to ensure they fall within the range of the curve. The range covered was 20 ng to 0.3125 ng. For the first standard (20 ng/3.6µl), 2 µl of HGDNA (Roche Applied Science, >50kb genomic DNA, isolated from buffy coat (human blood) from 98 individuals) was added to 70 µl of sterile water and mixed by pipetting up and down (Tube 1). 18 µl of sterile water was added to a further 6 Eppendorfs. Hereafter, 18 µl of DNA from Tube 1 was transferred to 18 µl H_2O in Tube 2. This was mixed well by pipetting up and down. This step was repeated until all 7 standards had been completed. The Primers/probe mix was prepared by adding 3 µl of both the forward and reverse primers (200 pmol/µl), 2 µl of MGB probe and 132 µl sterile H_2O to make the final volume of 140 µl. qPCR single reaction mastermix was prepared by adding 5 µl of TaqMan Fast Universal Mastermix and 1.4 µl of F/R/P mix. 6.4 µl of the reaction mastermix was applied to each well, then 3.6 µl DNA of each dilution was added in the appropriate wells. 3.6 µl sterile water was added to the No Template Control (NTC) wells, and 3.6 µl of the unknown samples were also added. All samples were downloaded in triplicate in 96-well PCR plate.

2.2.11 MicroRNA expression analysis in cell lines

2.2.11.1 TaqMan RT individual miRNA expression assay in cell lines

RNA was diluted in RNase-free water, so that 5 µl RNase-free water contained 10 ng RNA (applicable for every 15 µl RT reaction). Then, 10 µl of the +RT Mastermix was prepared per reaction by adding: 4.16 µl of nuclease-free water, 3 µl of RT primers (1.5 µl U6 and 1.5 µl miR mixed together), 1.5 µl of 10x RT buffer, 1 µl of RT enzyme (this was replaced with nuclease-free water for −RT), 0.19 µl of RNase inhibitor, and 0.15 µl of dNTPs (with dTTP). This was mixed thoroughly with a vortex. The total volume was amplified in order to prepare enough for all reactions. 10 µl of +RT Mastermix was added to each well, to which was then added 5 µl of RNA. 10 µl of –RT Mastermix was

added to one well then 5 µl of RNA was added. Those solutions were microfuged then placed on ice. The RT profile was: 16°C for 30 minutes, stem-loop specific; then 42°C for another 30 minutes; then 85°C for 5 minutes to kill enzyme. The final product was then stored at 4°C.

2.2.11.2 General PCR

CDNA was diluted with H_2O or RNAse-free water in a 1:5 ratio. Then, 2 Mastermixes (e.g. mir-21/U6) were prepared. For each reaction, the 7 µl Mastermix was prepared with 5 µl of TaqMan Fast Mastermix, 1.5 µl of H_2O, and 0.5 ul of forward/reverse/probe. This was vortexed then added to each well. Then, 3 µl of diluted cDNA was added to all wells except NTC wells (water), making a total volume of 10 µl per well.

2.2.12 MicroRNA expression analysis in plasma

To provide a method for TaqMan assay microRNA expression analysis from blood plasma.

2.2.12.1 Reverse transcription

The TaqMan microRNA RT Kit and Megaplex RT primers were prepared. First, all reagents were thawed on ice. Then each sample of Mastermix was prepared using the following: 0.8 µl of 10x RT primers, 0.25 µl of dNTPs, 1.5 µl of Reverse Transcriptase, 0.8 µl of 10x RT buffer, 1.03 µl of $MgCl_2$, and 0.125 µl of RNase inhibitor. This gave a total volume of 4.5 µl, to which 5.5 µl of 100 ng/3µl RNA was added, so as to have a total volume of 10 µl. This was then incubated on ice for 5 minutes. Afterwards, the Veriti thermal cycler was used in the following sequence: at 16°C for 2 minutes, at 42°C for 1 minute, 50°C for 1 second per cycle for 40 cycles, then 85°C for 5 minutes. Lastly, the temperature was set at 4°C. Similarly, the cDNA was stored at −20°C.

2.2.12.2 Pre-amplification

For this step, each sample of the Mastermix was prepared as follows: 12.5 µl of TaqMan Pre-amp Mastermix, 2.5 µl of Megaplex Pre-amp primers, and 5 µl of nuclease free water. This gave a total volume of 20 µl, to which 5 µl of cDNA was added, so as to have a total volume of 25 µl. This was then incubated on ice for 5 minutes. Afterwards, the Veriti thermal cycler was used on the reaction in the following cycling profile: 95°C for 10 minutes, 55°C for 2 minutes, 72°C for 2 minutes, 95°C for 15 seconds, 60°C for 4 minutes per cycle for 12 cycles, then 99.9°C for 10 minutes, 4°C. At this point, 75 µl of 0.1x TE with a pH of 8.0 was added to each sample. When this was done, the samples were stored at −20°C. Afterwards, the TaqMan microRNA 21 assay (pool A cDNA) was used to check the cDNA by qPCR. The pre-amplified cDNA was then diluted with a ratio of 1:5, with 3 µl utilised for each PCR. Meanwhile, the next batch of Mastermix was prepared. Each sample contained 5 µl of Fast TaqMan Mastermix and 0.5 µl TaqMan mir-21 probe assay. The 5.5 µl of Mastermix was then added to 4.5 µl of pre-amplified cDNA pool A, giving a total reaction volume of 10 µl.

2.2.12.3 TaqMan MicroRNA Array

The PCR Mastermix was prepared in a 1.5 ml Eppendorf as follows: 450 µl TaqMan Universal PCR Mastermix, no AmpErase UNG 2X, 9 µl diluted Pre-Amp product (cDNA) pool, and 441 µl nuclease free water. This gives a total volume of 900 µl. 100 µl MM was then added to each well. The 384-well TaqMan Low Density Array default thermal-cycling conditions were used to load and run the array. Analysis was carried out using SDS software. Refer to the Applied Biosystems TaqMan Array User Bulletin PN 4371129.

2.2.13 Determination of copy number alteration by qPCR in DNA isolated from plasma

qPCR based approach was applied to measure the relative gene level of different assays by analysing these in 6 breast cancer cell lines and then applied to the paired plasma cfDNA and healthy control (normal lymphocyte) samples. HGDNA was prepared and used as an internal control on each plate. It had been used as a reference sample for healthy lymphocytes and cell lines in qPCR analysis. In the CNV assay validation experiment using 6 breast cancer cell lines, both HGDNA and cell lines were diluted to 2.78 ng/µl (10 ng/3.6µl). For pre-amplification reaction, DNA from cell lines, lymphocytes and HGDNA were diluted to 0.278 ng/µl (1 ng/3.6µl). For plasma samples 10 µl of stock were used. 5 cycles pre-amplification reaction was carried out using lymphocytes and HGDNA. HGDNA was firstly diluted to 10 ng/µl, this was then used as stock for the second dilution 0.278 ng/µl, which was used for the analysis. However, as the amount of lymphocyte DNA and HGDNA is much higher than plasma cfDNA when pre-amplified for 10 cycles, DNA concentrations were further diluted to 1:5 from 0.278 ng/µl stock.

Pre-amplification primer pool (10x Pre-Amp Primers mix) mix was consisted of forward and reverse primers for 12 genes (Table 2.11) and was prepared by mixing 0.75 µl from each primer (200 pmol/µl stock), except for *CDKN2A* gene where 1.5 µl was added (100 pmol/µl stock), sterile water was then added to make the volume up to 500 µl.

Pre-amplification reaction was set up as follows:

Table 2.16 Pre-amplification reaction mixes preparation for CNV analysis using qPCR

Reagent	x1
TaqMan PreAmp Mastermix	12.5 µl
10x PreAmp Primer Mix	2.5 µl
DNA	10 µl

15 µl reaction mix was loaded into each tube (MicroAmp ™ Fast Reaction) then 10 µl of DNA was applied and placed into Gene AMp®PCR System 9700 version 3.02 thermal cycler, including the following programme: 10:00 minutes at 95°C (1 cycle), 0:15 seconds at 95°C and 4:00 minutes at 60°C (5/10 cycles) and 10 minutes at 99°C (1 cycle). The pre-amplified samples were then diluted 1:10 for qPCR analysis.

A primer and probe Mastermix (F/R/P) was prepared for the target and the reference genes as follows:

Table 2.17 Primers/probe mix preparation for CNV analysis using qPCR

Reagent	Volume
Forward primer (200 pmol/µl stock)	30 µl
Reverse primer (200 pmol/µl stock)	30 µl
MGB probe (as delivered)	20 µl
Sterile water	1320 µl

The F/R/P mix was prepared in advance, aliquotted into amber tubes and stored at −20°C. A reaction Mastermix was prepared as shown below:

Table 2.18 Reaction Mastermix preparation for CNV detection using qPCR

Reagents	X1
TaqMan Fast Mastermix	5 µl
F/R/P Mastermix	1.4 µl

A reagent Mastermix (6.4 µl) was then added to each well. Next, 3.6 µl of DNA was added to each well, and this resulted in a total volume of 10 µl. NTC was included. All samples were loaded in triplicate. The Applied Biosystems StepOne Plus machine was used for qPCR experiment and the CNV analysis setting were carried out using StepOne software v2.0'. A diagram of TaqMan PCR is shown in Figure (2.1).

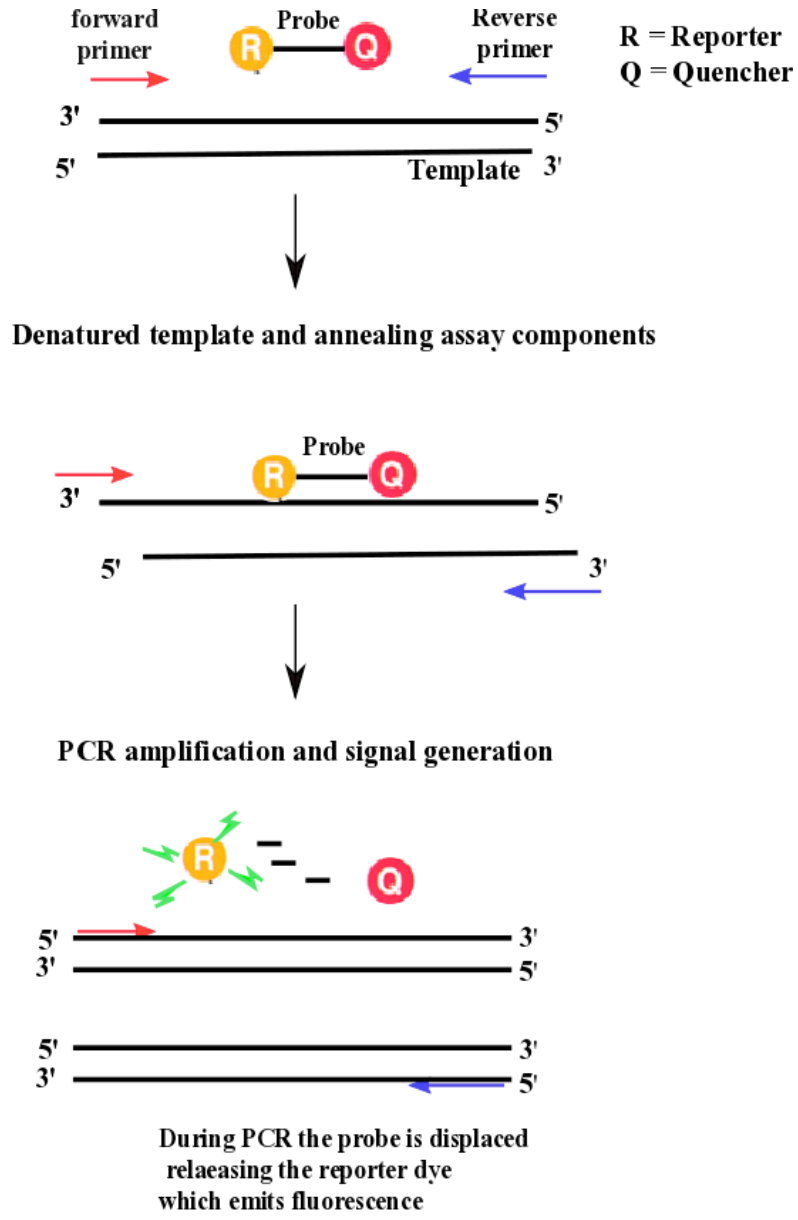


Figure 2.1 Mechanism of real time PCR Taqman assay chemistry

Adapted from Ali and Ali, 2011.

2.2.14 Determination of CNV using ddPCR

ddPCR method was performed to assess CN alterations detected by qPCR.

2.2.14.1 Sample Preparation

Each CNV target assay was run in a duplex reaction with *RPPH1* reference assay. Same preamplified plasma cfDNAs were used as for qPCR. Unamplified Lymphocyte DNAs (5 ng/5µl) were used as reference samples. HGDNA (5 ng/5µl) was used as a positive control, while no template control (NTC) was used as negative control. Briefly, a reagent Mastermix was prepared (Table 2.19). The reagents were thawed at room temperature, mixed and spun-down prior to usage.

Table 2.19 Show details of reaction mix for ddPCR

Reagents	X1
2x digital PCR supermix for probes	11 µl
20x Reference (Probe)/forward and reverse Primers	1.1 µl
20x Test (Probe)/forward and reverse Primers	1.1 µl
Sterile water	3.8 µl
Total volume	22 µl

Then, the Mastermix was vortexed for 10 seconds followed by pulse spins. 17 µl of reagent Mastermix was first aliquoted to each PCR single (0.2 ml) tube (8 tubes). 5 µl of DNA was added to the reagent Mastermix into each tube to make a total volume of 22 µl, and then all tubes were vortexed for 2-3 seconds followed by a quick spin. The eight wells must be filled when loading a cartridge, however a 1x control buffer (1:2 dilution) was used when the number of samples was less than 8.

2.2.14.2 Droplet Generation

To generate droplets, 20 µl of PCR reagents mix was transferred to the DG8^{TM} cartridge. After loading 20 µl of prepared PCR samples into individual 'sample' well (1 to 8) on the cartridge, 70 µl droplet generation oil for probes was added into the 'oil'

well. A gasket was attached to the cartridge and positioned on the QX200 droplet generator. Droplets were then transferred from the cartridge wells to a 96-well PCR plate using 5-50 µl multichannel pipette. The plate was sealed with pierceable foil sheet using Bio-Rad's PX1™ PCR plate sealer. Thereafter, the plate was positioned in a Bio-Rad's C1000 Touch™ Thermal Cycler using the following thermal cycling condition:

Table 2.20 Thermal cycling program for ddPCR

Stages and Temperature	Time	Ramp	Number of cycles
1. 95°C Enzyme activation	10 min		1
2. 94°C Denaturation	30 seconds	2°C/seconds	39
3. 59°C Annealing/ Extension	1 min	2°C/seconds	
4. Go to step 2, 39X	-	-	-
5. 98°C Enzyme deactivation	10 min	2°C/seconds	1
6. 4°C Holding	∞		

On Quantasoft software, the CNV experiment was selected as a type of experiment to enable measuring the concentration of a target relative to the concentration of a reference. The PCR plate was then placed in a droplet reader (QX 200). The QuanatSoft software was then used to analyse the information in the wells.

2.2.15 Sequencing of DNA with the Ion Torrent Personal Genome Machine

2.2.15.1 Generation of DNA library with Ion Torrent CNV Panel for Sequencing with Ion Torrent PGM

This method aims to give the methodology for the generation and multiplexing of DNA libraries for sequencing with the Ion Torrent PGM. DNA samples were quantified using Qubit® 2.0 Fluorometer.

2.2.15.2 Multiplex amplification of DNA with Ion AmpliSeq™ CNV Panel

The 5x Ion AmpliSeq™ HiFi Mix was thawed on ice, and kept on ice during the procedure and all other reagents were defrosted at room temperature. The reagents were

gently vortexed and spun-down before use. In a 0.2 ml PCR tube, the following mixture was prepared by adding reagents in the order indicated for each DNA sample being processed (Table 2.21).

Table 2.21 Mixture component for Multiplex DNA amplification with Ion AmpliSeq™ CNV Panel

Component	Cap colour	Volume (using 2x panel)
5X Ion AmpliSeq™ HiFi Master Mix	Red	4 µl
2x Ion AmpliSeq™ CNV Panel		10 µl
Genomic DNA		6 µl
Total		20 µl

The mixture was then mixed by pipetting up and down 5 times. The PCR tubes were then loaded in a PCR thermal cycler and run in the following program to amplify the target genomic region:

Table 2.22 PCR thermal cycler condition for Multiplex DNA amplification with Ion AmpliSeq™ CNV Panel

Step	Temperature	Time	Stage
Enzyme activation	99°C	2 min	Hold
Denaturation	99°C	15 sec	17 for lymphocytes 22 for plasma
Annealing and Extention	60°C	4 min	
Final Incubation	10°C	∞	Hold

PCR products could be stored at 10°C overnight.

2.2.15.3 Partial digestion of PCR primers

The FuPa reagent was thawed on ice and kept on ice throughout the procedure. 2 µl of the FuPa reagent was added to each of the amplified samples bringing the total volume up to 22 µl. The mixture was then mixed by pipetting up and down 5 times.

The PCR tubes containing the mixture were placed on a PCR thermal cycler using the following program:

Table 2.23 PCR thermal cycling program for PCR primers partial digestion

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

2.2.15.4 Ligation of adaptors

Multiple sample libraries were run on a single chip, and a unique barcode was assigned to each library using the Ion Xpress™ Barcode Adaptors 1-16 Kit. For each barcode X chosen, a mix of Ion P1 Adapter and Ion Xpress™ Barcode X was prepared at a final dilution of 1:4 for each adapter (as describes below):

Table 2.24 individual adapter mix component

Component	Volume
Nuclease-free water	4 µl
Ion P1 Adapter	2 µl
Ion Xpress™ Barcode X*	2 µl
Total volume	8 µl

The Switch Solution was then thawed at room temperature. 4 µl of the Switch Solution and 2 µl of individual adapter were combined then added to 22 µl of the digested sample bringing the total volume up to 28 µl per sample.

2 µl of DNA ligase was added to each tube. The samples were then mixed by pipette up and down 5 times.

The PCR tubes, were loaded on a thermal cycler and run with the following program:

Table 2.25 PCR thermal cycler condition for adaptors ligation

Temperature	Time
22°C	30 min
72°C	10 min
10°C	Hold (for up to 1 hour)

The procedure can be stopped at this point and samples should be stored at −20°C.

2.2.15.5 Purification of DNA libraries with AMPure beads

The AMPure® beads was brought to room temperature and vortexed thoroughly to disperse the beads before use. The solution was then pipetted slowly. Fresh 80% ethanol was prepared, by combining 310 µl of absolute ethanol with 135 µl of nuclease free water per sample. Ligated samples were removed from the thermal cycler and transferred to a 1.5 ml DNA Lo-Bind Eppendorf tube. Thereafter, 45 µl of AMPure® XP Reagent was added to each prepared library. The mixture was then pipetted up and down 5 times to thoroughly mix the beads suspension with the DNA. The mixture was incubated for 5 minutes at room temperature. The tubes were placed in a magnetic rack and incubated for 2 minutes or until the solution clears. The supernatant was carefully removed and discarded without disturbing the pellet. 200 µl of freshly prepared 80% ethanol was added and the tube was moved round while still on the magnetic rack to wash the beads, allow incubating for 2 minutes or until the solution clears. The supernatant was removed and discarded without disturbing the pellet. Those two steps were repeated for a second wash. Ensure that all ethanol droplets were removed from the tubes. The tubes were kept in the magnetic rack, air-dry the beads at room temperature for 5 minutes. The tubes were rotated in the magnetic rack to assess the dryness of the bead pellet. To maximise the recovery of the DNA from the beads, it is important not to over-dry the pellet.

2.2.15.6 Quantification and preparation of the 100 pM library

The tubes containing the libraries from the magnet (step) were removed and 50 µl of platinum® PCR SuperMix High Fidelity and 2 µl of Library Amplification Primer Mix were added to each pellet. The Library Amplification Primer Mix provided in the Ion AmpliSeq™ Library Kit, was used for multiple samples. The SuperMix and the primers could be combined before addition. The mixture was mixed thoroughly by pipetting up and down 5 times. The tubes were then placed back on the magnet for at least 2 minutes, then ~50 µl of supernatant was transferred into 200 µl PCR tubes that were placed in a thermal cycler and run with the following program:

Table 2.26 PCR thermal cycler conditions for libraries quantification

Step	Temperature	Time	Stage
Enzyme activation	98°C	2 min	Hold
Denaturation	98°C	15 sec	7 cycles
Annealing and Extension	60°C	1 min	
Final Incubation	10°C	∞	Hold

The sample could be stored at −20°C after this amplification stage.

2.2.15.7 Library Purification

The AMPure® beads were brought to room temperature and vortexed thoroughly to disperse the beads before use. The solution was pipetted slowly. Fresh 70% ethanol was prepared, by combining 315 µl of ethanol with 135 µl of nuclease–free water per sample. After amplification, the reaction was transferred to a 1.5 ml DNA Lo-Bind tube. 25 µl AMPure® XP beads was added to each 50 µl sample. The bead suspension with the DNA was mixed thoroughly by pipetting up and down 5 times. The mixtures were then incubated for 5 minutes at room temperature. The tubes were then placed in a magnetic rack for at least 5 minutes or until the solution was completely clear. The supernatant from each tube was carefully transferred to a new 1.5 ml DNA Lo-Bind tube. 60 µl of AMPure® XP beads was added to the supernatant and the bead suspension with the DNA was mixed thoroughly by pipetting up and down 5 times. The mixtures were incubated for 5 minutes at room temperature. The tubes were placed on the magnetic rack for 3 minutes or until the solution was clear. The supernatant was

carefully removed and discarded without disturbing the pellet. 150 µl of freshly prepared 70% ethanol was added and the tubes were moved round while still on the magnetic rack to wash the beads. The tubes were returned to the magnetic rack and allowed to incubate for 2 minutes or until the solution cleared. The supernatant was carefully removed and discarded without disturbing the pellet; those two steps were repeated for a second wash. All ethanol droplets were removed from the tubes and then the tubes were kept on the magnet to allow the beads to air-dry at room temperature for 5 minutes. The tubes were removed from the magnet and 50 µl of Low TE were added to the pellet to disperse the beads. The mixtures were mixed by pipetting up and down 5 times and then the tubes were placed back on the magnet for at least 2 minutes. The supernatant was removed and transferred to a clean 1.5 ml DNA Lo-Bind tube. Libraries were quantified using the Qubit® 2.0 Fluorometer as described in section (2.2.9). 10 µl of the library was used for quantification. Libraries may be stored short-term (4-8°C) or long-term (−20°C) at this stage or diluted ready for Template Preparations as follow: for each library prep, dilute 3 µl of the library to 0.013 ng/µl. For combined libraries, 10 µl of each individual library was pooled in a Lo-Bind Eppendorf. Diluted 100 pM libraries (combined and uncombined) can be stored at 4-8°C for short-term storage and −20°C for long-term storage. After that, DNA templates were prepared where amplified samples were clonally amplified around Ion Sphere Particles (ISPs) using the Ion One Touch™ 2 machine. The template positive ISPs were then amplified with the Ion One Touch™ ES machine. Subsequently, Sequencing of DNA-template ISPs was achieved using the Ion Torrent PGM. Analyses of DNA sequencing results were carried out by using the Torrent Browser.

2.3 Statistics

For this study, data obtained from qPCR were analysed to quantify gene expression levels for miRNAs and to determine CNV state by comparing the cycle threshold (CT) values of the target genes with the reference genes. These values were normalised to the reference as follows:

ΔCT= CT value of gene of interest – CT value of reference gene

In studies where several genes are selected to act as experimental controls (pooled cDNA), then the mean of these CT values normally acts as reference gene (Xia et al., 2010). Delta CT data are used to measure the relative gene expression fold change and relative quantitation value (RQ) using a reference sample (selected calibrator) (Xia et al., 2010):

$\Delta\Delta CT = \Delta CT$ sample $- \Delta CT$ calibrator, Fold Change $= 2^{-\Delta\Delta CT}$, $RQ = 2^{-\Delta\Delta CT}$

The comparative threshold (C_T) method (ΔΔCT method) was used to calculate the relative gene expression levels for miRNAs and CNV analysis. The lower CT values the higher gene expression. These analyses were performed using Microsoft Excel.

One-way analysis of variance (ANOVA) and Unpaired t-test were used to test for statistical significance (P value) between normal samples and breast cancer samples. These analyses were performed using Graphpad Prism 6.0 software.

Chapter 3

Circulating microRNA expression profiling

3.1 Introduction

The study by Volinia et al., (2006), was the first to report that small non-coding miRNAs, can serve as biomarkers of cancer because of their difference in expression between normal and cancerous cells. These miRNAs are currently being investigated not only for their cellular role but also whether they are useful circulating biomarkers. This chapter focuses on miRNA analysis in the plasma of women at high-risk of breast cancer due to an inherited *BRCA* mutation. The aim was to assess whether circulating miRNA profiles discriminate between cancers and healthy controls.

3.2 Results

3.2.1 Patients and samples

Plasma samples investigated in this chapter were from women with a family history of breast cancer, and screened for either *BRCA1* or *BRCA2* mutation. Patients were grouped by their mutation status and incidence of breast cancer. Women from both Leicester and London were also recruited as healthy female controls. The study cohort comprised of a total of 97 women with a family history of breast cancer (8 groups), 6 women with sporadic breast cancer, and 12 healthy controls (Table 3.1).

Table 3.1 Patients and samples analysed in this thesis. The cohort comprised a total of 97 women with a family history of breast cancer (53 women had *BRCA* family and 44 women who are negative for *BRCA* mutation or not tested for *BRCA* mutation), 6 women with sporadic breast cancer, and 12 healthy controls

Name of group	Description
Healthy controls	(12) Healthy female controls (5) from Leicester and (7) from London
BRCA1 carrier	Women with a *BRCA1* germline mutation, no evidence of breast cancer (n=13)
BRCA2 carrier	Women with a *BRCA2* germline mutation, no evidence of breast cancer (n=8)
BRCA1 affected	Women with *BRCA1* gene mutation, who have developed breast cancer. Blood sample taken after treatment (n=9)
BRCA2 affected	Women with *BRCA2* gene mutation, who have developed breast cancer. Blood sample taken after treatment (n=6)
Female pre-surgical BC (FPSX)	Sporadic primary breast cancer (no *BRCA* mutation), pre-surgical blood (n=6)
BRCA1 PNEG	*BRCA1* family, no germline mutation, no evidence of breast cancer (n=11) (Predictive negative= PNEG)

BRCA2 PNEG	*BRCA2* family, no germline mutation, no evidence of breast cancer (n=6) (Predictive negative= PNEG)
Controls	Not tested, no cancer (n=6); or no cancer, no mutation detected in relative (n=16)
Cancers	Have had cancer but no *BRCA* mutation (n=21); or have had cancer but not tested for *BRCA* (n=1)

3.2.2 TaqMan array discovery

The TaqMan array miRNA discovery study was carried out by Dr. Shona Elshaw as part of a large study funded by Hope Against Cancer. This analysis used TaqMan Microfluidic cards to assess expression of 384 miRNAs in pooled plasma from different at risk groups (Table 3.2).

Table 3.2 Details of pooled samples for each group

Sample Group	n samples per pool
Healthy controls	6
BRCA1 carrier	6
BRCA2 carrier	6
BRCA1 affected	6
BRCA2 affected	6
BRCA1 PNEG	6
BRCA2 PNEG	6
FPSX	6

Working with Dr. Shona Elshaw I carried out analysis of the miRNA card data as follows: CT values more than >34.999 were removed. Then, the average CT values were calculated for each pooled card and subtracted from each miRNA value to give a ΔCT for each pooled sample card and ΔΔCT was then calculated (ΔCT (*BRCA* affected) − ΔCT (healthy control)). Of 384 miRNAs analysed, 31 miRNAs were up-regulated in the *BRCA1* carrier and 125 miRNAs were up-regulated in the *BRCA1* affected group, compared to the healthy controls; overall 70 miRNAs were up-regulated in the *BRCA1* affected group compared to the *BRCA1* carrier group; 73 miRNAs were up-regulated in

the *BRCA2* carrier, and 111 miRNAs were up-regulated in the *BRCA2* affected group versus healthy controls; and 66 miRNAs were up-regulated in the *BRCA2* affected group compared to the *BRCA2* carrier group. Additionally, 31 miRNAs were up-regulated in all breast cancer groups including *BRCA1&2* PNEG, *BRCA1&2* carrier, and *BRCA1&2* affected compared to the healthy controls. Lastly, *BRCA1&2* PNEG, *BRCA1&2* carrier, and *BRCA1&2* affected data were combined to PNEG, *BRCA* carrier, and *BRCA* affected respectively, in order to have a larger set of samples by group which could give better results.

Table 3.3 MiRNAs identified as showing significant differences between groups

ΔCT for each sample was calculated as follows: CT value for individual miRNA − the average CT values for each group; ΔΔCT=ΔCT (*BRCA* affected group) − ΔCT (healthy controls); fold change=$2^{-\Delta\Delta CT}$ (>1-fold).

miRNA	Group/ Card					
	Healthy controls	*BRCA* carrier	*BRCA* affected	FPSX	MiRNAs up-regulated in *BRCA* affected vs. Healthy Control	
	ΔCT	ΔCT	ΔCT	ΔCT	ΔΔCT	Fold Change
hsa-mir-15b-000390	-4.2419	-3.0810	-5.7819	-5.2938	-1.5399	2.9
hsa-mir-20a-000580	-7.9359	-7.4916	-9.0780	-7.3779	-1.1421	2.2
hsa-mir-21-000397	-4.4928	-4.1868	-6.3309	-5.7006	-1.8381	3.6
hsa-mir-22-000398	2.8490	2.6534	-2.1029	-1.3724	-4.9519	31.0
hsa-mir-26a-000405	-5.5335	-5.0888	-7.7200	-6.2246	-2.1865	4.6
hsa-let-7a-000377	-4.1136	-3.9808	-6.0341	-4.1618	-1.9205	3.8
hsa-let-7f-000382	0.1720	-0.6407	-2.7839	-0.8482	-2.9559	7.8
hsa-mir-27a-000408	-2.3273	-2.5749	-5.3085	-3.2768	-2.9812	7.9
hsa-mir-27b-000409	-0.2453	-1.1348	-3.4740	-1.8993	-3.2287	9.4
hsa-mir-34a-000426	5.2287	1.5251	0.7613	2.4181	-4.4674	22.1
hsa-mir-130a-000454	-0.9951	-1.7316	-4.4370	-2.3801	-3.4418	10.9
hsa-mir-130b-000456	-1.0234	-0.8123	-2.6957	-1.3648	-1.6722	3.2
hsa-mir-142-5p-002248	-0.2565	0.9562	-2.3963	-1.7062	-2.1397	4.4
hsa-mir-221-000524	-6.5324	-6.0270	-7.6757	-5.7062	-1.1433	2.2
hsa-mir-324-3p-002161	-5.1311	-9.2222	-7.6780	-0.5050	-2.5468	5.8
hsa-mir-338-3p-002252	4.0738	4.9951	3.0273	2.2429	-1.0465	2.1
hsa-mir-361-000554	2.9999	0.8432	-2.0001	-0.9449	-4.9999	32.0
mmu-mir-374-5p-001319	-4.8389	-3.4357	-6.0331	-4.9526	-1.1942	2.3
hsa-mir-23b-000400	2.9451	4.8741	2.0611	1.1131	-0.8840	1.8
hsa-mir-26b-000407	-3.4017	-3.6632	-5.7509	-4.2897	-2.3492	5.1
hsa-mir-195-000494	3.7083	3.5502	3.0040	-2.3017	-0.7043	1.6
hsa-mir-181a-000480	0.6527	-0.4015	-2.1165	0.1131	-2.7692	6.8
hsa-mir-100-000437	3.9197	4.2673	0.2842	0.8896	-3.6354	12.4
hsa-mir-511-001111	9.8037	8.4609	5.4902	7.0370	-4.3135	19.9
hsa-mir-143-002249	-0.2514	-0.3351	-3.3089	-0.9564	-3.0575	8.3

25 miRNAs were significantly up-regulated in one or more cancer groups compared to the healthy controls (Table 3.3). Of these mir-22, mir-34a, mir-361, and mir-511 had the highest fold change (Table 3.3). A literature survey revealed a known cancer associated role for all 25 miRNAs as either tumour suppressor (18) or oncogenic role (7) (Table 3.4).

Table 3.4 25 miRNAs that were up-regulated in individuals with a family history of breast cancer versus healthy controls

miRNA	Chromosome location	Reference	Breast cancer	Samples
hsa-mir-15b-000390	13q14	Kedmi et al., 2015	Tumour suppressor - targets the *BCL2* and the metastasis suppressor protein 1 on mammary epithelia cells	Nontransformed mammary epithelial cell lines
hsa-mir-20a-000580	13q31.3	Li et al. 2012	Oncogene - negatively correlated with the target VEGF and HIF1A proteins	Breast tissues from 48 patients (32 patients with breast cancer)
hsa-mir-21-000397	17q23.1	Walter, 2011	Oncogene - associated with cell proliferation. Breast cancer progression and metastasis formation in PABC	Cancer tissues from 25 pregnancy-associated breast cancer (PABC)
hsa-mir-22-000398	17p13.3	Pandey et al., 2015	Oncogene – high-levels of mir-22 are associated with poor OS, facilitates pithelial-mesenchymal transition	Metastatic breast cancer cell line TIP60
hsa-mir-26a-000405	12q14.1	Gao et al., 2013	Tumour suppressor - mir-26a inhibits proliferation and migration of breast cancer through repression of *MCL1*	In 52 breast cancer specimens and 29 normal breast tissues. MDA-MB-231, MCF-7, MDA-MB-435, MDA-MB-468.
hsa-let-7a-000377	9q22.32	Kim et al., 2012	Tumour suppressor - suppressing migration and invasion of human breast cancer cells	Breast cancer cell lines MDA-MB-231 and MCF-7 and breast cancer patient tissues
hsa-let-7f-000382	9q21.32	Tao et al., 2015	Tumour suppressor - let-7f inhibition contributed to the up-regulation of *TSP-1* in paclitaxel LDM therapy, and apoptosis of breast cancer cells	LDM and MTD tissues in 4T1 mouse breast cancer model
hsa-mir-27a-000408	19p13.12	Han, 2015	Tumour suppressor - higher mir-27a expression in 2 TNBC cell lines than in human breast epithelial cell line. mir-27a could modulate proliferation and radiosensitivity of TNBC cells	TNBC cell lines MDA-MB-435 and MDA-MB-231 and in normal human breast epithelial cell line MCF10A
hsa-mir-27b-000409	9q22.32	Takahashi et al., 2014	Oncogene - T2D-associated gene plays an important role in tumour development	Normal and luminal-type breast cancer tissues
hsa-mir-34a-000426	1p36.22	Adams et al., 2015	Tumour suppressor - mir-34a in TNBC promotes anti-tumourigenic phenotypes both in vitro and in vivo, and could therefore be used as a therapeutic agent to treat the disease	TNBC cell lines compared to both a luminal cancer subtype as well as normal breast cells
hsa-mir-130a-000454	11q12.1	Stückrath et al., 2015	Oncogene - influenced the migration and invasion behavior of MCF-7 and MDA-MB-231 cell lines	Plasma of 111 breast cancer patients
hsa-mir-130b-	22q11.21	Chen et al.,	Tumour suppressor - alteration of the gene expression levels of neuropilin 1	Epithelial ovarian cancer cells

000456		2015	(*NRP1*), may contribute to MDR in epithelial ovarian cancer	
hsa-mir-142-5p-002248	17q22	Ghanbari et al., 2015	Tumour suppressor – down-regulation of plasma mir-142-3p and mir-26a-5p	Plasma samples of 37 colorectal cancer patients and 8 normal subjects using miRNA microarray
hsa-mir-221-000524	Xp11.3	Roscigno et al., 2015	Oncogene - transfection of mir-221 in T-47D cell lines increased the number of mammospheres and the expression of stem cell markers	Mammospheres/breast cancer cells from T-47D cell lines
hsa-mir-324-3p-002161	13q31.3	Hu et al., 2012	Tumour suppressor - diagnostic marker in breast cancer	Serum from 50 individual samples f different cancer traits and healthy controls
hsa-mir-338-3p-002252	17q25.3	Huang et al., 2011	Tumour suppressor - SK-HEP-1 cells suppressed cell migration and invasion, whereas inhibition of mir-338-3p in SMMC-7721 cells induced cell migration and invasion	Hepatocellular carcinoma cell lines
hsa-mir-361-000554	17p13.3	Sun et al., 2014; Inns & James, 2015	Tumour suppressor – involved in up-regulation of breast cancer	Serum from breast cancer cells
mmu-mir-374-5p-001319	13q31.3	Tan et al., 2015	Tumour suppressor - mir-671-5p reduces DNA repair capability in post-drug exposed breast cancer cells	Tissues from IDC
hsa-mir-23b-000400	9q22.32	Avci et al., 2015	Oncogene - mir-23b was up-regulated in MCF-7 breast cancer cell line after genistein treatment	MCF-7 breast cancer cell lines
hsa-mir-26b-000407	12q14.1	Verghese et al., 2013	Tumour suppressor - mir-26b in breast CAFs is a potent regulator of cancer behaviour in oestrogen receptor-positive cancers	MCF-7 breast cancer epithelial cell lines
hsa-mir-195-000494	17p13.1	Weng, 2013	Tumour suppressor - expression of mir-195 or knockdown of Raf-1 can similarly reduce tumour cell survival but increase apoptosis through down-regulation of Raf-1 and Bcl-2 and P-glycoprotein expression	Breast cancer cell lines and tissue specimens (obtained from chemotherapy-sensitive or resistant patients) as well as a normal breast cell line
hsa-mir-181a-000480	1q32.1	Zhang and Ma, 2012	Tumour suppressor - serum mir-181a may improve the sensitivity of BC screening	Serum mir-181a levels in breast cancer patients and healthy controls
hsa-mir-100-000437	11q24.1	Gong et al., 2015	Tumour suppression - overexpression of mir-100 led to the proliferation inhibition of the mir-100-down-regulated breast cancer cells	SK-BR-3 cell line compared with other human breast cancer cell lines (MCF7, MDA-MB-453, T-47D, HCC1954, and SUM149)
hsa-mir-511-001111	12q13.3	Zhang et al., 2012	Tumour suppressor - suppress A549 cell proliferation by suppressing *TRIB2* and further increasing C/EBPα expression	A549 cell lines
hsa-mir-143-002249	5q32	Jiang et al., 2012; Fang et al., 2012	Tumour suppressor - controls glycolysis by regulating hexokinase 2 in breast cancer cells	HEK293T, ZR-75-30, MDA-MB-231, MCF-7, MDA-MB-453, BT-474, and SK-BR-3 cell lines

In summary, 25 miRNAs were significantly up-regulated in the *BRCA* affected group compared to controls and considered as candidates for analysis in individual samples.

3.2.3 Card data Validation

In order to look at the variation between individual samples and pooled samples, Microfluidic card assays were run for plasma from 8 healthy controls and compared to the pooled control card using (xy) correlation analysis. Individual card assays and the pooled card were highly correlated, with little variability between different cards (Table 3.5; Figures 3.1; 3.2). Overall, the pooled samples were representative of the combined individual samples and suggest we can achieve data for individual or pooled plasma samples.

Table 3.5 Comparison between individual and pooled card data

Correlation analysis between individual card values and the pooled card; and the mean value (card 1-8) compared to the pooled card. There was a good correlation between 8 individuals/mean 1-8 card and pooled card.

Parameter	Card1	Card2	Card3	Card4	Card5	Card6	Card7	Card8	Mean 1-8
Pearson r	0.88	0.89	0.90	0.88	0.86	0.88	0.89	0.88	0.90
95% confidence interval	0.85 to 0.90	0.88 to 0.91	0.88 to 0.92	0.85 to 0.90	0.83 to 0.89	0.85 to 0.89	0.86 to 0.91	0.86 to 0.90	0.89 to 0.92
P value (two-tailed)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
P value summary	***	***	***	***	***	***	***	***	***
Is the correlation significant alpha=0.05	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
R squared	0.78	0.81	0.81	0.77	0.74	0.77	0.79	0.78	0.82

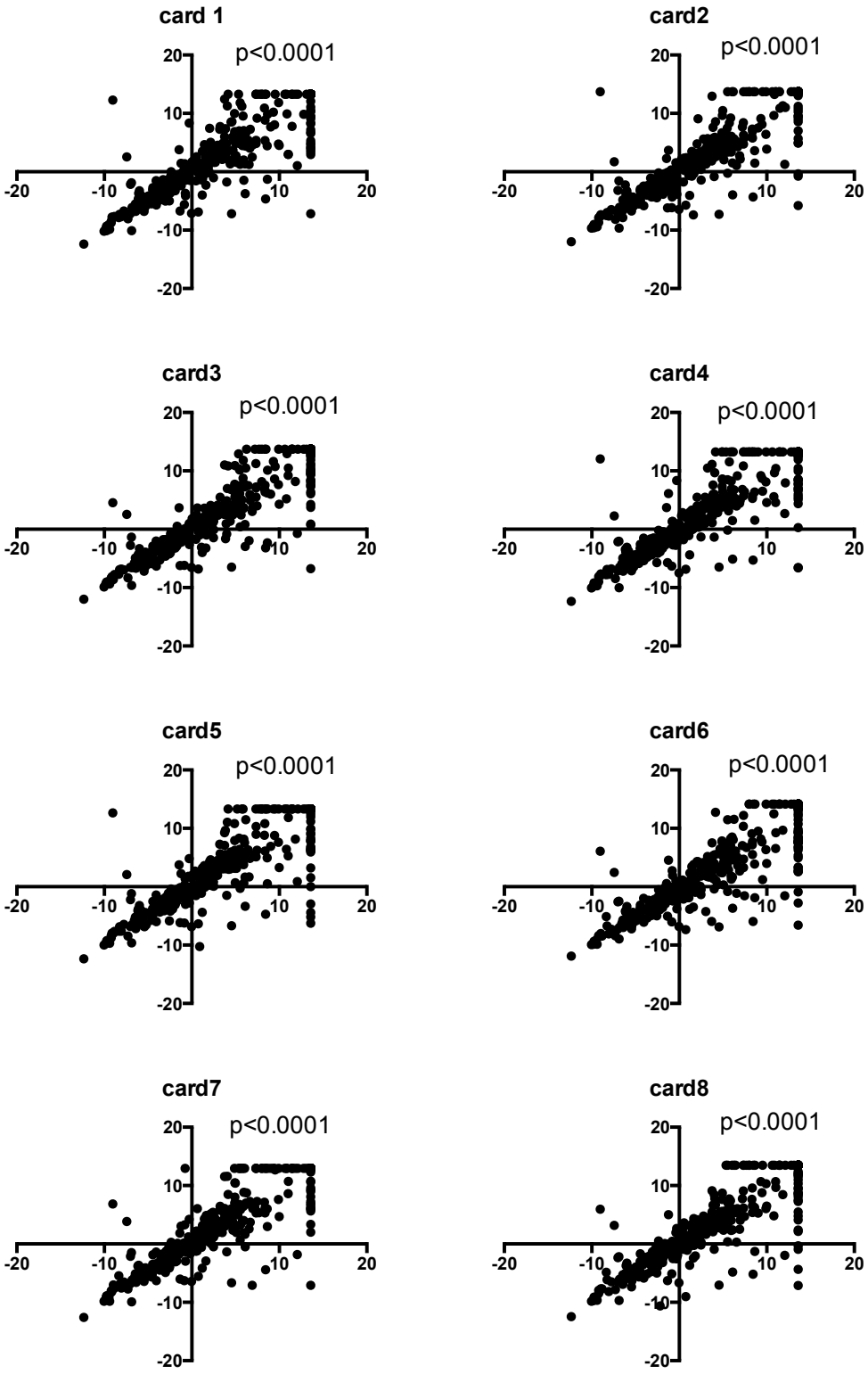


Figure 3.1 Correlation analysis between miRNA expression on each individual healthy control card and pooled controls card

8 healthy control samples were analysed individually using TaqMan miRNA cards. Card data was generated and compared to results from the healthy control pool using (xy) correlation analysis. X-axis= data from pooled normal card, Y-axis= data from each individual card. There was generally good correlation between miRNA expression of individual card data and data from the pooled normal card. Dots represent ΔCT values, $p < 0.05$ significant.

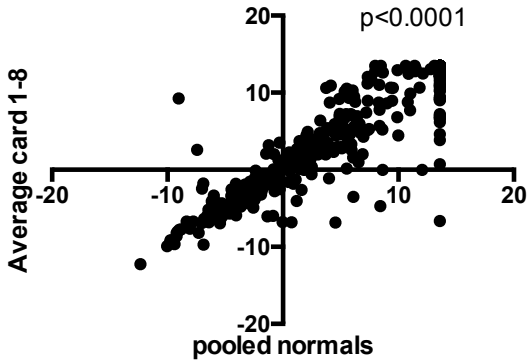


Figure 3.2 Correlation between miRNA expressions of the mean values of single cards versus pooled control values

Average card data of 8 healthy control samples were analysed after run individual samples using TaqMan miRNA cards. Average card data was compared to results from the healthy control pool using (xy) correlation analysis. X-axis= data from pooled normal card, Y-axis= average data for 8 individual card. There was generally good correlation between miRNA expressions of average card data and data from the pooled normal card. Dots represent ΔCT values, $p< 0.0001$.

In summary, a comparison of different card data suggests little variation between cards and individual samples in a pool.

3.2.4 qPCR assessment of single miRNA assays in cell lines

Eight candidate miRNAs (mir-20a, mir-21, mir-22, mir-27a, mir-27b, mir-130a, mir-130b, and mir-100) that were previously identified in pooled plasma samples were chosen for validation in the breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T47-D), using qPCR. RNA was extracted from the 6 breast cell lines using a phenol based extraction method and converted to cDNA applying reverse transcriptase. MiRNA assays were run in triplicate in order to assess reproducibility. The ΔCT for each cell line sample was calculated by taking the average CTs of the 3 replicates for U6 (reference gene) from individual CT for each miRNA (CT<35), where ΔΔCT= ΔCT (cancer) − ΔCT (control) (HBL-100 breast cell line was used as a control). The relative expression was calculated using the $(2^{-\Delta\Delta CT})$ method.

Results showed that CT values were consistent across triplicates for each miRNA assay indicating that those miRNA assays are reproducible (Table 3.7). All 8 miRNAs were previously identified as being significantly increased in the cancer groups compared to the control groups in pooled cDNA in plasma. However the expression levels in breast cell lines were different. The expressions of 6 miRNAs (mir-20a, mir-21, mir-27b, mir-130a, mir-130b, and mir-100) were significantly increased in the MDA-MB-468 cell line as compared to the normal breast cell line (HBL-100). Mir-22 was not detected in the MDA-MB-468 cell line, and mir-27a was not significantly expressed (Table 3.6; Figure 3.3).

In regards to the MDA-MB-231 breast cancer cell line, the expressions of 3 miRNAs (mir-21, mir-130a, and mir-130b) were down-regulated compared to the HBL-100 cell line. Two other miRNAs: mir-27b and mir-100 were significantly up-regulated compared to the HBL-100 cell line. Mir-20a, mir-22, and mir-27a were not significantly different.

Out of the 8 miRNAs investigated by qPCR in MCF-7 cell line, two (mir-20a and mir-21) showed a notable difference between normal and cancer cell line, as the expressions were higher in the MCF-7 cell line compared to the HBL-100 cell line. Five miRNAs (mir-22, mir-27a, mir-27b, mir-130b, and mir-100) were not significantly expressed. Mir-130a showed lower level of expression in the MCF-7 cell line compared to the HBL-100 cell line.

Mir-21 revealed higher levels of expression in the ZR-75-1 cell line, whereas mir-27b and mir-130a showed lower levels of expression compared to the HBL-100 cell line. The expressions of other miRNAs involving; mir-20a, mir-22, mir-27a, mir-130b, and mir-100 were not significantly different compared to the HBL-100 cell line.

The T-47D breast cancer cell line displayed different results, with some instances of up-regulation as shown in mir-130a and mir-130b. Down-regulation occurred in mir-21 and mir-27b. However, mir-20a, mir-22, mir-27a, and mir-100 did not show significant levels of expression. The expressions data is summarised in Table (3.6).

Table 3.6 miRNAs expression in breast cancer cell lines using qPCR

P values of the ANOVA test showing significant levels between breast cancer cell lines and normal breast cell line (HBL-100); ns: not significant; P<0.05 significant; ER: oestrogen receptor.

MiRNA	Cell line names / Change / Significant relative to HBL-100			
	ER− cell lines			
	231	Fold change	468	Fold change
hsa-mir-20a-000580	ns	0.78	Increase P<0.0001	11.45
hsa-mir-21-000397	Decrease P<0.0001	0.29	Increase P<0.05	1.88
hsa-mir-22-000398	ns	0.47	-	-
hsa-mir-27a-000408	ns	1.72	ns	12.44
hsa-mir-27b-000409	Increase P<0.0001	2.21	Increase P<0.0001	7.61
hsa-mir-130a-000454	Decrease P<0.0001	0.58	Increase P<0.0001	16.96
hsa-mir-130b-000456	Decrease P<0.001	0.34	Increase P<0.0001	5.60
hsa-mir-100-000437	Increase P<0.0001	72.92	Increase P<0.0001	7.73

MiRNA	ER+ cell lines					
	MCF7	Fold change	ZR75-1	Fold change	T-47D	Fold change
hsa-mir-20a-000580	Increase P<0.0001	4.11	ns	1.78	ns	1.41
hsa-mir-21-000397	Increase P<0.00001	20.19	Increase P<0.05	2.22	Decrease P<0.0001	0.28
hsa-mir-22-000398	ns	2.19	ns	0.74	ns	0.42
hsa-mir-27a-000408	ns	6.05	ns	2.84	ns	1.02
hsa-mir-27b-000409	ns	0.83	Decrease P<0.0001	0.52	Decrease P<0.05	0.73
hsa-mir-130a-000454	Decrease P<0.0001	0.27	Decrease P<0.00001	0.18	Increase P<0.0001	5.43
hsa-mir-130b-000456	ns	0.92	ns	0.58	Increase P<0.0001	3.02
hsa-mir-100-000437	ns	3.24	ns	1.11	ns	2.43

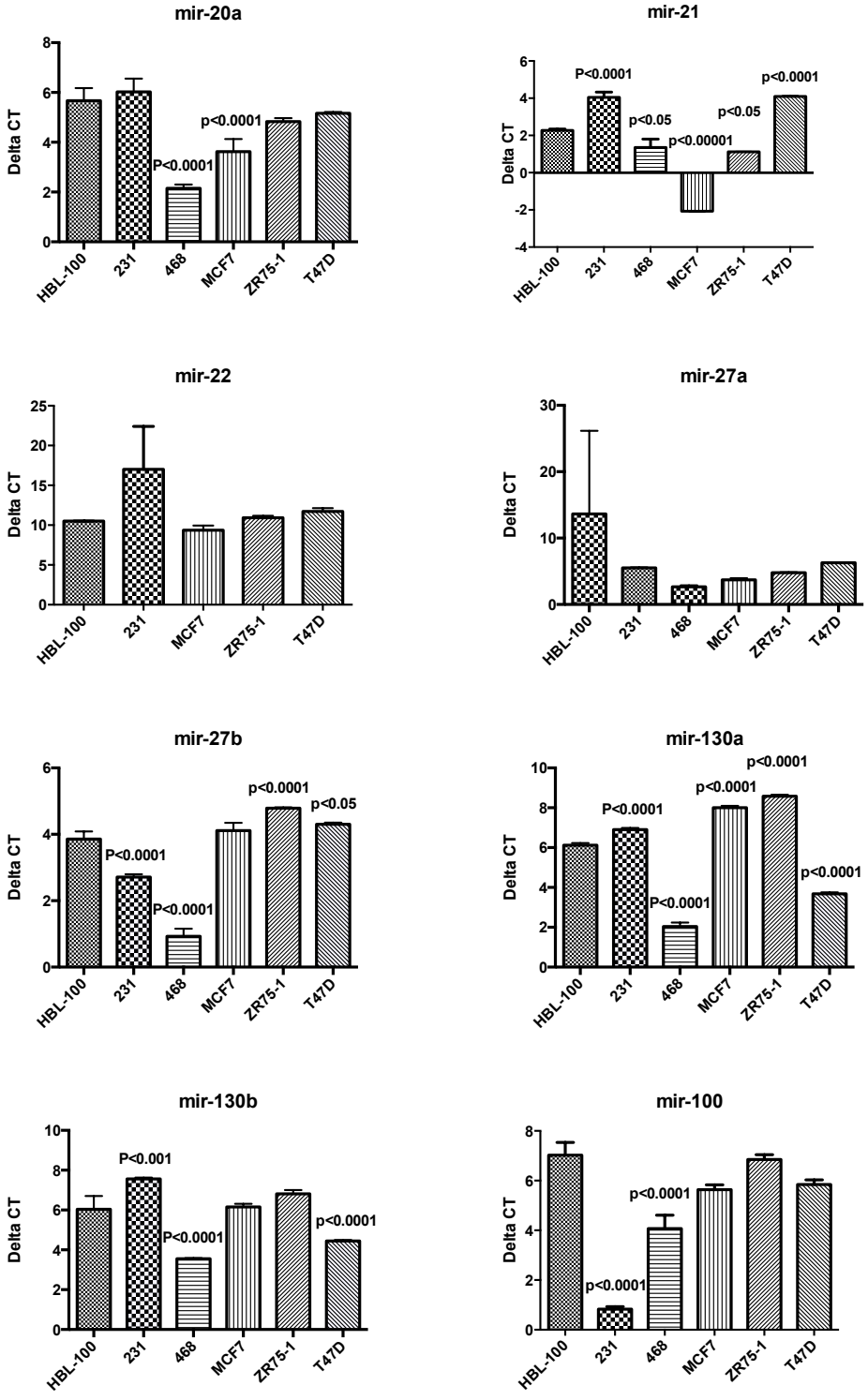


Figure 3.3 Expression of candidate miRNAs in breast cancer cell lines

ER+ cell lines (MCF7, ZR-75-1, and T-47D); ER− cell lines (MDA-MB-231 and MDA-MB-468); and normal breast cell line (HBL-100). P values of the ANOVA test show significant levels between breast cancer cell lines and normal breast cell line; the ΔCT for each sample were calculated by subtracting the average CT value of 3 replicates of U6 reference from each individual miRNA value; $p<0.05$ significant. Error bars represent the mean with SD.

Table 3.7 Validation of 8 selected miRNAs in 6 breast cell lines using qPCR

MiRNA	HBL-100	MDA-MB-231	MDA-MB-468	MCF-7	ZR75-1	T-47D
	CT	CT	CT	CT	CT	CT
Mir-20a	26.86	25.93	24.39	26.95	27.32	27.89
	27.76	26.96	24.53	26.76	27.16	27.88
	27.72	26.22	24.70	25.99	27.06	27.80
Mir-21	23.99	23.92	23.37	20.85	23.48	26.75
	24.21	24.35	23.22	20.95	23.43	26.85
	23.95	24.89	24.65	20.82	23.48	26.77
Mir-22	32.26	31.87	Undetermined	33.43	33.57	35.19
	32.29	31.92	Undetermined	31.55	32.79	33.77
	32.29	31.96	Undetermined	31.95	33.46	34.30
Mir-27a	28.33	25.94	25.32	26.94	27.07	28.97
	28.06	25.87	24.93	26.42	27.20	28.94
	27.87	25.80	24.94	26.58	27.16	28.99
Mir-27b	25.85	23.01	23.59	27.32	27.13	27.05
	25.67	23.01	23.17	26.97	27.11	26.95
	25.39	23.15	23.21	26.87	27.16	26.98
Mir-130a	27.97	27.31	24.30	30.94	31.01	26.29
	27.96	27.28	24.66	30.86	30.88	26.37
	27.77	27.15	24.33	31.04	30.89	26.44
Mir-130b	28.14	Undetermined	25.95	29.27	29.32	27.14
	28.26	27.95	25.97	29.00	29.20	27.08
	27.05	27.86	25.91	29.00	28.94	27.19
Mir-100	29.80	21.31	25.40	27.97	28.89	28.18
	28.51	21.23	27.13	28.63	29.17	28.31
	28.09	20.99	26.86	28.18	29.56	28.80

In summary, qPCR analysis of individual miRNA assays showed different levels of expression between breast cancer and non-tumourgenic cell lines. The results in cell lines confirmed that all tested miRNA assays were reproducible across 3 replicates (CT<35), and could be applied to individual plasma samples.

3.2.5 Stability of cDNA and plasma profiles

3.2.5.1 A comparison of plasma CT and ΔCT miRNA levels using two selected reference miRNAs

U6 and mir-484 were evaluated as reference genes and were selected based on our previous study by Dr. Shona Elshaw and other studies as the most commonly used and more stable reference genes for miRNA normalisation (Peltier and Latham, 2008; McDermott et al., 2013). The aim was to identify stably expressed reference miRNAs to measure candidate miRNAs expression levels that could differentiate between cancers and controls. As part of reference miRNA validation, I chose to compare 3 experiments at different time points to determine recovery of miRNAs in storage of cDNAs and plasma. MiRNA levels in the circulation of women with a known *BRCA* mutation (*BRCA* carrier (n=5) and *BRCA* affected (n=4)) and healthy mutation-negative control (PNEG (n=4)) were measured by qPCR. Plasma was stored for 12 months at −80°C before RNA extraction with the mirVana™ miRNA isolation Kit, reverse transcribed to cDNAs using the TaqMan Megaplex RT system followed by pre-amplification using the TaqMan Megaplex Pre-Amp system, then quantified by qPCR (first samples). CDNAs were then stored in the freezer at −20°C and re-run after 6 months (second samples). Afterwards, RNAs were re-extracted from the same sample numbers stored at −80°C for 2 years, and processed using the same approaches (third samples). The analysis was performed based on the CT values to examine the expression stability of U6 and mir-484. Next, miRNAs of interest were compared relative to references by ΔCT to see if there were any changes.

Little variability existed between the first samples and the second samples. Regarding mir-484, CT values were higher in the second samples compared to the first samples; whereas plasma levels in the U6 declined in the second samples compared to the first samples. Nonetheless, it is clear that the CT values after re-extraction (third samples) are coming out at a later stage for the two reference genes (U6 and mir-484) compared to the first and the second samples. Generally, no significant difference in expression existed among the three samples using ANOVA tests with the exception of second versus third cDNAs for U6 in the *BRCA* carrier group and first versus third cDNAs for

mir-484 in the *BRCA* affected group. A combination of U6 and mir-484 reference genes generated more reliable results than did using either miRNA alone (Figure 3.4; Table 3.8). However, because of the effects of plasma storage leading to lower recovery of miRNAs and therefore change in CTs, it was important to normalise data relative to reference miRNAs (U6 and mir-484).

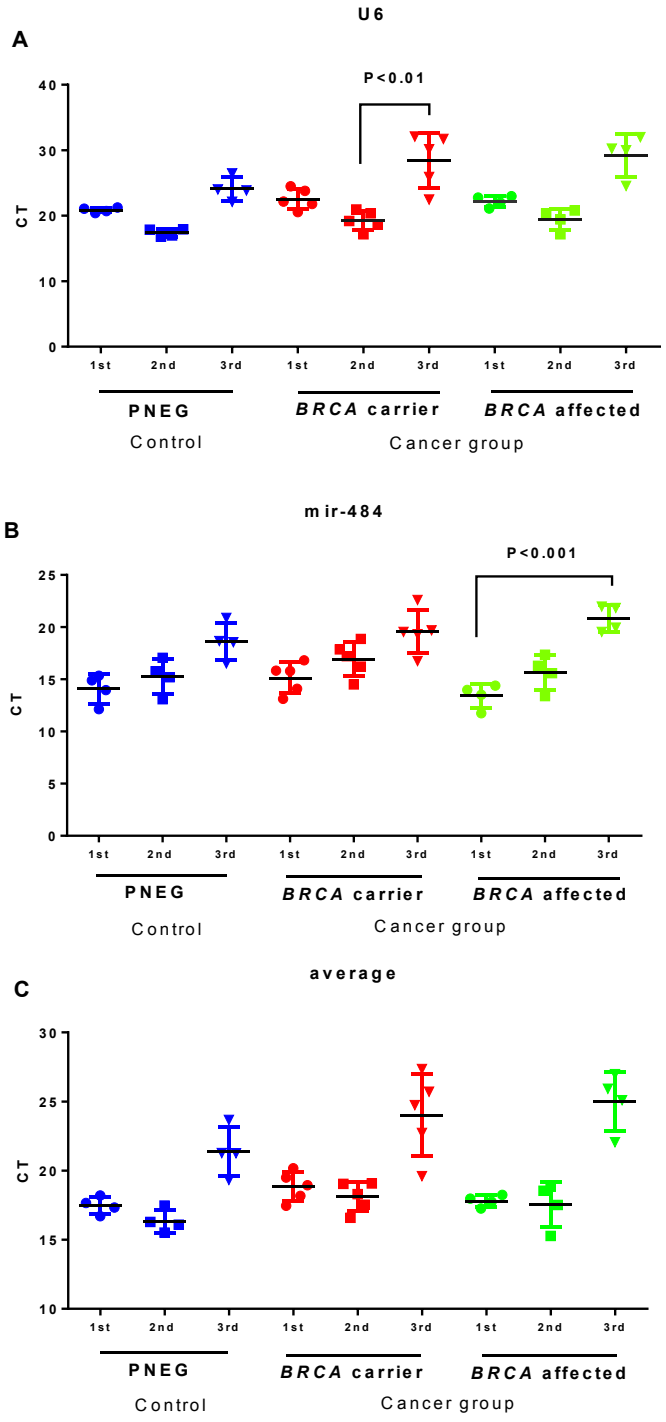


Figure 3.4 Validation of U6 and mir-484 reference genes across 3 experimental conditions

Scatter plots show un-normalised CT values of (A) U6, (B) mir-484, and (C) the average of both, across 3 experimental states in plasma samples from women from 3 risk groups (PNEG n=4, *BRCA* carrier n=5, and *BRCA* affected n=4). The ANOVA test was used to determine significant differences across the runs. 1st: first experiment (cDNA run after extraction); 2nd: same cDNAs were re-run after 6 months of storage (−20°C); 3rd: RNAs were re-prepped after 2 years of storage (−80°C), reverse transcribed to cDNAs, re-quantified by qPCR, and re-analysed using same cohort group and number, $p< 0.05$ significant. Error bars represent the mean with SD.

Table 3.8 Data for the CT was measured via the Kruskal-Wallis test across 3 different experiments for U6, mir-484, and the average of both in 3 risk groups

1st: first cDNAs run after extraction; 2nd: same cDNAs re-run after 6 months of storage; and 3rd: third cDNAs the data after re-extraction after 2 years of storage.

Group name	PNEG			*BRCA* CARRIER			*BRCA* AFFECTED		
Mir name	CT value			CT value			CT value		
	1st vs 2nd	1st vs 3rd	2nd vs 3rd	1st vs 2nd	1st vs 3rd	2nd vs 3rd	1st vs 2nd	1st vs 3rd	2nd vs 3rd
U6	Ns	Ns	Ns	Ns	Ns	P<0.01	Ns	Ns	Ns
Mir-484	Ns	Ns	Ns	Ns	Ns	Ns	Ns	P<0.001	Ns
Average	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns

Having shown that cDNA is stable but miRNAs degrade in plasma with time in storage, we then compared 8 selected miRNAs relative to the single references (mir-484 (Figure 3.5) and U6) and to the average of the two to determine which approach was best for normalisation of data between samples. The one-way ANOVA test was used to determine statistically significant differences between the 3 experiments (1st: first cDNA run after extraction), (2nd: second cDNA re-run 6 months later), and (3rd: third cDNA re-run after re-extraction). The analysis of 8 candidate miRNAs based on ΔCT gave more reliable data. The results for mir-484 and the combination of two reference genes (mir-484/U6) would be considered as demonstrating good abundance with relatively stable expression as no significant difference exists across different runs in all groups. The only significant difference was related to U6 in the *BRCA* carrier group for mir-21, mir-27b, and mir-100 (Table 3.9). These findings indicated that although the miRNAs declined on storage, the ratio of each miRNA, when normalised to the mean of two reference miRNAs and to mir-484 as a single reference, was stable with time (Figure 3.5). The only differences were using U6 as a single reference; mir-484 and the average of mir-484/U6 were stable. Since mir-484 was identified as stable reference miRNA, it was used for subsequent analysis in single plasma samples. Overall, when data is normalised the majority of results agree, as there were no significant differences in ΔCT between the first to second or third and the second to the third, which suggest mir-484 is stably expressed and could enable accurate data normalisation.

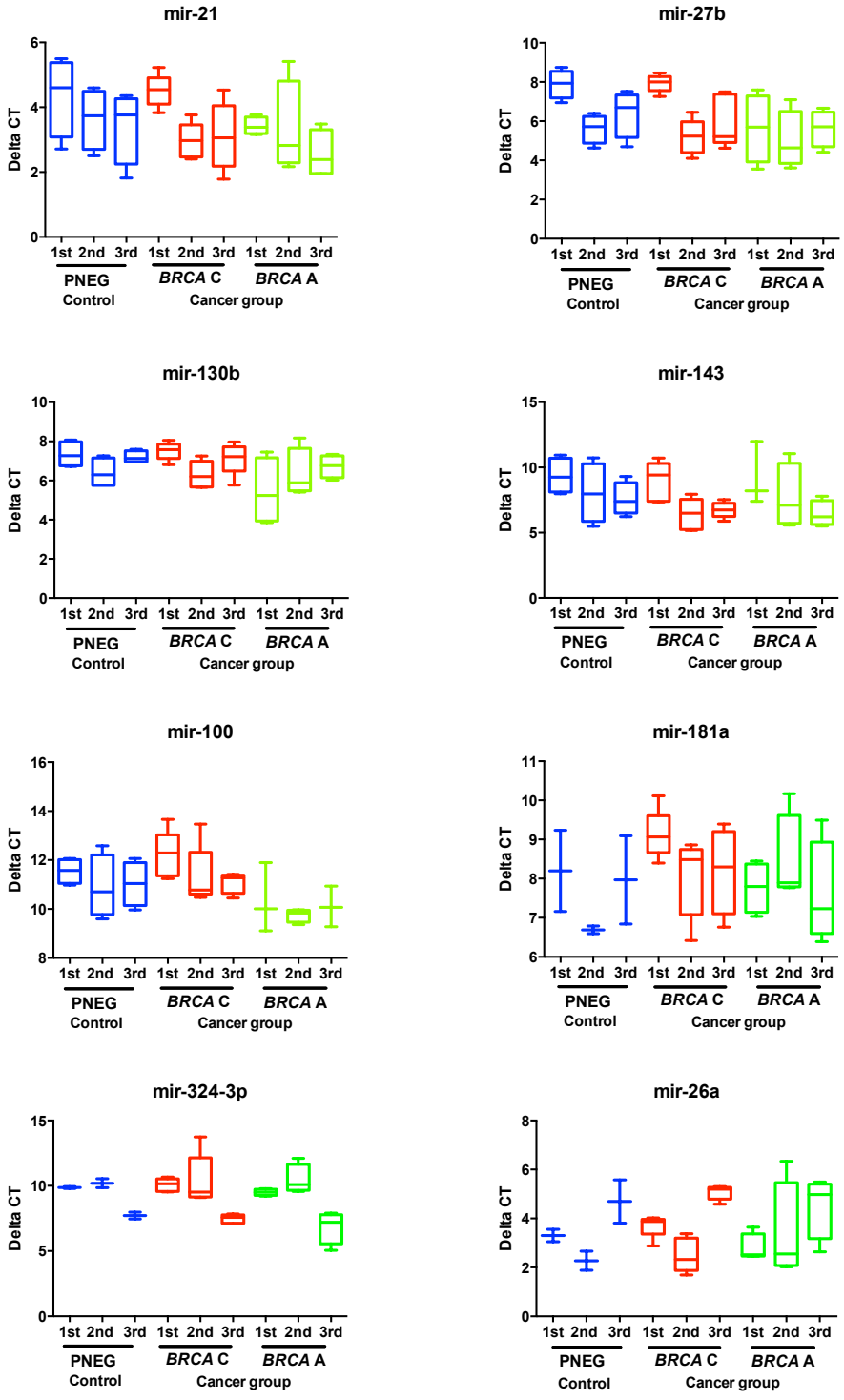


Figure 3.5 Box and whisker plots for eight miRNA expression data relative to mir-484

Evaluation expression stability relative to the mir-484. Data represents ΔCT for the 3 groups (PNEG n=4, *BRCA* carrier n=5, and *BRCA* affected n=4) across 3 different runs. ΔCT was calculated using the equation: CT value of each miRNA – CT value of mir-484. (*)=P<0.05 of the Kruskal-Wallis test. Analyses of 8 miRNAs revealed stable expression across the runs. 1st: first cDNAs run after extraction; 2nd: same cDNAs kept for 6 months at −20°C and then re-run; 3rd: RNAs were re-prepped after 2 years of storage (−80°C), reverse transcribed to cDNAs, re-quantified by qPCR, and re-analysed. Box plots represent lower quartile, upper quartile, and median; whiskers represent minimum and maximum values.

Table 3.9 Differently expressed miRNAs across 3 experimental states in different at risk groups

1st: first cDNAs run after extraction; 2nd: second cDNAs re-run 6 months later; and 3rd: third cDNAs the data after re-extraction. Delta CT relative to U6. P<0.05 (significant) of the Kruskal-Wallis test.

Group name	PNEG			*BRCA* CARRIER			*BRCA* AFFECTED		
MiRNA	ΔCT value			ΔCT value			ΔCT value		
	1st vs 2nd	1st vs 3rd	2nd vs 3rd	1st vs 2nd	1st vs 3rd	2nd vs 3rd	1st vs 2nd	1st vs 3rd	2nd vs 3rd
Mir-21	Ns	Ns	Ns	Ns	P<0.05	Ns	Ns	Ns	Ns
Mir-27b	Ns	Ns	Ns	Ns	P<0.05	Ns	Ns	Ns	Ns
Mir-130b	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Mir-143	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Mir-100	Ns	Ns	Ns	Ns	Ns	P<0.05	Ns	Ns	Ns
Mir-181a	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Mir-324-3p	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Mir-26a	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns

3.2.5.2 CDNA stability

As a part of my thesis, one objective was to assess the effect of time in storage of both plasma and isolated cDNA in a number of candidate miRNAs. After validation of reference miRNAs with sample storage, the levels of 3 candidate circulating miRNAs (mir-27b, mir-100, and mir-130b) were assessed in the plasma of women with a family history of breast disease, comprising healthy mutation negative controls (PNEG, n=17) and women with a known *BRCA* mutation (*BRCA* carrier, n=21 and *BRCA* affected, n=15) using a qPCR. Plasma was stored for 12 months at −80°C before RNA extraction with the mirVana™ miRNA isolation Kit, and reverse transcription to complementary DNAs (cDNAs) followed by pre-amplification and quantification by qPCR. CDNAs were then kept in the freezer (−20°C) and the reactions were re-run 3 months and 6 months later respectively. Results were compared using an analysis of variance (ANOVA) test. Generally, no significant difference in miRNA expression existed among the replicate cDNAs despite increasing time in storage (Figure 3.6). However, a higher level of expression for mir-27b manifested in the second and third replicates for

the PNEG and *BRCA* carrier groups compared to the first reaction; conversely, the levels were quite similar in the *BRCA* affected group. Delta CT values for mir-100 were relatively similar across the 3 replicate cDNAs reactions within all groups. Regarding mir-130b, there was little variability in miRNAs levels between replicate cDNA reactions in the PNEG and *BRCA* carrier groups. On the other hand, the *BRCA* affected group shows lower miRNA expression levels across the second replicate compared to the first and third replicate cDNAs. On the whole, there was little variability in miRNA levels between replicate cDNA reactions despite 6 months in storage.

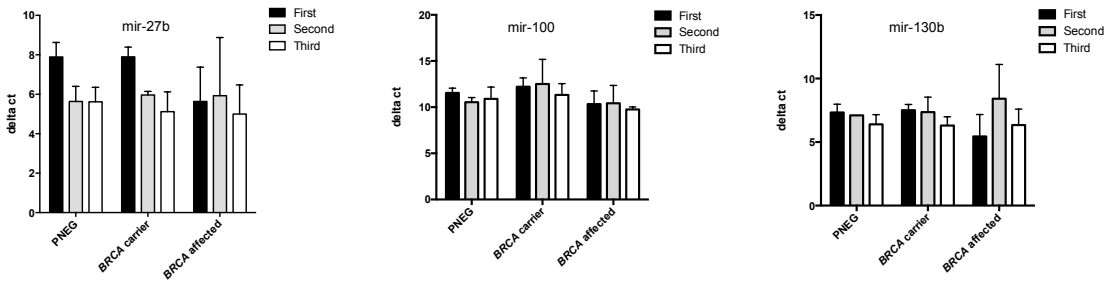


Figure 3.6 miRNA stability for mir-27b, mir-100, and mir-130b

Bar charts represent a comparison of miRNA expression profiles for PNEG (n=17), *BRCA* carrier (n=21), and *BRCA* affected (n=15) groups for the 3 analyses to evaluate cDNA stability with time in storage. Samples were run 3 different times (first cDNA: RNAs were extracted, reverse transcribed to cDNAs, and quantified by qPCR; second cDNA: qPCR was re-run 3 months later using the same cDNAs; third cDNA: qPCR was re-run 6 months later using the same cDNAs). The ANOVA test was used to determine significant differences across the runs. Error bars represent the mean with SD.

A correlation analysis was applied between the first to second and the first to third data using PRISM software and samples were highly and moderately correlated respectively (Figure 3.7; Table 3.10). In summary, results show that cDNA is stable in storage for at least 6 months as there were no significant differences between the experimental repeats.

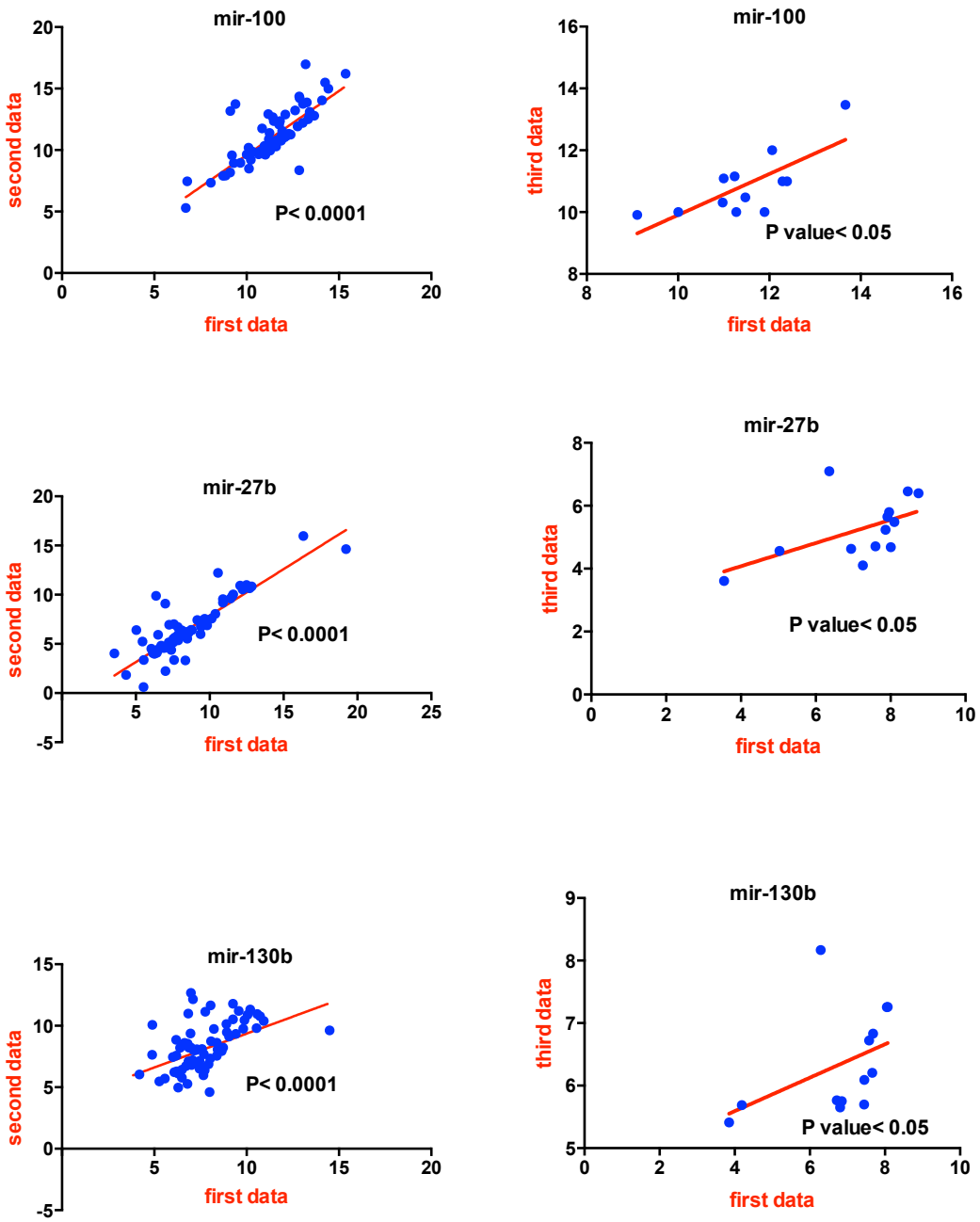


Figure 3.7 Correlation analysis between the miRNA expression in 3 experimental replicates

There was generally good correlation between miRNA expressions, $p < 0.05$ significant. Samples were run 3 different times (first data: first cDNAs run after extraction; Second data: same cDNAs re-run 3 months later; and third data: qPCR re-run 6 months later using the same cDNAs). The lines represent a linear regression fit.

Table 3.10 Correlation of miRNA expression between the first and the second cDNA

First cDNA: run after extraction; second cDNA: the same cDNA re-run 3 months later.

Parameter	MiRNA		
First vs. Second cDNA	Mir-100	Mir-27b	Mir-130b
Spearman r	0.7623	0.7929	0.5392
95% confidence interval	0.6360 to 0.8489	0.6829 to 0.8678	0.3421 to 0.6909
P value P (two-tailed)	< 0.0001	< 0.0001	< 0.0001
P value summary	****	****	****
Significant? (alpha=0.05)	Yes	Yes	Yes
Number of XY Pairs	68	71	70

3.2.5.3 Plasma storage

Having established that the cDNA is stable in storage for at least 6 months, I next assessed whether the time in storage of the plasma has an effect on RNA recovery and miRNA profiles in a larger number of miRNAs. Eight candidates' miRNAs (mir-21, mir-27b, mir-130b, mir-143, mir-100, mir-181a, mir-324-3p, and mir-26a) were selected for analysis. RNA was re-extracted after 2 years of storage from 13 selected plasma samples (PNEG (n=4), *BRCA* carrier (n=5), and *BRCA* affected (n=4)) using the Life Technologies™ miRVana™ miRNA isolation Kit. A TaqMan megaplex RT reaction was carried out to transcribe miRNAs, followed by TaqMan pre-amplification of cDNA. A new aliquot of TaqMan individual MicroRNA assays was then used to quantify miRNA expression in the individual samples of cDNA. qPCR was run in triplicate. At this stage we were comparing re-extracted plasma samples stored for 2 years at −80°C (second samples) to the first plasma samples stored for 12 months at −80°C (Figure 3.8). The analyses were performed based on the CT values of the first and the second cDNAs in order to determine whether miRNAs were recoverable from stored plasma. Generally, all 8 miRNAs showed lower levels of expression in the second samples, when the same set of samples was re-extracted after 2 years of storage (−80°C). This is because the CT values were higher, which indicates less miRNA. Significant differences in expression between the first and the second cDNAs were found in the *BRCA* affected group for mir-21, mir-27b, mir-130b, mir-181a, and mir-26a using the ANOVA test (Figure 3.8; Table 3.11).

In Summary, the results showed that the cDNA could be stable in the freezer for a long time after RNAs extraction and reverse transcription to the cDNAs, but longer storage of plasma led to the degradation of some miRNAs, so fewer miRNAs could be obtained. There were some differences in miRNA expression between samples, however, to reduce differences in expression between samples, the normalisation approach using stable reference genes (mir-484) is generally an accepted method enabling accurate data interpretation (Derveaux et al., 2010).

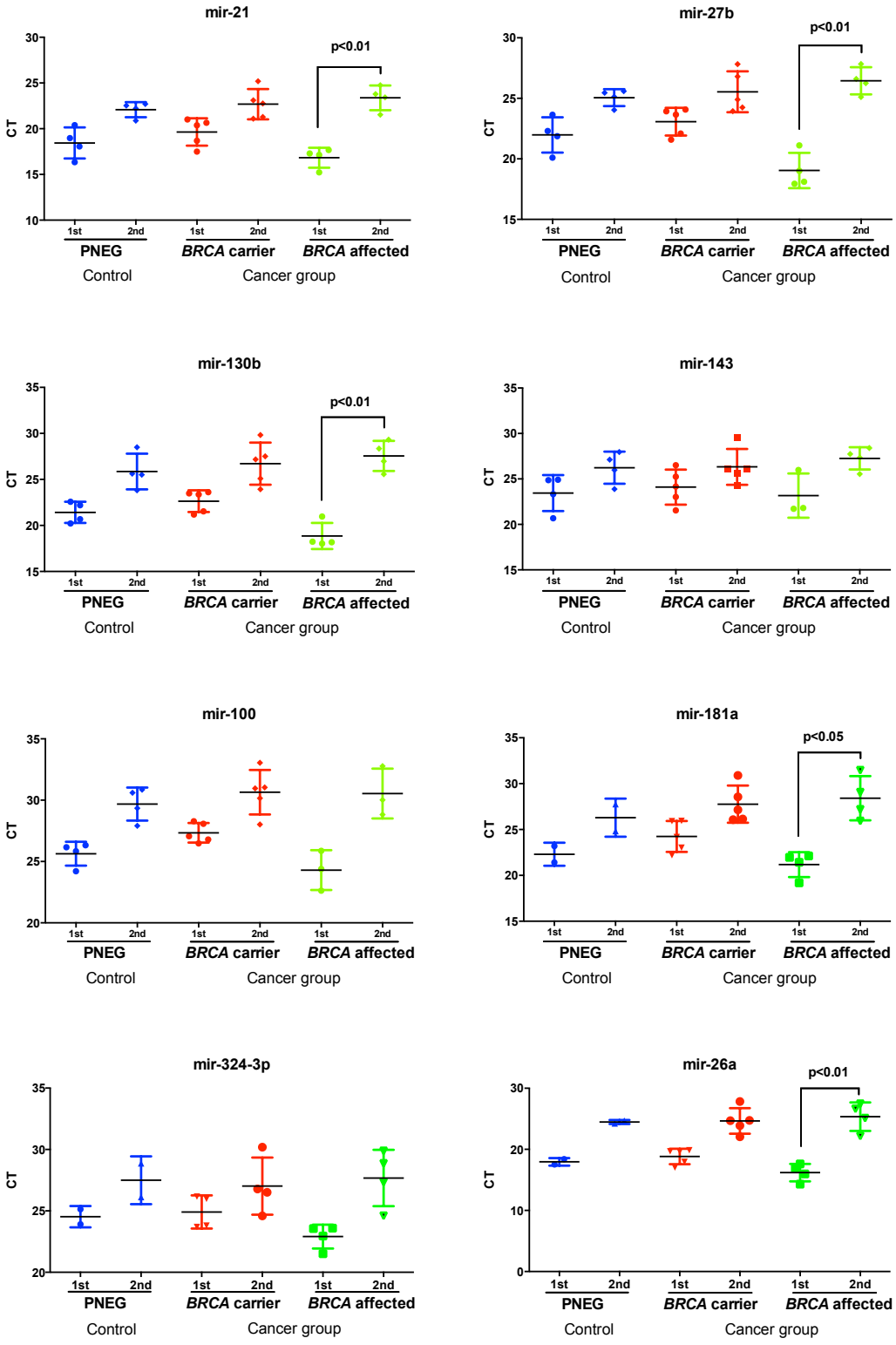


Figure 3.8 Variation of miRNA expression with time in storage

Un-normalised CT values for the discriminate variability in miRNA expression across the 2 runs are presented for 8 candidate miRNAs of the control group (PNEG n=4) and the cancer groups (*BRCA* carrier n=5 and *BRCA* affected n=4). The ANOVA test was used to determine significant differences between the control group and the cancer groups across the runs. 1st: first cDNA run after extraction; 2nd: second cDNA run after re-extraction following one year of storage (−80°C) and re-quantified by qPCR, Error bars represent the mean with SD, P< 0.05 significant.

Table 3.11 Data from the Kruskal-Wallis test for the three groups (PNEG, *BRCA* carrier, and *BRCA* affected) showing the significance levels between the 2 runs

1st: first cDNAs run after extraction (samples stored for 12 months at −80°C); 2nd: second cDNAs the data after re-extraction (samples stored for 2 years at −80°C). *P< 0.05, **P< 0.01 significant.

Group name	PNEG	*BRCA* CARRIER	*BRCA* AFFECTED
Test	Kruskal-Wallis test	Kruskal-Wallis test	Kruskal-Wallis test
Significance	P value	P value	P value
MiRNA	1st vs 2nd	1st vs 2nd	1st vs 2nd
Mir-21	Ns	Ns	**
Mir-27b	Ns	Ns	**
Mir-130b	Ns	Ns	**
Mir-143	Ns	Ns	Ns
Mir-100	Ns	Ns	Ns
Mir-181a	Ns	Ns	*
Mir-324-3p	Ns	Ns	Ns
Mir-26a	Ns	Ns	**

3.2.6 MiRNA screening in individual samples

Having first confirmed that stored samples are suitable for analysis as long as individual miRNA levels are related to a suitable reference (mir-484), I next chose to investigate candidate miRNAs as a biomarker of breast cancer in a cohort of 71 samples. 12 healthy controls were split into London (n=7) and Leicester (n=5) healthy females in order to see if there was any difference by sample groups. Generally, no significant differences in expression between London and Leicester controls were noticed using Unpaired t-test. These samples were taken in different places by different people, but processed to the same SOP and I saw that they were very similar in all candidate miRNAs investigated. Accordingly, they were combined together into one pooled control (healthy controls) (Figure 3.9 shows 4 examples of selected miRNAs). In this study, we measured the level of circulating miRNA in the plasma of healthy controls and women with a family history of breast diseases using qPCR approach and TaqMan MicroRNA individual assays. CTs were normalised to mir-484.

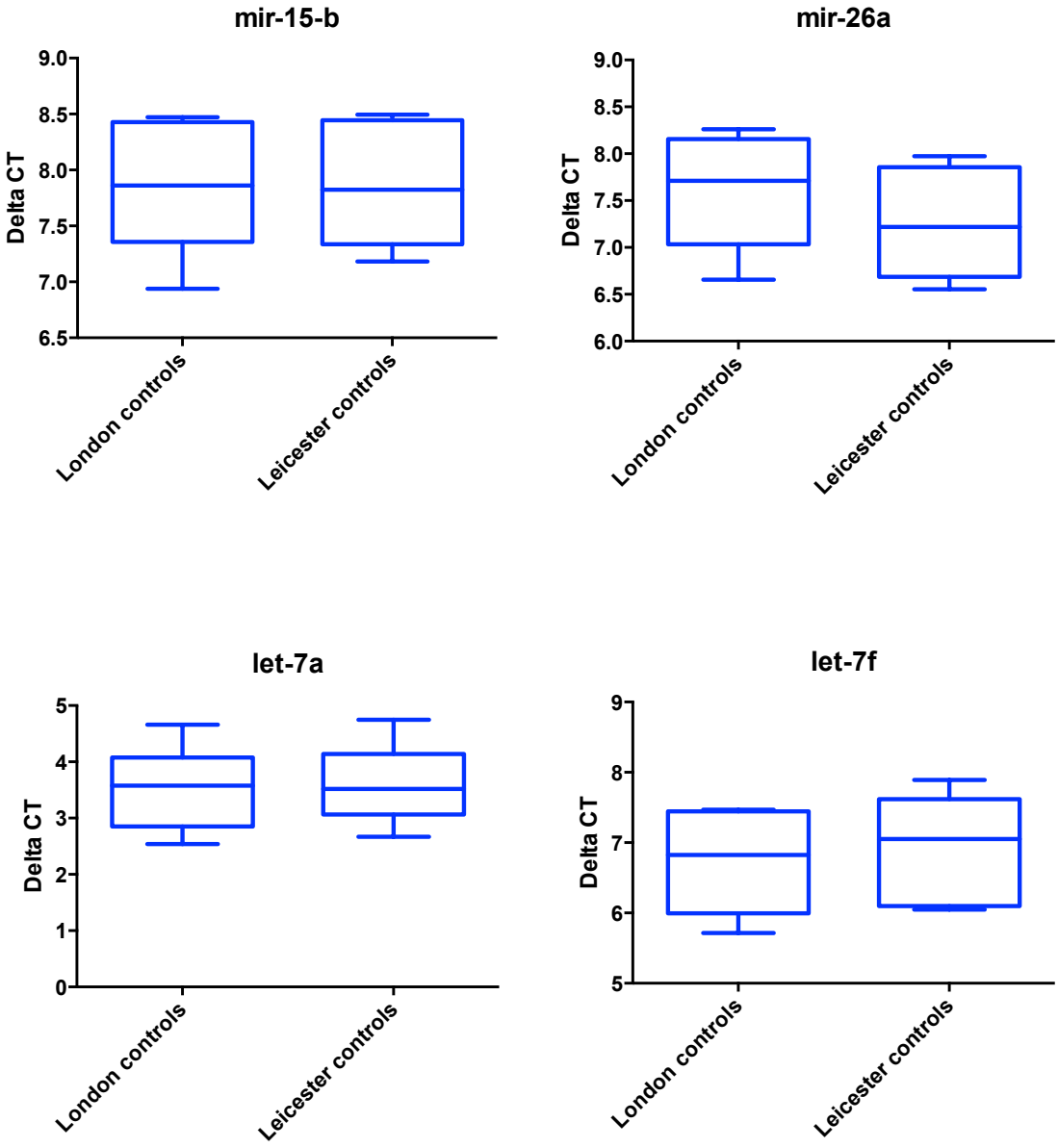
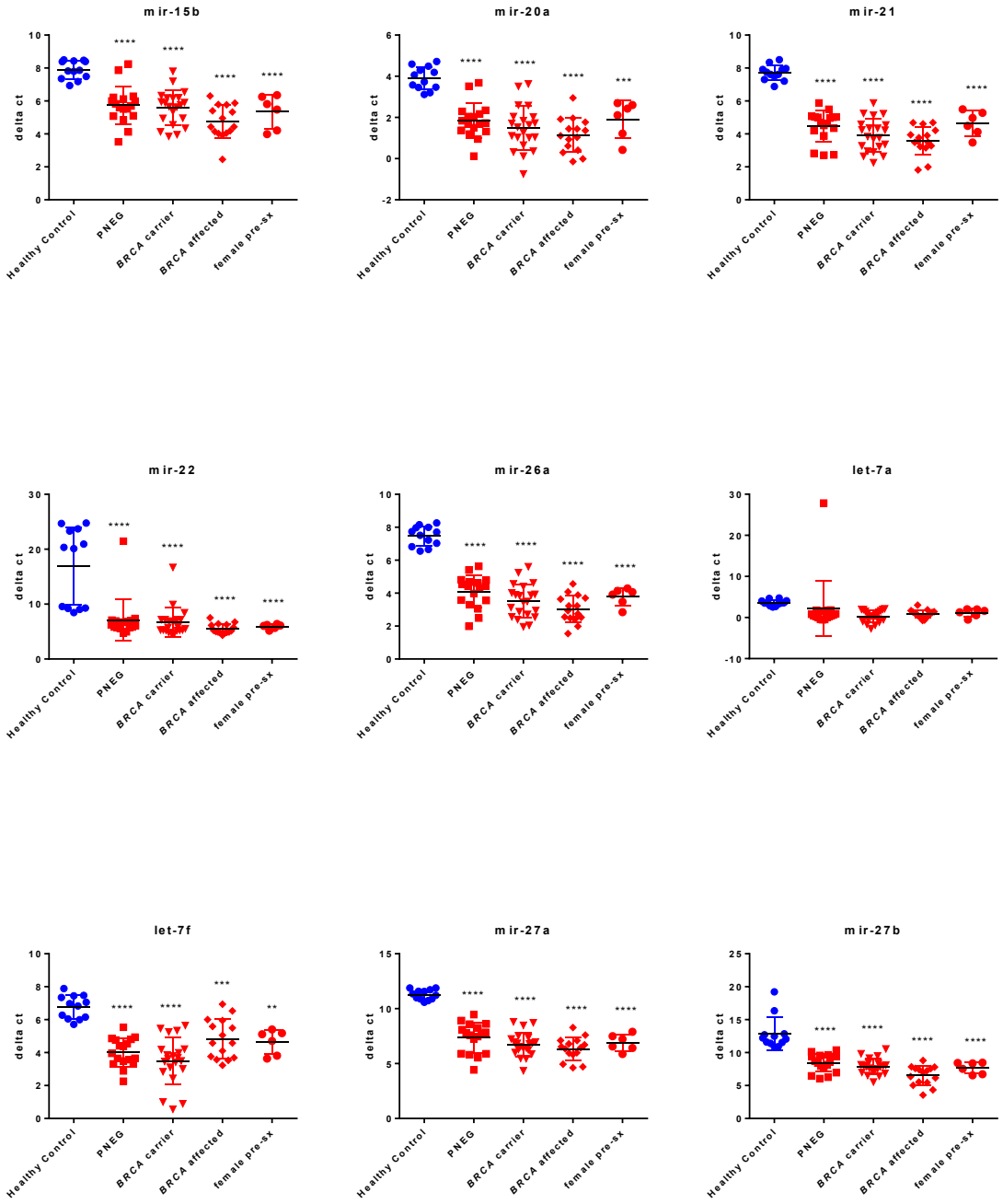
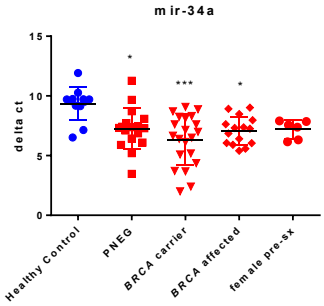


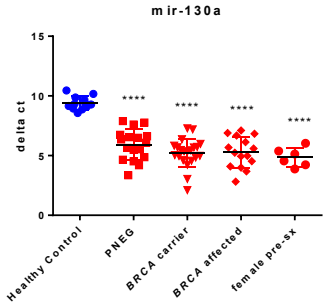
Figure 3.9 Examples of 4 candidates showing a comparison of miRNA expression levels in the plasma of two study cohorts of controls including London and Leicester controls

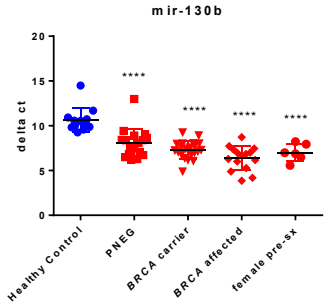
Box and whisker plots showing plasma miRNA levels for London and Leicester controls of 4 selected miRNAs that were determined by qPCR. There were no statistically significant differences determined using Unpaired t-test. Delta CT were normalised to mir-484. Whiskers of the box-plot represent minimum and maximum values. Box plots represent median with lower and upper quartiles.

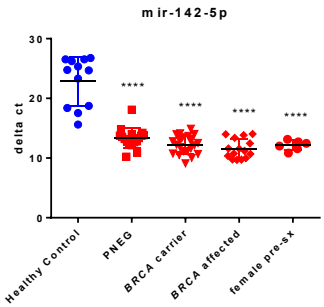
By investigating in a validation cohort (n=71) using Tukey's multiple comparisons test, we noticed that the expression levels of 25 circulating miRNAs were present at higher levels in the plasma of breast cancer patients when compared with healthy controls (Figure 3.10).

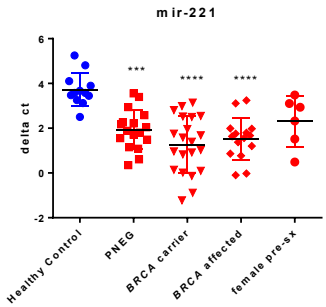


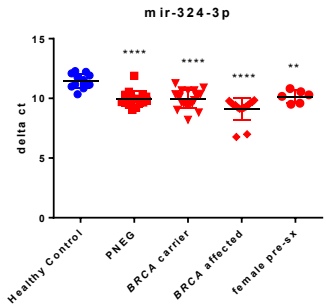

mir-34a
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx

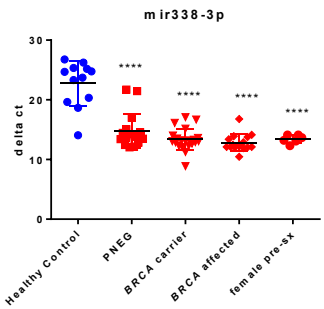

mir-130a
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx


mir-130b
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx


mir-142-5p
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx


mir-221
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx


mir-324-3p
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx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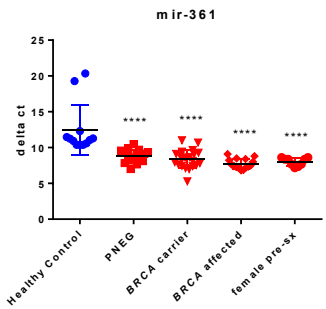
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx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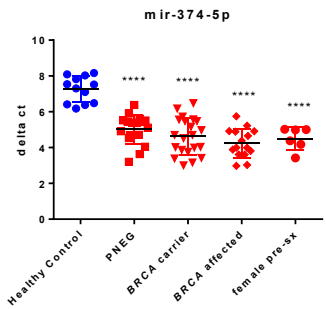
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx


mir-374-5p
delta ct
Healthy Control
PNEG
BRCA carrier
BRCA affected
female pre-sx

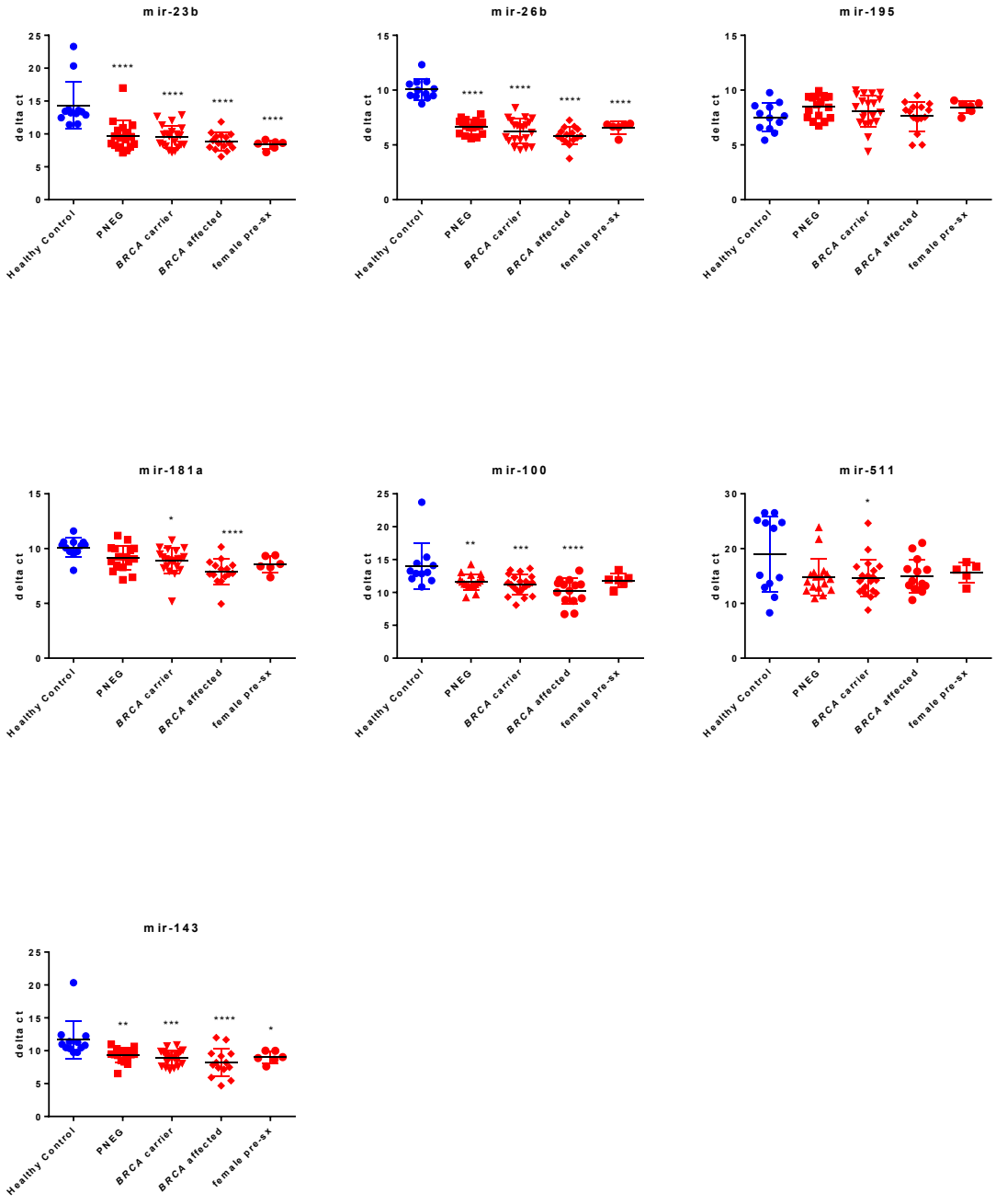


Figure 3.10 Expression levels of 25 selected miRNAs in the validation cohort

Validation of miRNAs in plasma samples (n=71). Circulating miRNAs are present at an elevated level in the plasma of women with a family history of breast cancer (in red) including PNEG (n=17), *BRCA* carrier (n=21), *BRCA* affected (n=15), and female pre-sx (n=6) when compared to healthy controls (n=12) (in blue) using the ANOVA test. Mir-let-7a, mir-195, and mir-511 levels in the breast of cancer patients are not statistically significantly different to the healthy controls. Expression levels of candidate miRNAs were determined by TaqMan miRNA assays, delta CT were normalised to mir-484. Error bars represent the mean with SD.*P< 0.05, **P< 0.01, ***P< 0.001, **** P< 0.0001 compared to control group.

At this stage it was noticeable that most breast cancer groups seemed to be significantly different to the healthy controls (combined control) with the exception of 3 miRNAs (let7a, mir-195, and mir-511) (Figure 3.10) and so could not be considered as good controls. The PNEG group are women with a family history of breast cancer but they have no *BRCA* mutation. Consequently, the ANOVA analysis was run again after removing the combined controls, using PNEG as the control group. In the larger set of samples, a significant difference between the PNEG group and other breast cancer groups was no longer noticed, for example, there was no significant difference between carrier and PNEG, though some miRNAs showed differences in post-surgical RNA (women who have had breast cancer in the affected group). Out of the 25 miRNAs investigated there were only 5 miRNAs (mir-26a, mir-27b, mir-130b, mir-324-3p, and mir-181a) that showed a significant different in expression between the PNEG group and the *BRCA* affected group (Figure 3.11; Table 3.12).

In conclusion, within this cohort when we use PNEGs as controls, there were 5 miRNAs (mir-26a, mir-27b, mir-130b, mir-324-3p, and mir-181a) that were able to distinguish between breast cancers and controls, suggesting that those miRNAs could have a role in the monitoring of patients with breast cancer, and in determining risk in women with a family history. However, those miRNAs could be only important in this group of samples and may not be so in others; nevertheless, larger validation studies are needed to test this. The female pre-surgical group were women with sporadic breast cancer and no *BRCA* mutation. Their miRNA profiles were similar to the *BRCA* carrier group and these were also similar to the PNEG controls. Hence, either miRNA changes only when someone has cancer; whether cancer is present or has been removed (*BRCA* affected). Of note the *BRCA* carriers had all had mastectomies to reduce their risk of developing breast cancer. This study would support the fact that their risk has been reduced as miRNA profiles overlapped with controls. However for the *BRCA* affected group, these women have had cancer and treatment, post-surgery and are disease free, but their miRNA profiles suggest differences, and may reflect the presence of minimal residual disease.

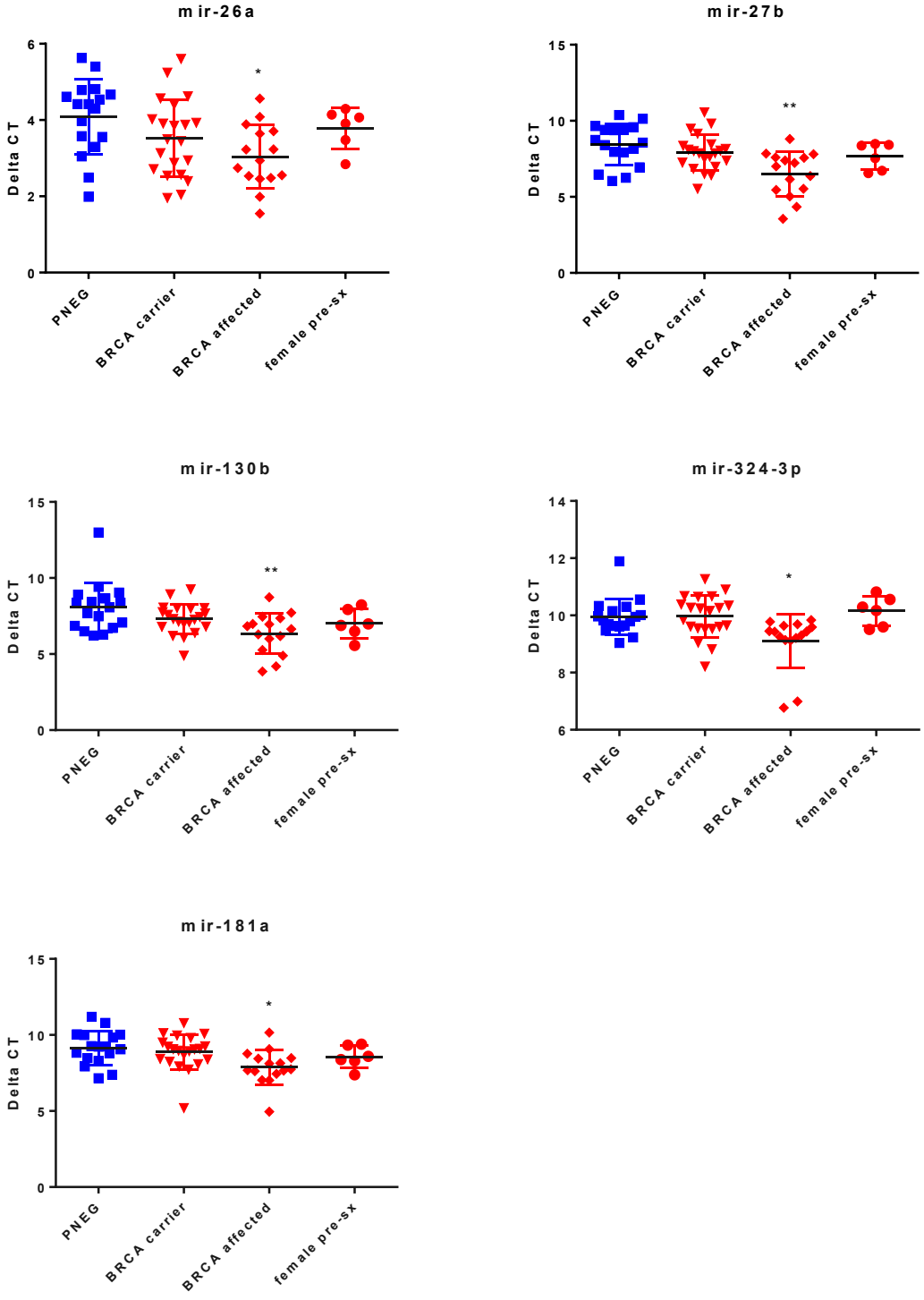


Figure 3.11 The scatter plot differentiates five selected miRNAs showing significant levels in expression between PNEG group (control) and *BRCA* affected group (cancer group)

The plasma level was analysed in the PNEG group (n=17) (control), the *BRCA* carrier (n=21), the *BRCA* affected (n=15), and female pre-sx (n=6) (cancer group) using the ANOVA test. Expression levels of mir-26a, mir-27b, mir-130b, mir-324-3p, and mir-181a were determined by TaqMan miRNA assays and delta CT were normalised to mir-484. Error bars represent the mean with SD. *P< 0.05, **P< 0.01 compared to control group.

Table 3.12 The ANOVA multiple comparison test and Linear trend analysis between the control group (PNEG) and cancer groups (*BRCA* carrier, *BRCA* affected, and female pre-sx) of five circulating miRNAs

P< 0.05 significant.

MiRNA	PNEG vs. *BRCA* affected	PNEG vs. *BRCA* carrier &PNEG vs. female pre-sx	Trend Significance
Mir-26a	P<0.01	No	0.3074
Mir-27b	P<0.001	No	0.0581
Mir-130b	P<0.001	No	0.0337
Mir- 324-3p	P<0.01	No	0.8388
Mir-181a	P<0.01	No	0.0956

3.2.7 MiRNA analysis in other high-risk family groups

Having established selected miRNAs in plasma of women with a family history of breast cancer who have had *BRCA* mutation, as validation we wanted to look at miRNA expression in an independent cohort of women with a family history of breast cancer, but who were not *BRCA* mutated or not tested for *BRCA* mutation (Table 3.1). In this analysis plasma samples were combined into one cancer group and one control group in order to have a bigger cohort of samples for more accurate results.

Six miRNAs were selected for assessment, 2 miRNAs from Table (3.12) that showed the most significant difference between affected and control groups (mir-27b and mir-130b), and 4 others from the 25 miRNAs (Table 3.3), with known cancer profiles published by other groups (mir-20a, mir-21, mir-27a, and mir-130a). These miRNAs (mir-20a, mir-21, mir-27a, mir-27b, mir-130a, and mir-130b) were validated in 44 patients with a family history of breast cancer (Table 3.1) using TaqMan MicroRNA assays, applying qPCR. Gene expression levels were normalised using a reference gene (mir-484). Delta CT was calculated by subtracting the CT value of mir-484 (reference gene) from the individual CT for each miRNA. The ANOVA multiple comparisons test was performed to compare the PNEG group, which was used as a control in the

validation of 5 candidate miRNAs, with this cohort. The expression levels of those miRNAs were slightly higher in the cancer group than in the controls except for mir-130a. The Unpaired t-test showed that the expression levels of 5 miRNAs (mir-20a, mir-21, mir-27b, mir-130a, and mir-130b) between cancer and control groups were not statistically significant, excluding mir-27a. However, all 6 miRNAs were significantly elevated in the cancer and control groups compared to the PNEG group (Figure 3.12).

In summary, these data showed that miRNA profiles of healthy female controls were similar to those seen in patients who have had breast cancer and from a high-risk family but who are negative for *BRCA* gene mutation (cancer group). This suggest that miRNA profile returnes to that of normal controls in women who have had surgery and treatment. However, when the PNEG group was used as controls both cancer and healthy controls showed significant differences in the miRNA profile. This suggests that results vary by sample type and group.

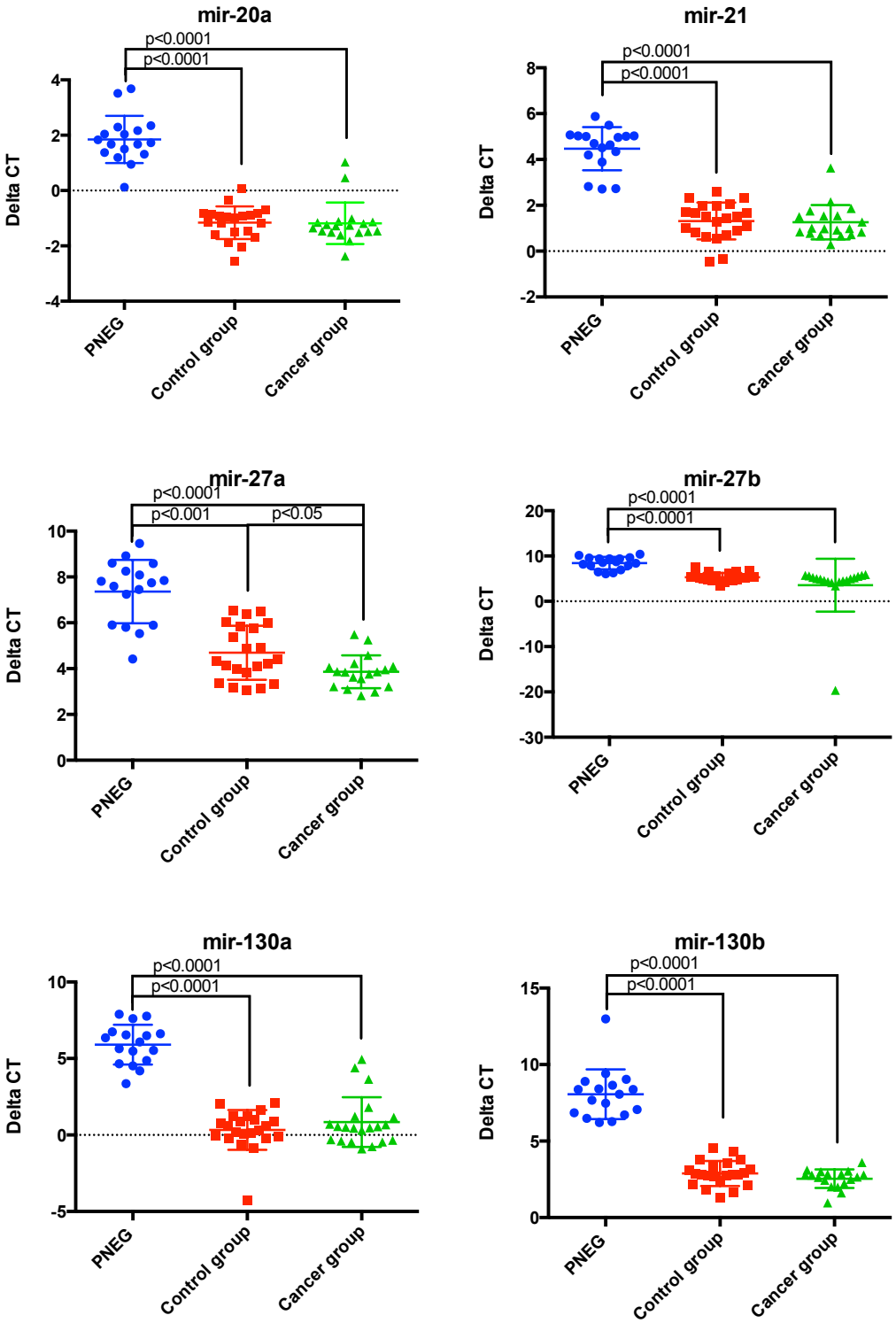


Figure 3.12 Validation of 6 candidate miRNAs in plasma of women with a family history of breast cancer who are negative for *BRCA* gene mutation

Analysis of 6 miRNAs show higher expression in cancer (n=22) and control (n=22) groups than PNEG (n=17) group; there were no significant differences in miRNA expression between the cancer group and the control group except mir-27a; ΔCT values (CT for each miRNA – CT for mir-484). Error bars represent the mean with SD.*P< 0.05, ***P< 0.001, **** P< 0.0001 compared to the control and PNEG group.

3.3 Discussion

Circulating miRNAs are under investigation as non-invasive biomarkers of cancer. The aim of this chapter was to identify any tumour-associated miRNAs among women with an elevated risk of developing breast cancer based on their family history (*BRCA* mutation carrier) using qPCR.

3.3.1 Card data Validation

Through this analysis the expression of miRNAs of TaqMan array cards for 8 healthy individuals were compared to the pooled card. Our results clearly demonstrated a high-level of reproducibility between TaqMan array cards for 8 healthy individuals and the pooled card since the miRNA expressions of individual array cards and the pooled card were highly correlated (Table 3.5; Figures 3.1 and 3.2). The TaqMan array card method demonstrated reliable and accurate data and showed good reproducibility, specificity, and sensitivity. Diaz et al., (2013) reported that TaqMan array card has provided a successful strategy for pathogen detection, which would lead to more comprehensive clinical assessment, less potential for contamination, requiring minimal volume of specimen, and simplicity of use.

In general, all technical steps included such as sampling, RNA extraction, reverse transcription, pre-amplification of the target gene, use of a newer generation enzyme mix, and TaqMan Array Card (TAC) design and production (Soong and Ladanyi, 2003; Chen et al., 2009; Diaz et al., 2013; Ferlinz et al., 2013; Mestdagh et al., 2014) should be carefully optimised for accurate data. Reverse transcription reaction contributes to a high variation particularly for low amount miRNAs (Mestdagh et al., 2008; Stahlberg et al., 2004). A RT-negative control was run to evaluate whether the RT reaction primed by other molecules present in the sample or possibly by dNTPs, as false primers used, contributes to higher RT yields than the negative control (Stahlberg et al., 2004; Agranovsky, 1992). Mestdagh et al., (2008) have proved that high sensitivity and accuracy of reverse transcription followed by pre-amplification could be due to the fact

that more miRNAs are detected when performing the pre-amplification. The pre-amplification technique has been widely used especially with a limited quantity of RNAs as with blood plasma, as it enables the identification of regulated miRNAs below the detection rate and increases sensitivity of qPCR. A major issue regarding pre-amplification approach is the probability that not all miRNA transcripts could be equally amplified (Chen et al., 2009). Though the pre-amplification efficiency could be influenced by probe and primer sequence/length, variation might also increase towards low expression level and high CT values of miRNAs (Mestdagh et al., 2014). Overall, numerous factors could influence reproducibility, specificity and sensitivity of pre-amplification reaction including: template/primer concentration, number of assays, number of cycles, and annealing time/temperature (Andersson et al., 2015). NTC was included as a negative control to ensure that pre-amplification will not be affected by primer dimer formation or by assays that would amplify genomic DNA (Korenková et al., 2015). To conclude, results in this thesis show that the TaqMan Array Card method has proven that pooled plasma samples are representative of single plasmas.

3.3.2 MiRNA assessment in breast cancer cell lines

In this study, we assessed a number of miRNA assays before recruitment to validation cohort. qPCR is an accurate and sensitive technique that is widely used for detecting RNA levels in cell lines (Bustin, 2002). We examined the expression of 8 miRNAs in 5 breast cancer cell lines (MCF-7, ZR-75-1, T-47D, MDA-MB-231, and MDA-MB-468).

The expression of those miRNAs demonstrated a high degree of reproducibility since qPCR was achieved in triplicate with relatively low variation across replicates (CT<35), thus it is likely that those miRNA assays are good and could be applied to plasma samples (Table 3.7). Moreover, the expression levels of selected miRNAs in individual cell lines were differently expressed between breast cancer cell lines and the non-tumourgenic cell line (HBL-100) (Table 3.6; Figure 3.3). Additionally, results were in agreement with some studies but not with others. Having variable results could be explained by Jarry et al., (2014) who studied the expression of 154 miRNAs in 26

different tumour types. Additionally, O'Donnell et al., (2005) suggested that the expression as well as the role of miRNA depend and vary as per cell type. Also, it has been observed that the expression of miRNA in several tumourous groups is categorised depending upon several bio-pathological characteristics including hormone receptor expression, stage and grade of the disease, proliferation index as well as vascular invasion (Iorio et al., 2005; Blenkiron et al., 2007).

Results by Zhu et al., (2006) and Cicatiello et al., (2010) demonstrated that the use of BC cell models, manifesting sensitivity to oestrogen, has been highly beneficial in this case due to the fact that the ER α-expressing breast tumours and cell lines exhibit transcriptomes that are remarkably similar. Sempere et al., (2007) further identified the link between molecular expression of miRNA and biomarkers and indicators, which include progesterone receptor, oestrogen receptor, and HER2. These miRNAs are vital for the identification of tumour subtypes at the molecular level (Kosaka et al., 2010). Generally, there are a number of possible explanations for the differences between miRNA expressions of normal/cancer breast cell lines, plasma, and tissues samples, which include: A) heterogeneity of breast cancers, B) numbers of samples analysed and C) data normalisation. In conclusion, we have shown that cDNA quantification by qPCR generated equivalent results across each cell line (triplicate CT values (<35) were consistent) suggesting that those miRNA assays were reproducible overall.

3.3.3 Stability of miRNAs in storage

MiRNAs are promising biomarkers since they can be examined directly from plasma (Saldanha et al., 2013). Despite studies that are very encouraging, reliable measurement of circulating miRNAs bears some inherent challenges and requires careful standardisation of pre-analytical variables (Becker and Lockwood, 2013). As part of miRNA validation on the sample in storage it was important to identify a stably expressed reference miRNA to normalise qPCR data. The mean of two reference miRNAs (mir-484 and U6) and mir-484 (Figure 3.5) were stably expressed since no significant differences were found by ΔCT across samples using the ANOVA test.

Since mir-484 was widely used and found in the literature as the most stable reference gene for miRNA normalisation (McDermott et al., 2013), and was also approved by our group as the most constantly expressed across the samples, it was validated accordingly, and used in this analysis. Mir-484 displayed similar levels between the first and second samples as there were no significant differences, but the CT values were higher after re-extraction (third samples), which indicated miRNAs were degraded in the plasma stored at −80°C (Table 3.8; Figure 3.4). However, the way data were presented and calculated relative to mir-484 (ΔCT) (Figure 3.5) strongly support that the level of degradation of the plasma can increase the CT values but it could not influence the ΔCT expression as approved by Antonov et al., (2005). As when miRNA expressions were normalised relative to mir-484, there were no significant differences found across the 3 samples for all 8 miRNAs and so the results would be arguably more reliable and accurate. Several reviews showed that data normalisation using stable reference is an important strategy to avoid differences in miRNA expression between different samples (Mestdagh et al., 2009). However, a combination of reference genes for normalisation was confirmed previously for reliability of the data produced (Andersen et al., 2004; Chang et al., 2010; McDermott et al., 2013).

Profiling of 3 miRNAs (mir-27b, mir100, and mir-130b) showed little variability with increasing storage time of their cDNAs (at −20°C), between first (initial run after extraction), second (first replicate 3 months later) and third samples (second replicate 6 months later), analysed in women with a family history of breast cancer which indicated that cDNA storage time could have a minimal effect on miRNA concentration since no significant difference was found using the ANOVA test (Figure 3.6). Moreover, correlation analysis also showed high/moderate relationship between first and second/third samples analysed (Figure 3.7; Table 3.10). McDonald et al., (2011) reported stability of miRNAs of up to 72 h, when stored at −20°C. This is similar to Mitchell et al., (2008) who demonstrated that plasma storage for 24h at room temperature had a minimal effect on miRNAs. Moreover, one study by Grasedieck et al., (2012) showed miRNAs stability after a long time of storage for 2-4 years at −20°C had minimal effects on miRNAs. However, results by Bravo et al., (2007) confirmed instability of miRNAs and their cDNAs in storage.

In order to validate miRNA instability in plasma storage at −80°C, RNA was re-isolated from 13 blood plasma samples from 3 independent groups (PNEG (n=4), *BRCA* carrier (n=5), and *BRCA* affected (n=4)) 12 months after the initial run to find altered level of circulating miRNA in plasma samples. Data for eight miRNAs (mir-21, mir-27b, mir-130b, mir-143, mir-100, mir-181a, mir-324-3p, and mir-26a) (Figure 3.8; Table 3.11) and two references (mir-484 and U6) (Figure 3.4; Table 3.8) showed a similar trend as the raw CTs were higher after isolation in comparison to the first analysis when RNAs were early extracted, reverse transcribed, then quantified using qPCR. Overall, according to the previous finding several of the pre-analytical and analytical variables affect the quantification of miRNA in plasma (Becker and Lockwood, 2013). A possible explanation for this variability could be due to the fact that plasma samples have been longer in the freezer, which might change miRNA profile. This is in agreement with a previous study which showed that miRNAs could be lost in the stored plasma at −80°C, when they did a comparison between a total of 177 miRNAs detected in the stored pool, with 202 miRNAs detected in the fresh pool (Page et al., 2013). The stability of miRNA is largely influenced by several variables, including the collection method along with specimen processing, storage conditions, and specimen type and handling, such as freezing (Duttagupta et al., 2011; Becker and Lockwood, 2013; Cheng et al., 2013).

The finding of this study confirmed previous results regarding miRNA stability issue on storage of cDNA and plasma (Bravo et al., 2007; Page et al., 2013). In summary, miRNAs' levels were stable in cDNA stored at −20°C despite 6 months of storage. However, their expression was unstable with increasing time of storage of plasma at −80°C. The mir-484 was used as a reference since it showed no significant difference and was approved by our group as the most stable in plasma overall.

3.3.4 MiRNA expression in plasma samples

Circulating miRNAs have increasingly been touted as promising biomarkers, especially for certain types of cancer. The general premise behind them is that tumours 'secrete'

tumour-specific miRNAs, which can be detected in readily accessible body fluids, such as blood (Cuk et al., 2013).

In this study, we used TaqMan miRNA card data of pooled plasma to identify candidate miRNAs. Thus, 25 miRNAs were confirmed as being significantly increased in young women with a family history of breast cancer (*BRCA* affected) versus the combined healthy controls (Table 3.3; Figure 3.10). However, because most breast cancer groups looked to be significantly different to the healthy controls (combined control), they could not be considered as reliable controls. Sample processing might affect the results of miRNA profiling on healthy controls (Pritchard et al., 2012). The PNEG group has a family history of breast cancer but they have no *BRCA* mutation or cancer and were subsequently used as a control. Accordingly, five miRNAs showed a significant difference between the PNEG control group and the *BRCA* affected group (mir-26a, mir-27b, mir-130b, mir-324-3p, and mir-181a), which can reliably distinguish between patients with breast cancer after surgery and healthy controls and could be used as a potential biomarker for patients with breast cancer (Table 3.12; Figure 3.11). Some of those miRNAs have been previously identified as being involved in breast cancer as markers in addition to targets for novel therapeutics (Tang et al., 2012). Mir-26a was frequently down-regulated in breast cancer tissues and cell lines (Zhang et al., 2011), as it inhibited cell proliferation, colony formation and migration, but promoted apoptosis in breast cancer cells. These effects suggested mir-26a is a potential tumour suppressor in breast cancer. The investigation of the function mechanism indicated that mir-26a inhibited the tumour growth by at least partially targeting *MCL1*. Furthermore, mir-26a effectively sensitised breast cancer cells to paclitaxel (Gao et al., 2013).

Mir-27b regulates the generation of a side-population (SP) fraction, which indicates high tumourigenicity and docetaxel resistance. The gene encoding ENPP1, a negative modulator of insulin receptor activation that is associated with type II diabetes (T2D) development, was identified as a novel target of mir-27b. ENPP1 stimulated the generation of the SP fraction by up regulation of the ABCG2 transporter. Additionally, mammosphere culture conditions that enrich breast cancer stem cells (CSCs) induced

down-regulation of mir-27b and suppression of proteasome activity, leading to the accumulation of ENPP1 (Takahashi et al., 2015). Mir-27b functions as an oncogene in breast cancer cells and is associated with poor prognosis in triple negative breast cancers (TNBC) (Jin et al., 2013; Shen et al., 2014; Wang et al., 2009).

TAp63 is a p53 family member and tumour suppressor gene, which might control Dicer by binding to and transactivating the Dicer promoter (Su et al., 2010). *Dicer1* expression regulations in TAp63 mouse embryonic fibroblasts reduced and inhibited the ability of these cells to invade whilst *Dicer1* knockdown in wild-type mouse embryonic fibroblasts might increase invasiveness in vitro, indicating that a decrease of Dicer expression may increase cell invasion. TAp63 was found to transactivate mir-130b that correlated with metastatic and was processed by Dicer. As up-regulation the expression of *Dicer1* and mir-130b might affect the metastatis of cells lacking TAp63. TAp63 suppresses tumourigenesis and metastasis through the coordinate transcriptional regulation of Dicer and mir-130b (Wang and Wang, 2012).

Cookson et al., (2012) reported that mir-324-3p might potentially serve as a tumour biomarker in the plasma of breast cancer patients since it was down-regulated after surgery. It was detected at relatively high-levels before surgery in all four patients, and was undetectable after surgery in 3 patients at two weeks, and 4 patients after six months. Hu et al., (2012) have found that mir-324-3p was consistently differentially expressed between breast cancer cases and controls.

A study by D'lppolito and Iorio, (2013) has reported that mir-181a, which is overexpressed in TNBC, belongs to the mir-181 family and is positively regulated by TGF-β. Down-regulation of mir-181a inhibits TGF-β mediated EMT, migration and invasion, and can also revert anoikis resistance in breast cancer cells; high-levels were correlated with shorter DFS of patients with breast cancer, who were negative for *HER2*

amplification (Taylor et al., 2013). Moreover, it plays a role in TNBC, both as a marker and therapeutic target (Bisso et al., 2013).

3.3.5 MiRNA analysis in other high-risk family groups

In this study, the analysis of 6 miRNAs (mir-20a, mir-21, mir-27a, mir-27b, mir-130a, and mir-130b) showed no significant differences between the control group and the cancer group in the plasma of women with a family history of breast cancer who are either negative for *BRCA* mutation and/or not tested for *BRCA* mutation (Figure 3.12). Because the control group seemed to be very similar to the cancer group, they could not be considered as reliable controls. Generally, miRNA expression profiling of the control group could be influenced by several factors including: the number of samples analysed (n=22 cancers, n=22 controls), which could have an effect on data analysis; technical variables that could introduce systematic bias (Nelson et al., 2008); pre-analytical variables (i.e. collection procedure, specimen processing) (Becker and Lockwood, 2013; Pritchard et al., 2012); and normalisation (Davison et al., 2006).

However, when we used PNEG as a control, significant differences were noticed across the 6 miRNAs (Figure 3.12). The PNEG group has a family history of breast cancer but they have no *BRCA* mutation, it gave reliable and sufficient data with the previous cohort of women with a family history of breast cancer, and was also used with this cohort. Two miRNAs (mir-27b and mir-130b) that were picked from the 5 miRNAs that were significantly up-regulated in the cancer group (*BRCA* affected) compared to the PNEG group (Table 3.12; Figure 3.11), again showed a similar trend. The 4 other miRNAs (mir-20a, mir-21, mir-27a, and mir-130a) from the 25 miRNAs have been observed in several studies and have potential roles in cancer as either tumour suppressors or oncogenes (Tables 3.3; 3.4) and also showed a significant increase in expression in the cancer group compared to the PNEG group. On the whole those miRNAs can distinguish between the cancer group and PNEG group and may be considered as biomarkers in this cohort of patients.

In summary, detection of miRNA expression in plasma has revealed that there are significant differences in miRNA expression between sample groups, but that some of these differences are likely to be due to differences in pre-analytical factors. In addition, there are other technical difficulties mostly due to their small size, and the relative poor sensitivity and low comparative power of the current methods used for their detection, low abundance of miRNAs in body fluids and high-level of homology among miRNA family members (Barad et al., 2004; Mestdagh et al., 2014). Overall, results in this chapter suggest that analysis of circulating miRNA could aid in the follow-up of women from a *BRCA1* or *BRCA2* family who have had cancer but this need investigation in a larger series.

Chapter 4

Copy Number Variation in specific chromosomal intervals as screening biomarkers in circulating free DNA in plasma

4.1 Introduction

This chapter focuses on CNV analysis in cfDNA at eight selected loci in the plasma of women who carry an inherited mutation in the *BRCA* gene. Patients included healthy *BRCA* carrier (n=19) with no evidence of cancer as well as post-surgical breast cancer patients (*BRCA* affected (n=15)). These were compared with healthy females from *BRCA* families, but who had not inherited a *BRCA* gene mutation termed "predictive negative" (PNEG (n=14)). Eight genes/markers were selected for screening of specific CNVs (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2* (*ERBB2*), *CYP19A1*, *PBX1*, and *DMXL2*) based on published data and our previous study (Shaw et al., 2012). Assays were developed in-house and analysed by qPCR. Initially, breast cancer cell lines models were used for assay validation by qPCR and then the approaches were applied in paired plasma cfDNA and lymphocyte DNA as experimental controls. 5 selected CN assays with CN changes detected in a number of plasma samples (by qPCR) were then validated using ddPCR. Lastly, the Ion Torrent PGM was applied to 9 plasma cfDNA samples and matched lymphocyte DNAs to confirm CNV analysis based on qPCR and ddPCR methods and to detect further mutations.

4.2 Results

4.2.1 qPCR CNV reference and target assay optimisation

A Standard curve qPCR was applied to examine the efficiency of the reference and the target assays to be used for CNV analysis, using HGDNA for comparative results. Standard curves were generated for 4-reference genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), contactin-associated protein 1 (*CNTNAP1*), ribonuclease P RNA component H1 (*RPPH1*), and KDEL motif-containing protein 2 (*KDELC2*)) (Figure 4.1) and for 8 target genes (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) (see Appendix 1). The PCR efficiency of each individual assay was determined by measuring 7 serial dilutions of HGDNA starting from 20 ng/3.6µl (1:2 dilution factor) and all the reactions were carried out in triplicate. The efficiency was calculated to determine the performance of the PCR assay (ideally 90–105%); the coefficient of determination (R2) of the standard curve (ideally >0.980);

and the CT values of the replicates.

All standard curves showed a linear correlation between the concentration of the DNA template (HGDNA) and the resulted CT values. The PCR efficiency was calculated from the standard curve; *GAPDH* and *KDELC2* had an efficiency of 97%, *RPPH1* 101% and *CNTNAP1* 100 % (Figure 4.1). All standard curves of the reference genes showed R2>0.98 and slope values were between −3.297 and −3.513. On the other hand, for the target genes standard curve slopes were between −3.431 and −3.555; the R2>0.98 and the efficiency values between 96%-92%. The PCR efficiency, R2 and slope values for the reference and the target assays are summarised in Table (4.1).

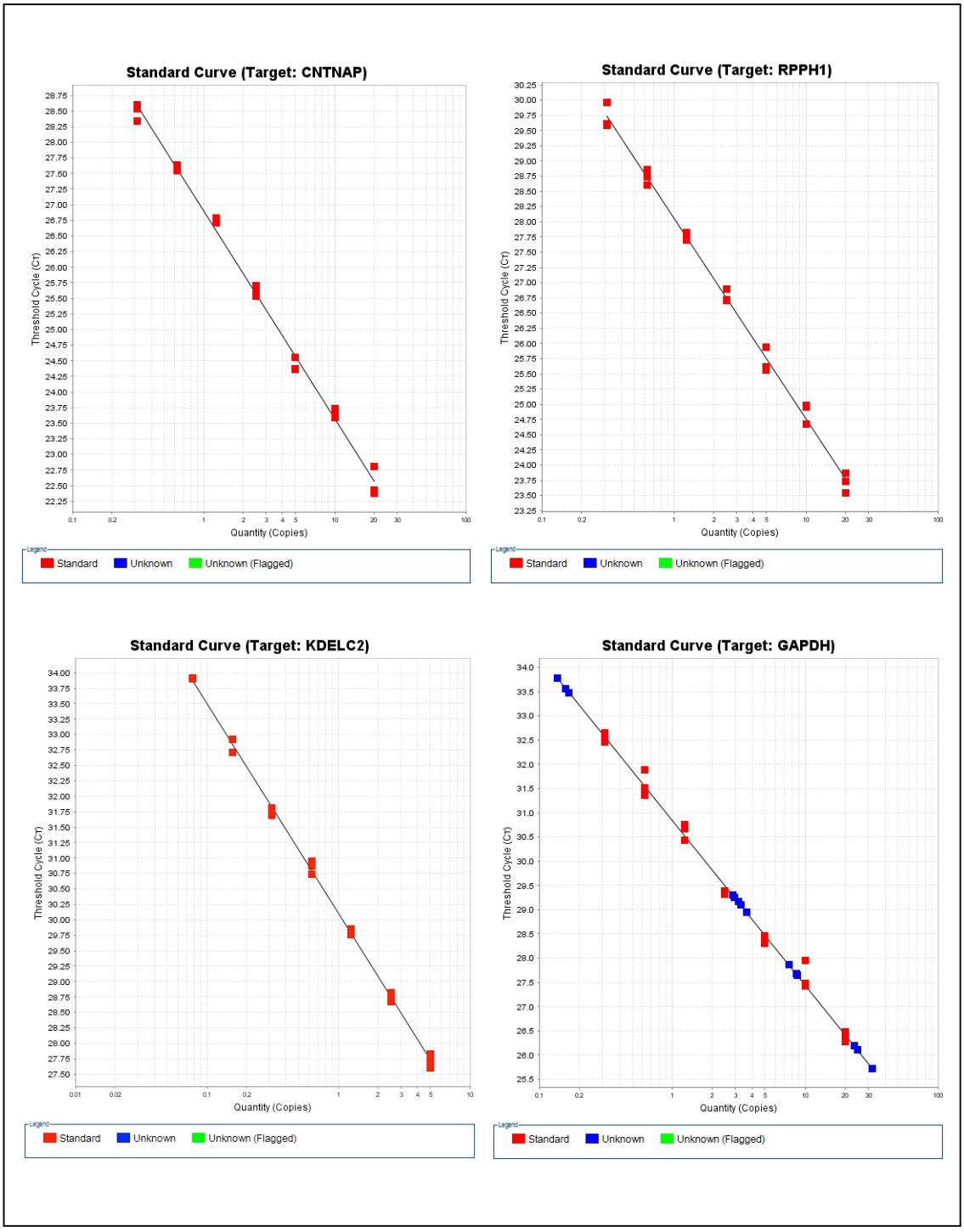


Figure 4.1 Standard curves of *CNTNAP1*, *RPPH1*, *KDELC2*, and *GAPDH* reference assays

The efficiencies of the primers and probes were determined using 7 serial dilutions of HGDNA, 1:2 dilution factors. PCR efficiencies: 100%, 101%, 97%, and 97% respectively; the coefficient of determination (R2): 0.995, 0.995, 0.998, and 0.998 respectively.

Table 4.1 Summary of slope, coefficient of determination (R2) and PCR efficiency of the reference and target assays

Gene name	Slope	R^2	Efficiency%
CNTNAP1	**−3.325**	**0.995**	**100%**
RPPH1	**−3.297**	**0.995**	**101%**
GAPDH	**−3.513**	**0.998**	**97%**
KDELC2	**−3.403**	**0.998**	**97%**
MYC	**−3.513**	**0.997**	**93%***
FGFR1	**−3.501**	**0.997**	**93%***
CDKN2A	**−3.555**	**0.994**	**93%***
CCND1	**−3.452**	**0.998**	**95%**
HER2	**−3.517**	**0.998**	**92%***
CYP19A1	**−3.431**	**0.996**	**96%**
PBX1	**−3.51**	**0.997**	**93%***
DMXL2	**−3.463**	**0.995**	**94%***

* Efficiency below 95%

4.2.2 Cell line assay development and validation

4.2.2.1 Reference gene selection

The four reference genes (*GAPDH*, *CNTNAP1*, *RPPH1*, and *KDELC2*) were compared for optimum normalisation of qPCR data to identify the reference gene with minimal variability using six breast cancer cell lines (SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D). Starting from 0.278 ng/µl of total DNA, the (10 cycles) Pre-amplification method was applied according to the protocol (method section 2.2.13). The unamplified DNA (10 ng/3.6µl) from the same breast cancer cell lines was used in order to determine if the preamplified DNA introduced any bias.

The CT values were plotted for individual reference genes in both unamplified and preamplified template (Figure 4.2). The CT values were also plotted using the average of two references ((*GAPDH* and *CNTNAP1*), (*GAPDH* and *RPPH1*), and (*CNTNAP1* and *RPPH1*)) and three references ((*GAPDH*, *CNTNAP1*, and *RPPH1*) and (*KDELC2, CNTNAP1*, and *RPPH1*)) (Figure 4.2). Notably, the average of two and/or three reference genes decreased the variation in the level of all genes across cell lines. The overall trend was similar between preamplified and unamplified DNA, though the

overall levels were slightly different.

Thereafter, The CT values were statistically analysed and compared using one-way ANOVA, followed by Kruskal-Wallis multiple comparisons test, which showed no significant difference between the reference genes. The only significant differences were between *GAPDH* and *RPPH1* ($P<0.05$) in the unamplified cell lines and *GAPDH* and *CNTNAP1* ($p<0.001$) in the preamplified cell lines. The *GAPDH* reference gene showed a lower level (higher CT value) compared to other reference genes (Figure 4.2). *CNTNAP1*, *RPPH1*, and *KDELC2* reference genes showed approximately similar CT values. As a result *GAPDH* was considered inadequate for normalisation since it showed the most variability across samples.

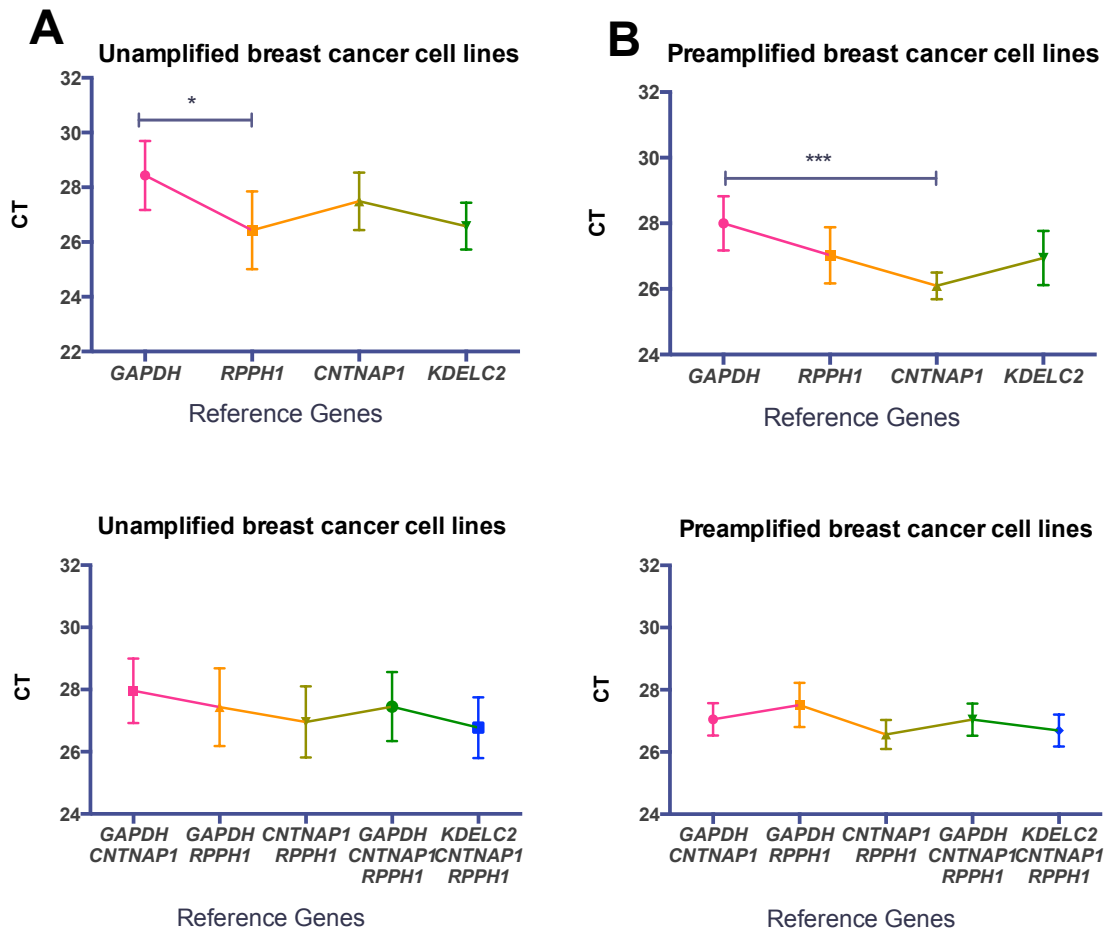


Figure 4.2 Validation of reference genes

A: Raw CT values in 6 unamplified cell lines for 4 reference genes individually (top), and in combination with two and three references (bottom). B: Raw CT values in 6 preamplified cell lines for 4 reference genes individually (top), and the average of two and three references (bottom). *P< 0.05, ***P< 0.001. Error bars represent the mean with SD.

4.2.2.2 qPCR CNV validation

Eight qPCR assays (*MYC*, *FGFR2*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) were validated with DNA extracted from six breast cancer cell lines (SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D) using HGDNA as a control. The levels of target genes was normalised against the three selected reference genes *CNTNAP1*, *RPPH1*, and *KDELC2*. As cfDNA levels in plasma are relatively low, pre-amplification is needed to generate an appropriate quantity of DNA for accurate CNV analysis. In order to validate this, the analysis was conducted in unamplified versus 10 cycles preamplified cell lines to see if pre-amplification introduced any bias

to the results.

All reactions were carried out in triplicate. A no template control (NTC) was used as a negative control. Accordingly, the comparative C_T method, which is also referred to as the ΔΔCT method was used for relative quantification. ΔCT for each cell line was calculated by taking the mean of the triplicate CT values of each reference gene from the mean of the triplicate CT values of each target, CT (<35). ΔΔCT data were then obtained by subtracting ΔCT of HGDNA from ΔCT for each cell line sample to determine the relative gene level, $RQ = 2^{-\Delta\Delta CT}$. Target gene status was based on the RQ of each gene in DNA isolated from the breast cancer cell lines in comparison to HGDNA. RQ values of ⩾2.1 were considered to be amplified while RQ values of <0.5 were considered to be deleted.

The target levels were measured and plotted relative to individual reference genes, the average of two reference genes, and the average of three reference genes, which will be discussed in the following sections.

4.2.2.2.1 Expression relative to individual reference genes

CNTNAP1 as a reference gene

RQ values for eight candidates (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) were normalised to the *CNTNAP1* reference gene (Figure 4.3). Preamplified and unamplified cell lines were used (SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D). The *MYC* gene was highly amplified in SK-BR-3 cell line. ZR-75-1 cell line showed a low-level amplification. Additionally, unamplified DNA from MCF-7 cell line indicated amplification. The only evidence of CN alteration at *FGFR1* was a potential deletion in the preamplified MCF-7 and ZR-75-1 cell lines. *CDKN2A* deletion was detected in 3 cell lines (MDA-MB-231, MCF-7, and ZR-75-1). MCF-7 cell line showed *CCND1* amplification, however SK-BR-3 cell line showed amplification in the unamplified status. The *HER2* locus had high-level amplification in SK-BR-3. MDA-MB-468, MCF-7, and ZR-75-1 cell lines had *DMXL2* and *CYP19A1* amplification. *DMXL2* amplification was also seen in the preamplified DNA of T-47D cell line. *PBX1* amplification was detected in the MDA-MB-468 and T-47D cell lines. Moreover, unamplified ZR-75-1 and preamplified MDA-MB-231 and MCF-7 cell lines had *PBX1* duplication. Overall, the only genes showing significant differences between unamplified and preamplified DNA were *PBX1* in the MCF-7 and T-47D cell lines and *DMXL2* in the MCF-7 cell line (Figure 4.3).

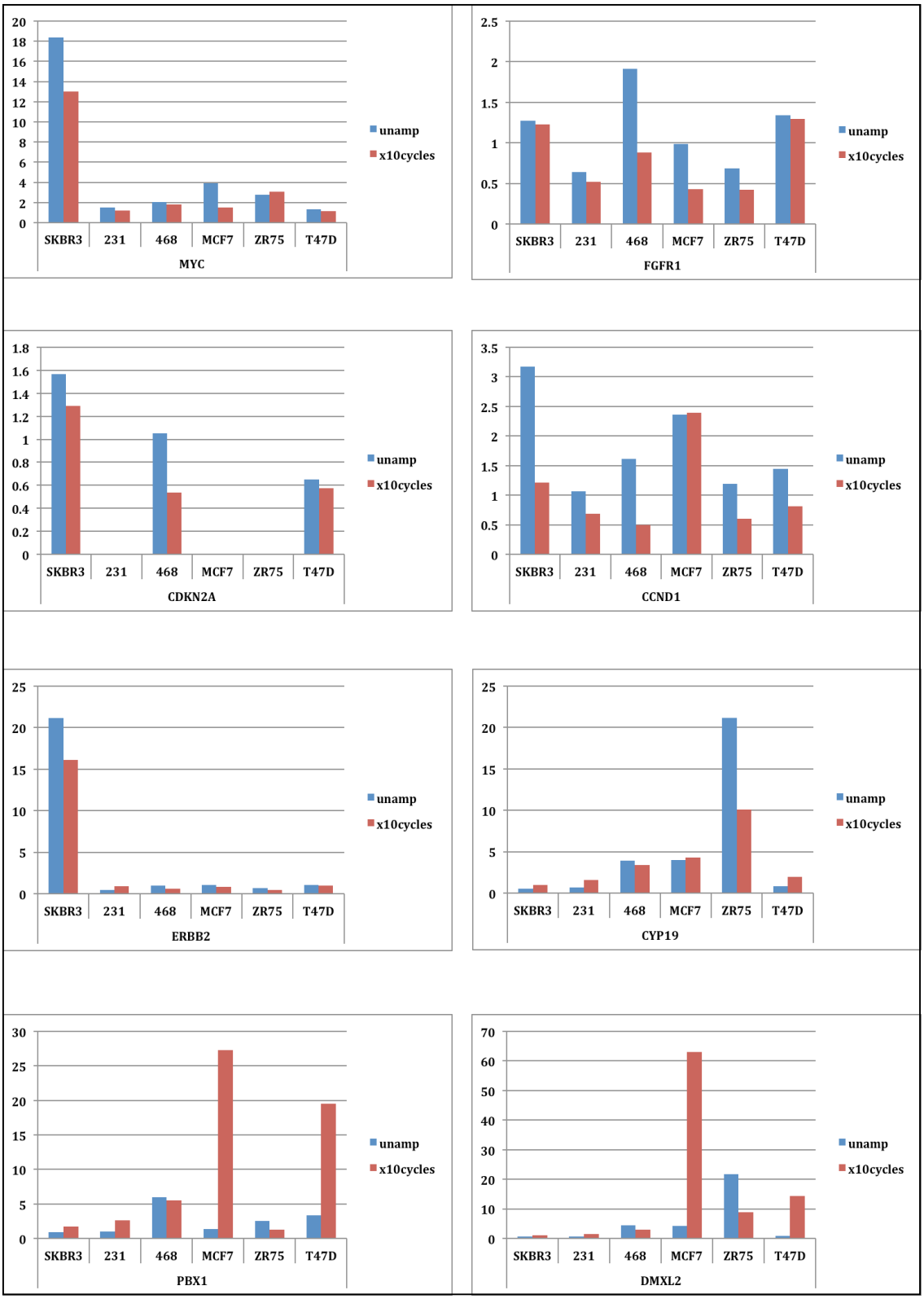


Figure 4.3 Validation of CNV status of 8 genes in unamplified and preamplified breast cell lines

Each graph represents the RQ values (Y-axis) of individual target gene relative to *CNTNAP1* reference gene as part of reference gene validation. CNV status for each target was determined based on the RQ values. RQ for each sample was calculated as follows: ΔCT= the mean of the triplicate CT values for each target − the mean of the triplicate CT values for *CNTNAP1*; this was then used to calculate ΔΔCT=ΔCT for each cell line − ΔCT of HGDNA; RQ=$2^{-\Delta\Delta CT}$. RQ≥2.1 (amplification), RQ<0.5 (deletion), unamp (unamplified DNA), x10cycles (10cycles preamplified DNA).

RPPH1 as a reference gene

The results were expressed relative to the *RPPH1* reference gene, using preamplified and unamplified 6 breast cancer cell lines (Figure 4.4). Generally, all of the genes did not show a significant difference between unamplified and preamplified assays across 6 cell lines, excluding *PBX1* and *DMXL2* genes, for which significant differences were observed in T-47D and (T-47D and MCF-7) cell lines respectively, as the pre-amplification method resulted in higher RQ values in the preamplified samples compared to unamplified samples (Figure 4.4).

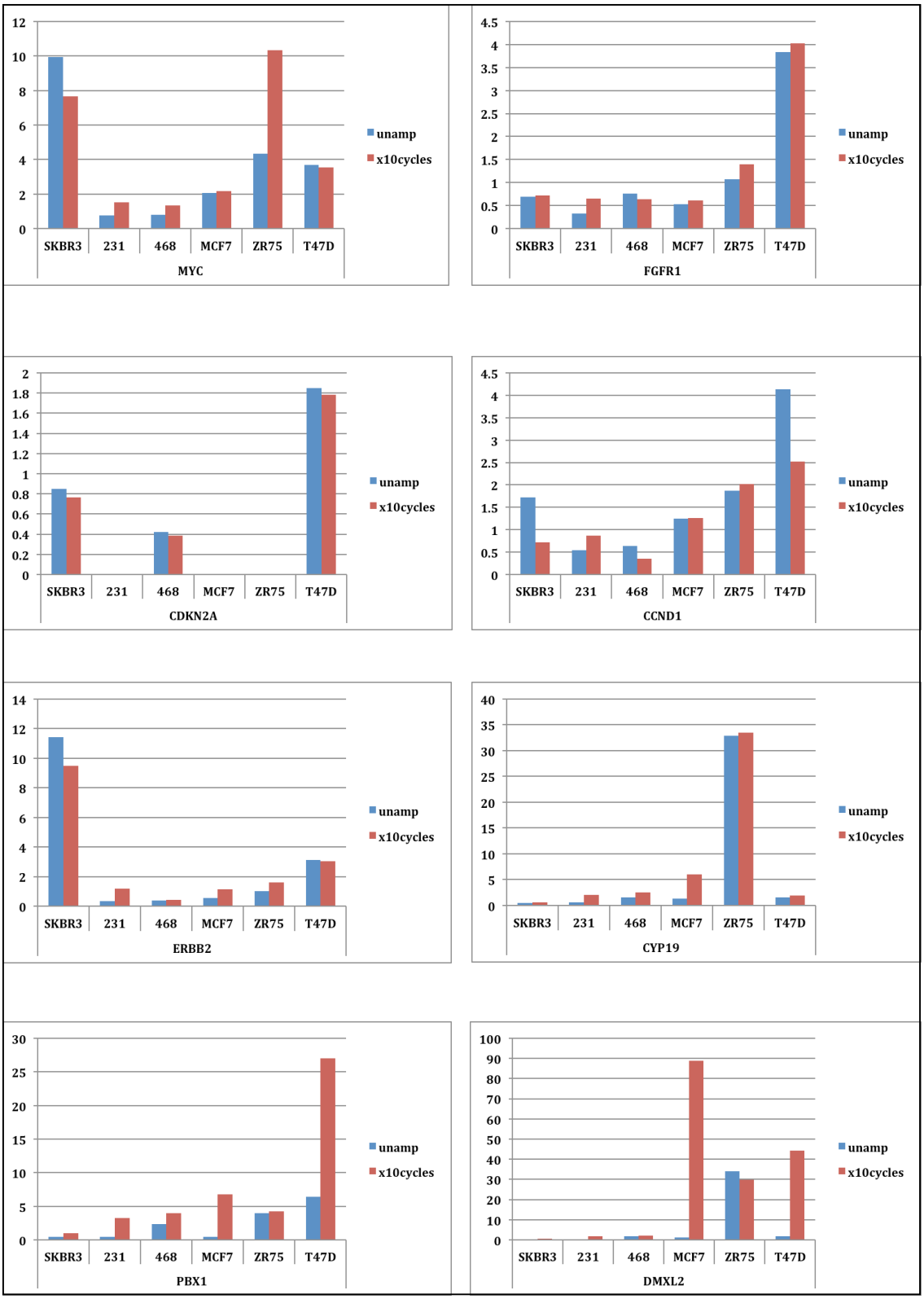


Figure 4.4 Validation of CNV status of 8 genes in unamplified and preamplified breast cell lines

Bars represent the RQ results relative to *RPPH1* reference gene (Y-axis) across cell lines. CNV status for each target was determined based on the RQ values. RQ for each sample was calculated as follows: ΔCT= the mean of the triplicate CT values for each target − the mean of the triplicate CT values for *RPPH1*; this was then used to calculate ΔΔCT=ΔCT for each cell line − ΔCT of HGDNA; RQ=$2^{-\Delta\Delta CT}$. RQ≥2.1 (amplification), RQ<0.5 (deletion), unamp (unamplified DNA), x10cycles (10cycles preamplified DNA).

KDELC2 as a reference gene

CNV status of 8 target genes was determined relative to the *KDELC2* reference gene across preamplified and unamplified breast cancer cell lines (Figure 4.5). The RQ data relative to *KDELC2* reference gene were different to the two other references (*RPPH1* and *CNTNAP1*). *MYC* and *CCND1* genes showed high-level differences in the MCF-7 and T-47D cell lines between preamplified and unamplified DNA, whereas *FGFR1* and *CDKN2A* also showed major differences in the T-47D cell line. The pre-amplification reaction seemed to reduce the amplification using this reference gene (Figure 4.5).

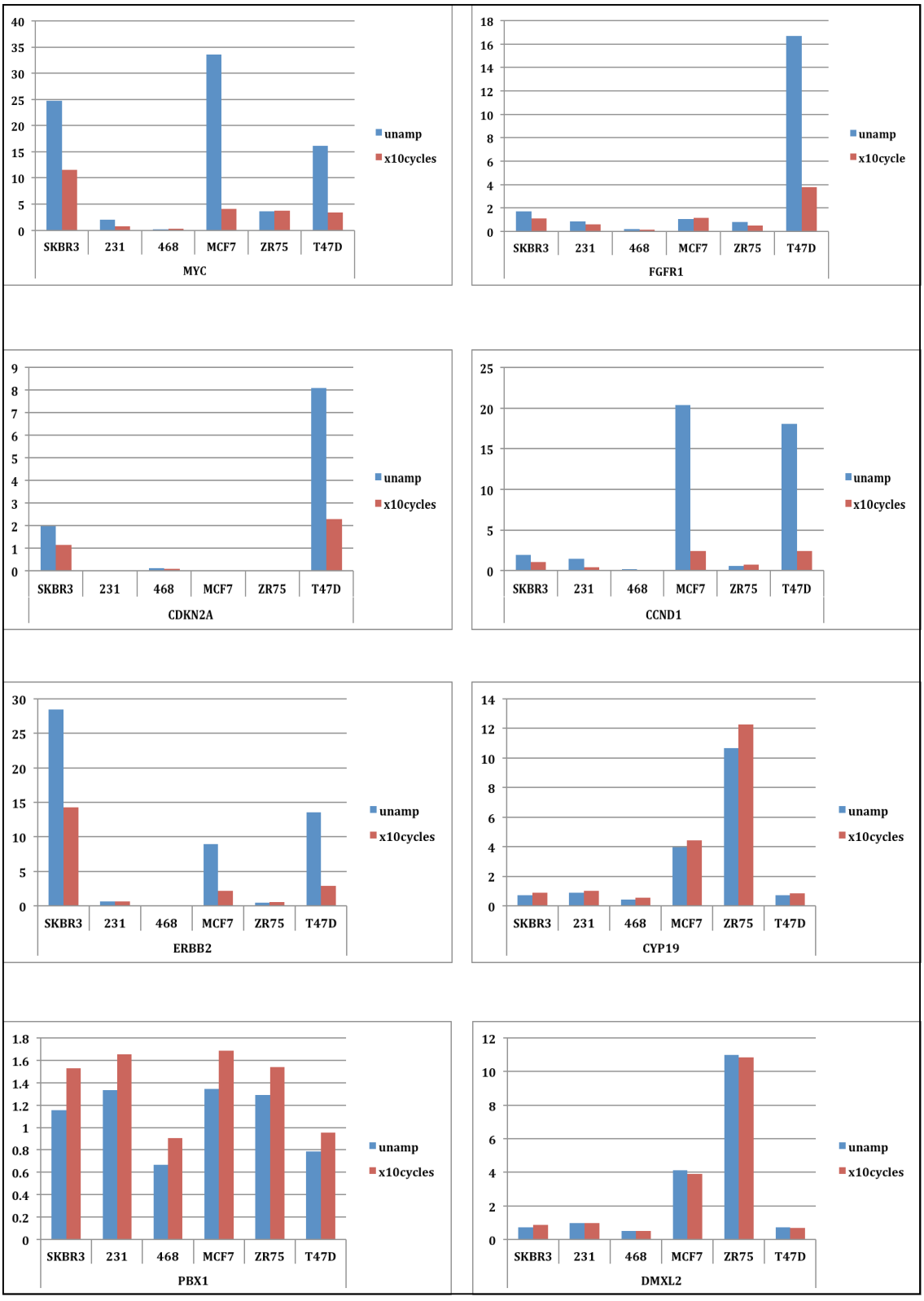


Figure 4.5 Validation of CNV status of 8 genes in unamplified and preamplified breast cell lines

The Y-axis represents RQ values relative to *KDELC2* reference gene; CNV status for each target was determined based on the RQ values. RQ for each sample was calculated as follows: ΔCT= the mean of the triplicate CT values for each target − the mean of the triplicate CT values for *KDELC2*; this was then used to calculate ΔΔCT=ΔCT for each cell line − ΔCT of HGDNA; RQ=$2^{-\Delta\Delta CT}$. RQ≥2.1 (amplification), RQ<0.5 (deletion), 10 cycles preamplified DNA (x10cycles), unamplified DNA (unamp).

4.2.2.2.2 Expression relative to the average of two reference genes

Average of CNTNAP1 and RPPH1 as a reference gene

CNV status of eight target genes was based on the RQ data relative to the average of *RPPH1* and *CNTNAP1* references in DNA isolated from breast cancer cell lines (Figure 4.6). Generally, the average of two references showed a similar issue that was observed with individual references in the fact that *PBX1* and *DMXL2* genes showed a marked difference between the two states (preamplified and unamplified DNA) and had significant differences in MCF-7 and T-47D cell lines, while the other genes did not (Figure 4.6).

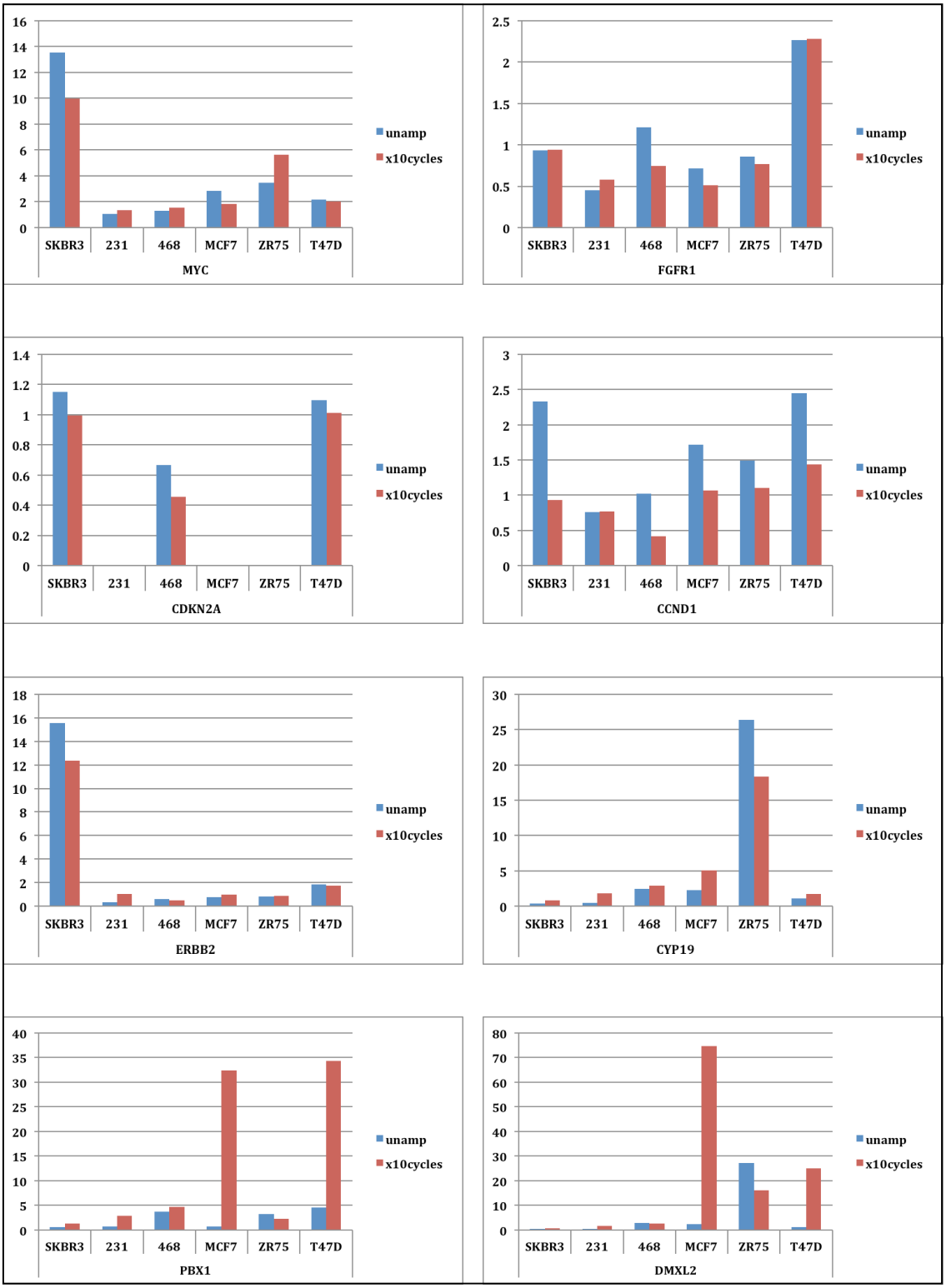


Figure 4.6 Validation of CNV status of 8 genes in unamplified and preamplified breast cell lines

RQ values on the Y-axis represent levels of target genes that normalised to the average of *CNTNAP1* and *RPPH1*. CNV status for each target was determined based on the RQ values. RQ for each sample was calculated as follows: ΔCT= the mean of the triplicate CT values for each target − the mean of the triplicate CT values for average of *RPPH1* and *CNTNAP1*; this was then used to calculate ΔΔCT=ΔCT for each cell line − ΔCT of HGDNA; RQ=2 $^{-\Delta\Delta CT}$. RQ≥2.1 amplification, RQ<0.5 deletion, unamp= unamplified DNA, x10cylces= 10 cycles preamplified DNA.

4.2.2.2.3 Expression relative to the average of three reference genes

Average of KDELC2, CNTNAP1, and RPPH1 as a reference gene

The CN changes at eight different loci were represented relative to the average of *KDELC2*, *RPPH1*, and *CNTNAP1*. Preamplified and unamplified breast cancer cell lines were normalised against HGDNA (Figure 4.7). The average of the 3 references was the most reliable overall, as single references showed bias in some of the pre-amplification reactions, which were resolved by averaging. However, there was still a noticeable difference between preamplified and unamplified MCF-7 DNA for *PBX1* and *DMXL2* targets (Figure 4.7).

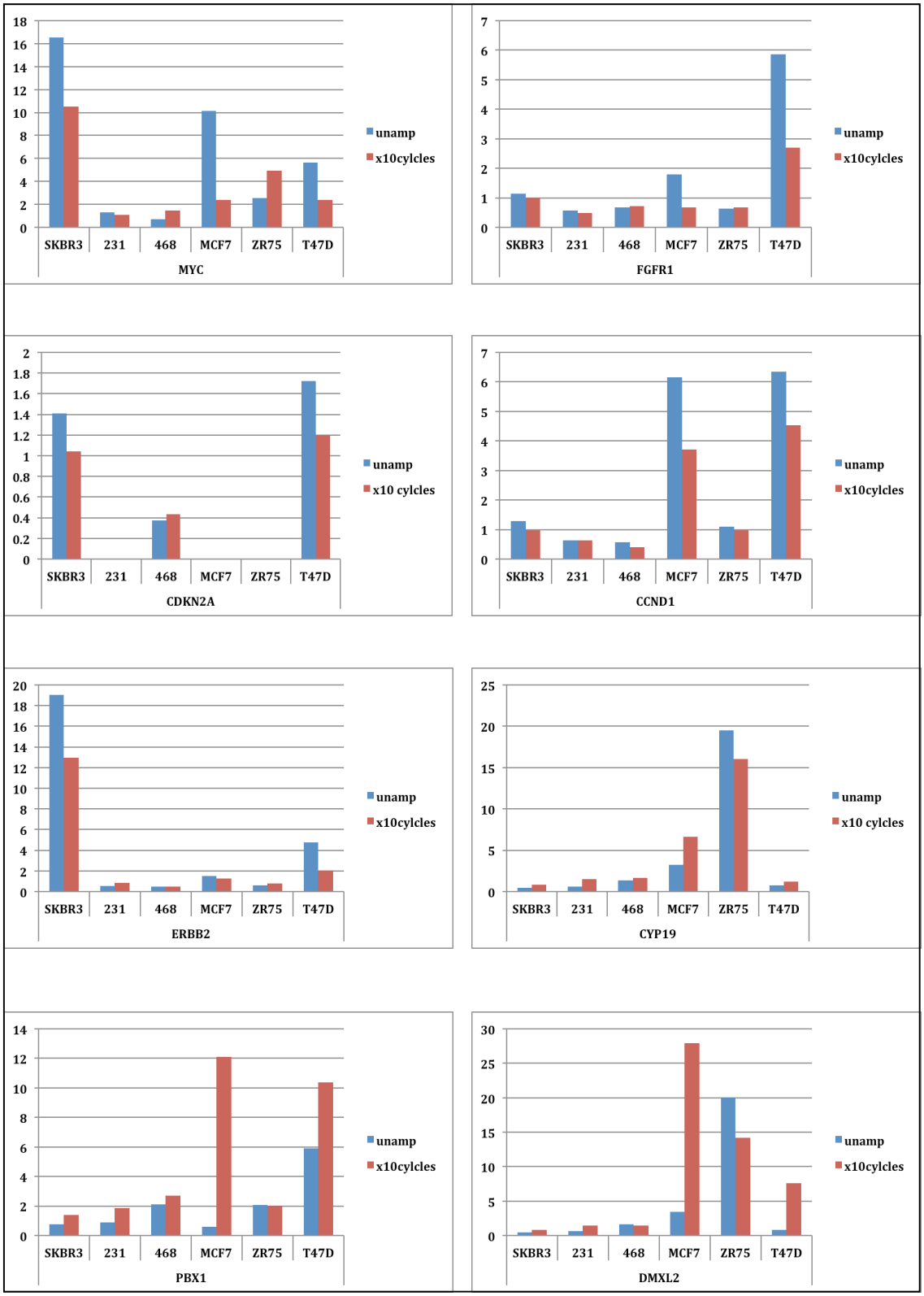


Figure 4.7 Validation of CNV status of 8 genes in unamplified and preamplified breast cell lines

RQ data on Y-axis showed levels of the target genes in unamplified and preamplified cell lines relative to the average of *KDELC2*, *CNTNAP1*, and *RPPH1*. CNV status for each target was determined based on the RQ values. RQ for each sample was calculated as follows: ΔCT= the mean of the triplicate CT values for each target − the mean of the triplicate CT values for the average of 3 references; this was then used to calculate ΔΔCT=ΔCT for each cell line − ΔCT of HGDNA; RQ=$2^{-\Delta\Delta CT}$. RQ≥2.1= amplification, RQ<0.5= deletion, unamp= unamplified cell line DNA, x10cylces= 10cylces preamplified cell line DNA.

4.2.2.3 CNV analysis comparison in preamplified (10 cycles) and unamplified breast cancer cell lines across different reference genes

After investigating target CNV statuses relative to the different references separately, RQ results among all various references used were next compared and analysed. CNV of eight targets (*MYC*, *FGFR2*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) in the preamplified and unamplified DNA from SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D were measured and plotted relative to individual reference (*CNTNAP1*, *RPPH1*, and *KDELC2* separately), average of two references (*CNTNAP1* and *RPPH1*), and the average of three references (*KDELC2*, *CNTNAP1*, and *RPPH1*) (Figure 4.9).

The results of CNV analysis obtained support other studies e.g (ref, COSMIC) (Table 4.11). There were high-level gains of *MYC* and *HER2* in SK-BR-3 and that was agreed between all reference genes. *CDKN2A* loss was consistent in two cell lines MDA-MB-231 and MCF-7. RQ data relative to *RPPH1*, *KDELC2*, and the average of *KDELC2*, *CNTNAP1*, and *RPPH1* showed deletion of *CDKN2A* in MDA-MB-468. Gain of *MYC* can also be seen in MCF-7 and T-47D when normalised to *RPPH1*, *KDELC2*, and to the average of *KDELC2*, *CNTNAP1*, and *RPPH1*. *FGFR1* gain was detected in T-47D across all reference candidates with the exception of *CNTNAP1* (no evidence of alteration). *CCND1* gain was found in MCF-7 when normalised to *CNTNAP1*, *KDELC2*, and average of *KDELC2*, *CNTNAP1*, and *RPPH1*. Similarly T-47D revealed *CCND1* gain against *RPPH1*, *KDELC2*, and average of *KDELC2*, *CNTNAP1*, and *RPPH1*. Levels relative to *CNTNAP1*, *KDELC2*, average of *CNTNAP1* and *RPPH1*, and average of *KDELC2*, *CNTNAP1*, and *RPPH1*, showed *CYP19A1* and *DMXL2* amplification in MCF-7. *PBX1* amplification was observed in T-47D when normalised to *CNTNAP1, RPPH1*, average of *CNTNAP1* and *RPPH1*, and average of *KDELC2*, *CNTNAP1*, and *RPPH1*. The RQ relative to the average of the 3 references showed the most consistent results when matched with previous studies. In summary, when a gene was amplified/deleted, the sample was usually positive either for unamplified or preamplified DNA but the absolute copy number values varied by RQ (Figure 4.9); for example, pre-amplification seemed to reduce the magnitude of amplification/deletion in

some samples while increasing it in others. Unpaired Student's t-test and ANOVA multiple comparison tests were performed to compare RQ data of 8 targets relative to single, average of two and average of three references of pre- and unamplified DNA samples (no significant differences were found). A high correlation of RQ data between the two conditions was detected as the values scored between (0.6 and 0.8) on the Pearson scale (Figure 4.8), suggesting the pre-amplification technique introduced no or little bias.

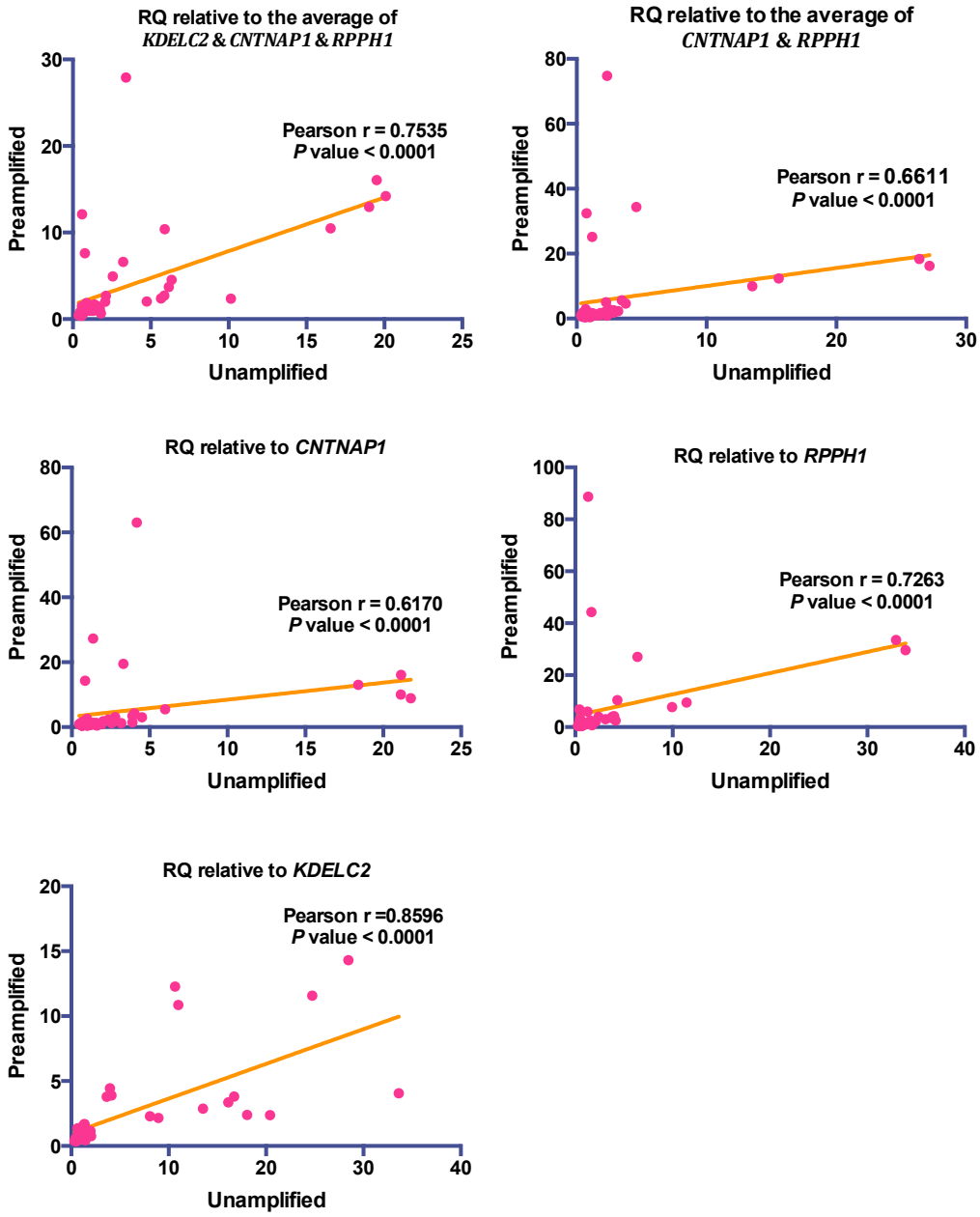


Figure 4.8 Correlation analysis of RQ data between preamplified and unamplified breast caner cell lines relative to different references

Pearson correlation analysis between preamplified and unamplified DNA from SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D cell lines. A high correlation was obtained between the two conditions. Data shown is RQ values of 8 targets (*MYC*, *FGFR2*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) relative to reference genes. RQ for each sample was calculated as follows: ΔCT= the mean of the triplicate CT values for each target − the mean of the triplicate CT values for each reference gene; this was then used to calculate ΔΔCT=ΔCT for each cell line − ΔCT of HGDNA; RQ=$2^{-\Delta\Delta CT}$. The lines represent a linear regression fit, Pearson r, and *P* values are indicated on each graph.

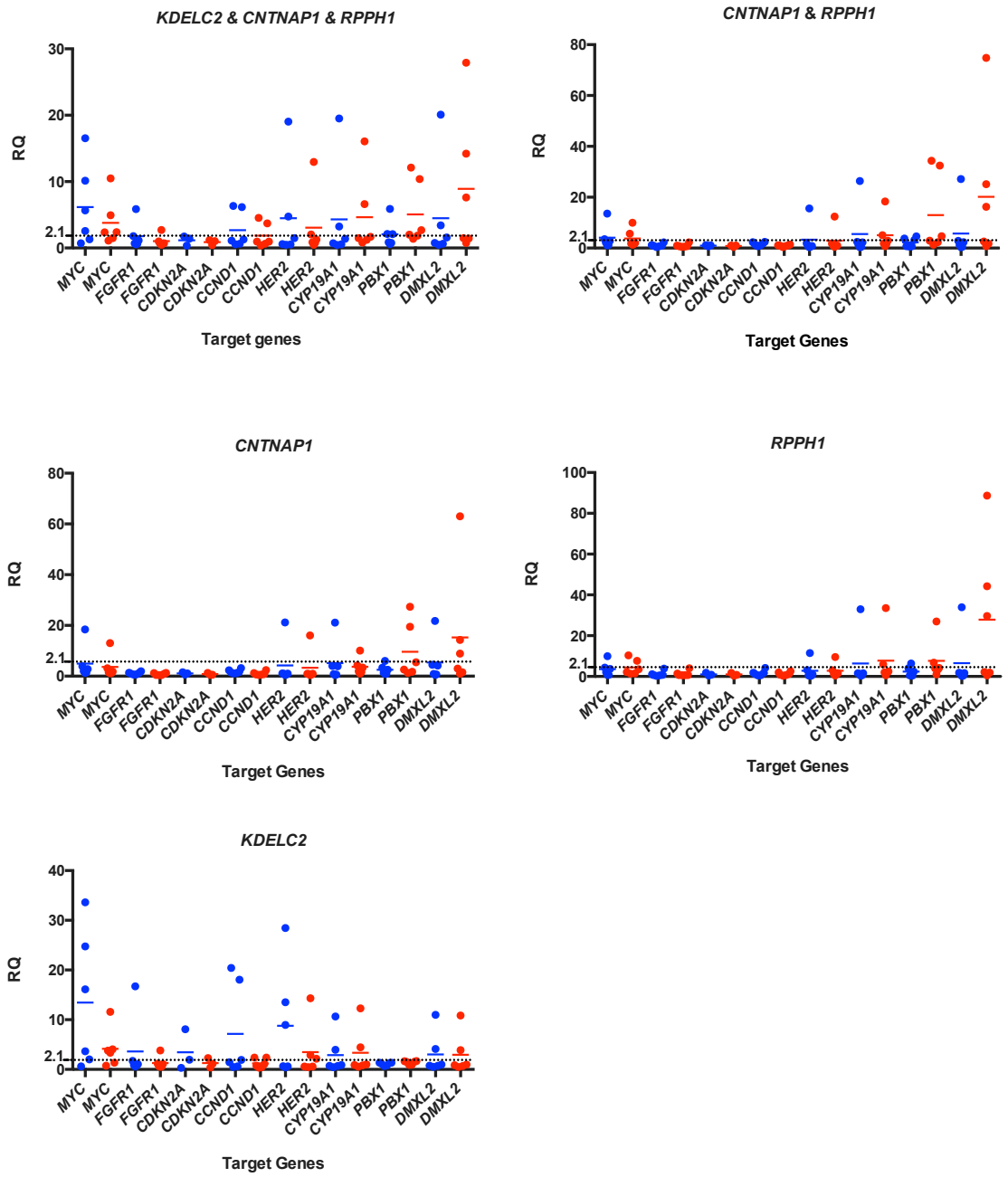


Figure 4.9 Scatter plots of Unpaired t-test and ANOVA multiple comparison test of eight targets between unamplified and 10 cycles preamplified breast cancer cell lines

CNV analysis comparison using single references, average of two references, and average of three references. ΔCT for each cell line was calculated by subtracting the mean of the triplicate CT values of each reference gene from the mean of the triplicate CT values of each target. ΔΔCT data were then obtained by subtracting ΔCT of HGDNA from ΔCT for each cell line sample to determine the relative gene level, RQ = $2^{-\Delta\Delta CT}$. RQ values of ≥2.1 and <0.5 are used as thresholds for gain and loss respectively. Unamplified DNAs (blue dots); 10 cycles preamplified DNAs (red dots). Bars represent the mean.

4.2.3 CNV analysis in plasma cfDNA of women with a family history of breast cancer and matched lymphocyte DNA

After validating the assays using breast cancer cell lines, we next assayed CNVs in normal lymphocyte DNA isolated from the buffy coat (white blood cells) of 14 patients on follow-up after surgical removal of their primary tumour (*BRCA* affected) and 3 patients of women with *BRCA* gene mutation who have no cancer (*BRCA* carrier). These served as an important relative control for subsequent CNV targets analysis in plasma cfDNA since they give the germline CNV data for each individual. Since plasma cfDNA concentration is relatively low, 5 and 10 cycles pre-amplification reactions were first validated in lymphocytes and HGDNA, which was used as a calibrator in order to generate a sufficient amount of DNA for more accurate CNV analysis. 10 cycles pre-amplification reaction was then performed to the cfDNA plasma samples. CT values (<35) of the reference and the target genes were measured using qPCR. To determine CNVs, target ΔCT values were calculated (mean CT of each target gene − mean CT of the average of reference genes) and used to obtain ΔΔCT data (ΔCT of lymphocyte/plasma DNA – ΔCT of the HGDNA/lymphocyte DNA) to determine the relative gene level. The target level (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) was normalised to the average of the three reference genes (*KDELC2*, *CNTNAP1*, and *RPPH1*). The CNV status of the targets was documented according to the RQ values of each plasma sample in comparison to the matched lymphocyte DNA. RQ values of ≥2.1 and <0.5 were used as threshold for amplification and deletion respectively. RQ values of ~1 reflect normal diploid CN.

4.2.3.1 CNV validation in 5 and 10 cycles preamplified lymphocyte DNAs

The RQ values of lymphocytes DNA against HGDNA (control) were measured across 17 patients (14 cases of *BRCA* affected group and 3 cases of *BRCA* carrier group) using qPCR. RQ values of ~1 was detected for each gene (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*), which confirmed normal diploid CN (Tables 4.2; 4.3). Figure (4.10) revealed normal CN obtained for 5 and 10 cycles pre-amplification reaction, the mean RQ values for the target genes ranged between (1.15 to 0.95) and (1.18 to 0.87) respectively. 10 cycles pre-amplification reaction showed more

noise with some samples drifting from 1, while 5 cycles pre-amplification reaction displayed the lowest variation. However, Unpaired t-test showed no significant differences between 5 and 10 cycles pre-amplification reaction. Moreover, a high correlation was found between the 2 conditions (data scored 0.7 by Spearman's correlation (Figure 4.11)). Together, those results indicated that matched lymphocyte DNAs could be served as reliable references for plasma cfDNAs, since they showed normal CN levels at the 8 different loci under investigation for CNV.

Table 4.2 A normal copy number state (RQ values ~1) of 8 genes across 5-cycle preamplified lymphocyte controls

Group Name	Sample No.	*MYC*	*FGFR1*	*CDKN2A*	*CCND1*	*HER2*	*CYP19A1*	*PBX1*	*DMXL2*
***BRCA* affected**	**82 lym**	0.98	0.9	1.11	0.95	0.89	1.18	1.22	0.99
***BRCA* affected**	**80 lym**	0.92	1.03	1.1	0.93	1.01	1.08	1.03	1.21
***BRCA* affected**	**78 lym**	0.95	1.08	1.23	0.85	1.06	0.95	1	1.18
***BRCA* affected**	**16 lym**	0.9	0.95	1.06	0.84	0.85	0.95	1	0.86
***BRCA* affected**	**48 lym**	1	1.15	1.13	0.96	1.04	0.99	1.1	1.26
***BRCA* affected**	**62 lym**	1.01	0.99	0.98	0.89	0.92	1.14	1.11	1.01
***BRCA* affected**	**67 lym**	1.04	1.06	1.1	0.93	1.03	1.22	1.19	1.22
***BRCA* affected**	**85 lym**	1.08	0.91	1.09	0.99	0.85	0.96	1.1	0.95
***BRCA* affected**	**86 lym**	1.1	0.9	1.01	0.75	0.89	0.95	1.03	0.95
***BRCA* affected**	**99 lym**	1.02	1.1	1.24	1.06	0.89	1.01	0.92	1.13
***BRCA* affected**	**18 lym**	0.98	0.97	1.38	1.08	0.87	0.98	0.9	1.16
***BRCA* affected**	**54 lym**	0.91	1.03	1.26	0.92	0.93	1.01	1.26	1.21
***BRCA* affected**	**60 lym**	0.91	1.16	1.2	0.95	0.87	1.05	1.2	1.26
***BRCA* affected**	**90 lym**	0.91	1.09	1.19	0.96	0.89	1.1	1.17	1.04
***BRCA* carrier**	**23 lym**	1.05	1.03	1.19	1.04	1.11	0.92	0.97	0.98
***BRCA* carrier**	**35 lym**	0.91	0.95	1.13	1.11	1.1	0.89	0.94	0.99
***BRCA* carrier**	**37 lym**	0.98	1.06	1.11	1.01	1.1	1	1	1.02
Average mean		0.98	1.02	1.15	0.95	0.96	1.02	1.07	1.08
Maximum		1.1	1.16	1.38	1.11	1.11	1.22	1.26	1.26
Minimum		0.9	0.9	0.98	0.84	0.85	0.89	0.9	0.86

Table 4.3 A normal copy number state (RQ values ~1) of 8 genes across 10-cycle preamplified lymphocyte controls

Group Name	Sample No.	*MYC*	*FGFR1*	*CDKN2A*	*CCND1*	*HER2*	*CYP19A1*	*PBX1*	*DMXL2*
***BRCA* affected**	**82 lym**	1.07	0.87	1.3	0.93	1.03	1.05	1.15	1.28
***BRCA* affected**	**80 lym**	0.8	1.03	1.05	0.92	0.92	0.94	0.98	1.19
***BRCA* affected**	**78 lym**	0.83	0.91	0.9	0.6	0.83	1.01	1	1.03
***BRCA* affected**	**16 lym**	0.89	0.97	1.42	0.84	0.88	1.13	1.08	0.91
***BRCA* affected**	**48 lym**	0.86	1.1	1.15	1.29	1.47	1.14	1.3	1.28
***BRCA* affected**	**62 lym**	0.88	1.11	0.88	1.13	1.19	1.23	1.17	1.09
***BRCA* affected**	**67 lym**	0.89	0.78	1.49	1.44	1.25	1.42	0.97	0.98
***BRCA* affected**	**85 lym**	1.02	0.89	1.46	1.08	0.99	1.04	1.05	0.93
***BRCA* affected**	**86 lym**	1.1	1.02	1.22	1.33	0.84	0.95	1.04	1.02
***BRCA* affected**	**99 lym**	1.05	0.75	1.05	0.97	0.58	0.88	0.91	0.97
***BRCA* affected**	**18 lym**	0.87	0.89	0.89	0.88	0.99	1.1	1.12	1.03
***BRCA* affected**	**54 lym**	0.81	1.05	0.84	0.8	0.95	1.07	0.95	0.82
***BRCA* affected**	**60 lym**	0.66	1.05	1.69	0.96	1.24	1.07	0.83	0.93
***BRCA* affected**	**90 lym**	0.84	1.04	1.3	0.9	1.24	1.09	0.87	0.95
***BRCA* carrier**	**23 lym**	0.84	0.93	0.88	0.77	1.13	1.13	1	0.91
***BRCA* carrier**	**35 lym**	0.74	0.72	0.81	0.55	1.01	1.32	0.87	0.63
***BRCA* carrier**	**37 lym**	0.63	0.61	1.66	1.08	0.99	0.83	0.95	0.64
Average mean		0.87	0.92	1.18	0.97	1.03	1.08	1.01	0.98
Maximum		1.1	1.11	1.69	1.44	1.47	1.42	1.3	1.28
Minimum		0.63	0.61	0.81	0.55	0.58	0.83	0.83	0.63

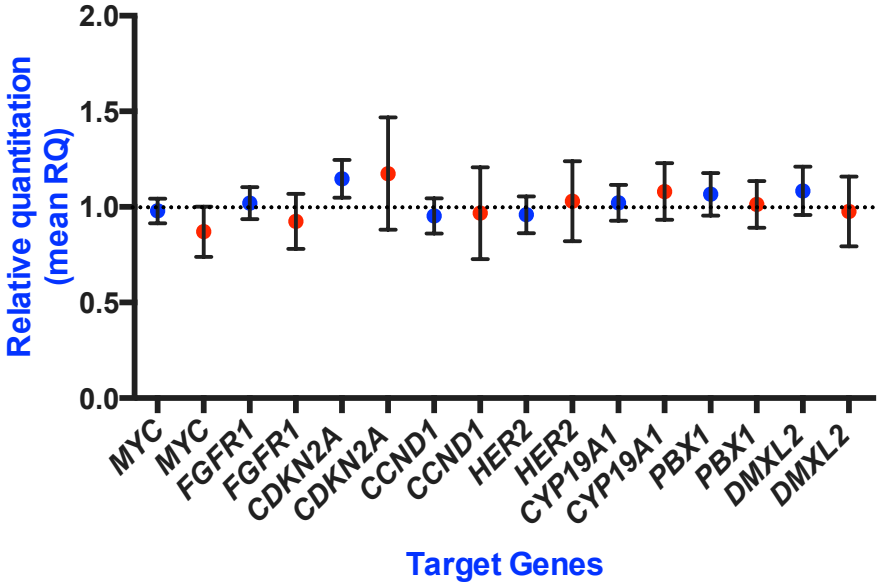


Figure 4.10 The mean RQ data for 8 CNV assays in 17 preamplified lymphocyte controls

Scatter-plots represent normal CN (~1) of qPCR data in lymphocyte DNAs; samples were preamplified for 5 (blue dots) and 10 (red dots) cycles. Error bars represent the mean with SD.

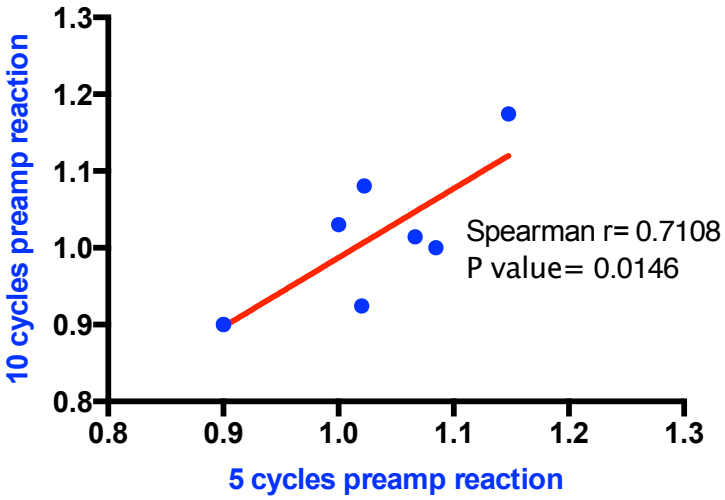


Figure 4.11 Correlation analysis between 5 and 10 cycles preamplified lymphocyte controls

The mean RQ data of 8 target genes (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) among 17 lymphocyte samples. A high correlation was found between 5 and 10 cycles reaction. The line represents a linear regression fit, Spearman r, and *P* value are indicated.

4.2.3.2 10 cycles preamplified plasma cfDNA against 10 cycles preamplified HGDNA

A total of 17 cfDNA samples isolated from the plasma of 14 women with breast cancer (with *BRCA* mutation) on follow-up after surgery (*BRCA* affected group) and from 3 healthy women with *BRCA* mutation (*BRCA* carrier group) were surveyed. 10 cycles of pre-amplification were applied to plasma and HGDNA. Among the 8 candidate genes, only *CCND1* showed CN gain in (♯85) and (♯35) plasma samples from *BRCA* affected and *BRCA* carrier groups, respectively. Deletion was observed in the *BRCA* affected group at *PBX1* (82 plasma sample) and *DMXL2* (82, 80, 16, 48, and 85 plasma samples) loci (Table 4.4; Figure 4.12). Overall, CN alteration was detectable in the plasma of women with *BRCA* mutation (*BRCA* affected and *BRCA* carrier group) relative to HGDNA, whereas it was not detected in lymphocyte DNA relative to HGDNA in the same group of patients.

Table 4.4 CNV analysis of 8 targets in the plasma of 17 women with a family history of breast cancer

RQ values ≥2.1 amplification (in red); RQ values <0.5 deletion (in green).

Group Name	Sample No.	*MYC*	*FGFR1*	*CDKN2A*	*CCND1*	*HER2*	*CYP19A1*	*PBX1*	*DMXL2*
***BRCA* affected**	**82 pl**	1.07	0.87	1	1.26	1.35	0.75	0.41	0.39
***BRCA* affected**	**80 pl**	1.19	0.9	1.19	1.49	1.25	0.77	0.64	0.42
***BRCA* affected**	**78 pl**	1.28	0.89	1.24	1.45	1.46	0.73	0.62	0.53
***BRCA* affected**	**16 pl**	1.15	0.82	1.2	1.61	1.05	0.64	0.61	0.46
***BRCA* affected**	**48 pl**	1.17	0.81	1.34	1.62	1.31	0.73	0.51	0.42
***BRCA* affected**	**62 pl**	1.4	0.92	1.28	1.4	1.55	1.03	0.62	0.56
***BRCA* affected**	**67 pl**	1.53	0.82	1.33	1.37	1.48	0.91	0.62	0.52
***BRCA* affected**	**85 pl**	1.61	0.92	1.5	2.16	1.36	0.92	0.66	0.46
***BRCA* affected**	**86 pl**	1.11	0.78	1.06	0.69	1.17	0.77	0.99	0.73
***BRCA* affected**	**99 pl**	1.35	1.05	1.38	1.2	1.54	0.86	0.85	0.64
***BRCA* affected**	**18 pl**	1.39	0.96	1.35	1.49	1.33	0.88	0.65	0.54
***BRCA* affected**	**54 pl**	1.42	1.07	1.17	1.32	1.23	0.95	1.05	0.84
***BRCA* affected**	**60 pl**	1.17	1.21	1.33	1.57	1.43	0.92	0.73	0.77
***BRCA* affected**	**90 pl**	1.15	0.75	1.09	0.91	1.14	0.71	0.6	0.55
***BRCA* carrier**	**23 pl**	1.26	0.92	1.1	1.82	1.2	0.83	0.58	0.58
***BRCA* carrier**	**35 pl**	1.33	1.09	1.56	2.54	1.58	0.9	0.65	0.53
***BRCA* carrier**	**37 pl**	1.27	0.73	1.2	1.67	1.33	0.93	0.54	0.5

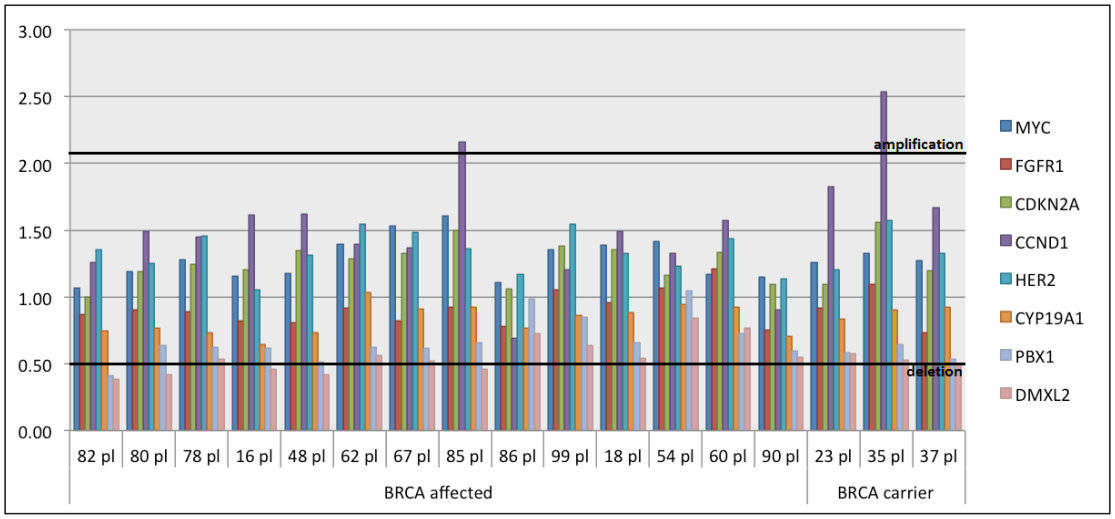


Figure 4.12 CNV analysis in 10 cycles preamplified plasma cfDNA samples

RQ values relative to HGDNA; pl (plasma sample number); RQ≥2.1 (amplification); RQ<0.5 (deletion).

4.2.3.3 10 cycles preamplified plasma cfDNA against 10 cycles preamplified lymphocyte DNA

Paired plasma cfDNA and matched lymphocyte DNA from 14 healthy female controls with a family history of breast cancer but who had not inherited a *BRCA* mutation (*BRCA* predictive negative (PNEG)), 19 healthy female who had inherited *BRCA* mutation (*BRCA* carrier), and 15 women with a family history who had inherited *BRCA* mutation on follow-up after surgical removal of their primary tumour (*BRCA* affected group) were first screened for CN alteration using qPCR after 10 cycles of pre-amplification. Matched lymphocyte DNA for each patient was used as the experimental calibrator and used to calculate the RQ values. RQ values ≥2.1 and <0.5 were used as thresholds for amplification and deletion, respectively.

CNVs were found in the six genes: *MYC*, *CDKN2A*, *CCND1*, *HER2*, *PBX1*, and *DMXL2*. Two genes *CYP19A1* and *FGFR1* did not show any evidence of CN alteration. *CCND1* and *HER2* amplification occurs frequently in breast cancer (OP Kallioniemi et al., 1992; Berns et al., 1992) and was also observed in this study. *MYC*, *CDKN2A*, *PBX1*, and *DMXL2* CN amplification were less frequent within this group of patients. For example, *HER2* amplification was seen in the plasma cfDNA of 7 patients (PNEG

(n=2), *BRCA* carrier (n=1), and *BRCA* affected (n=4) group) and *CCND1* gain was found in 13 samples (PNEG (n=3), *BRCA* carrier (n=4), and *BRCA* affected (n=6) group). There were 2 patients harbouring *MYC* amplification from the *BRCA* affected group, and 3 patients with *CDKN2A* amplification (*BRCA* carrier (n=2) and *BRCA* affected (n=1) group). In contrast, *MYC* deletion was seen in 2 patients from the *BRCA* affected group; 16 patients had evidence of deletion in *PBX1* (PNEG (n=5), *BRCA* carrier (n=7), and *BRCA* affected (n=4) group). Additionally, *DMXL2* loss was widely detected from PNEG (n=6), *BRCA* carrier (n=8), and *BRCA* affected (n=10) group (Table 4.5; Figure 4.13).

Analysis of plasma cfDNA has shown that CNV was less common in the plasma of healthy females with a family history who did not have a *BRCA* mutation (with 28.6% gain and 50% loss) and in healthy women who carried an inherited *BRCA* mutation (31.6% gain and 53% loss) than in the plasma of women who carried an inherited *BRCA* mutation after treatment in the follow-up of primary breast cancer (60% gain and 66% loss) (Figure 4.14-A). Dunn's multiple comparison test revealed no significant difference between the RQ values of breast cancer patients and controls (Figure 4.13). *PBX1* and *DMXL2* loss seems to be common in all plasmas compared to lymphocyte controls, which may suggest an issue with pre-amplification (Table 4.5; Figure 4.13). Accordingly, they were excluded from the analysis (Figure 4.14-B). Overall, CN alterations were discovered in all groups but the greatest numbers of patients where CN alteration was detected was in women, post-surgery and on follow-up of primary breast cancer.

Table 4.5 CNV analysis of 8 targets in the plasma of women with a family history of breast cancer

Data shown is RQ values. RQ ≥2.1 gain (in red); RQ <0.5 loss (in green).

Group name	Sample No.	*MYC*	*FGFR1*	*CDKN2A*	*CCND1*	*HER2*	*CYP19A1*	*PBX1*	*DMXL2*
***BRCA1* PNEG**	**116 plasma**	1.49	0.85	0.85	1.76	1.54	1.16	0.49	0.42
***BRCA1* PNEG**	**115 plasma**	1.13	0.89	1.35	1.71	1.27	0.97	0.38	0.36
***BRCA1* PNEG**	**113 plasma**	1.44	0.94	0.99	2.42	1.41	0.74	0.54	0.5
***BRCA1* PNEG**	**109 plasma**	1.4	0.99	0.92	2.92	2.21	1.42	0.43	0.39
***BRCA1* PNEG**	**91 plasma**	1.76	1.24	1.08	2.14	1.79	1.16	0.63	0.65
***BRCA1* PNEG**	**63 plasma**	1.17	0.85	0.94	1.73	1.3	0.81	0.52	0.56
***BRCA1* PNEG**	**70 plasma**	1.38	1.21	1.19	1.7	0.98	0.99	0.77	0.58
***BRCA1* PNEG**	**103 plasma**	1.44	0.77	1.12	0.86	1.27	0.89	0.43	0.46
***BRCA1* PNEG**	**94 plasma**	1.8	0.94	0.97	1.28	2.23	1.12	0.67	0.47
***BRCA2* PNEG**	**28 plasma**	1.48	1.29	0.99	1.28	1.34	1.25	0.42	0.76
***BRCA2* PNEG**	**100 plasma**	1.97	1.25	1.7	1.54	1.83	0.99	0.87	0.74
***BRCA2* PNEG**	**105 plasma**	1.09	0.69	1.23	1.03	1.21	0.93	0.59	0.43
***BRCA2* PNEG**	**75 plasma**	1.12	0.88	1.13	1.32	1.12	0.81	0.55	0.6
***BRCA2* PNEG**	**98 plasma**	1.27	0.83	1.22	1.06	1.13	0.85	0.81	0.83
***BRCA1* carrier**	**23 plasma**	0.88	1.11	2.07	1.74	1.24	0.79	0.46	0.71
***BRCA1* carrier**	**35 plasma**	1.04	1.7	3.18	3.43	1.82	0.73	0.59	0.94
***BRCA1* carrier**	**37 plasma**	1.38	0.94	1.92	2.1	1.13	1.16	0.58	0.75
***BRCA1* carrier**	**53 plasma**	1.38	1.02	1.15	1.44	1.25	1.08	0.56	0.66
***BRCA1* carrier**	**69 plasma**	1.41	0.75	1.04	1.38	1.36	0.83	0.36	0.39
***BRCA1* carrier**	**84 plasma**	1.09	0.68	0.99	1.57	1.33	0.74	0.46	0.38
***BRCA1* carrier**	**95 plasma**	1.28	0.86	0.96	2.1	1.6	0.68	0.63	0.71
***BRCA1* carrier**	**107 plasma**	1.24	0.98	0.74	1.76	1.33	0.97	0.56	0.63
***BRCA1* carrier**	**108 plasma**	1.85	0.81	0.96	1.8	1.36	0.82	0.64	0.38
***BRCA1* carrier**	**112 plasma**	1.55	1.3	1.1	1.01	1.48	1.01	0.47	0.55
***BRCA1* carrier**	**114 plasma**	1.11	0.77	1.08	1.8	1.57	0.82	0.45	0.29
***BRCA2* carrier**	**42 plasma**	1.12	1.58	1.2	1.66	2.59	1.37	0.68	0.59
***BRCA2* carrier**	**24 plasma**	1.47	0.97	1.3	1.88	1.55	0.84	0.56	0.42
***BRCA2* carrier**	**17 plasma**	1.38	0.89	1.05	1.96	1.64	0.74	0.41	0.33
***BRCA2* carrier**	**19 plasma**	1	0.71	0.78	1.6	1.2	0.78	0.49	0.45
***BRCA2* carrier**	**21 plasma**	1.45	1.16	0.86	2.5	1.51	0.58	0.52	0.5
***BRCA2* carrier**	**39 plasma**	1.08	0.69	0.95	1.6	1.29	0.83	0.57	0.66
***BRCA2* carrier**	**89 plasma**	1.69	0.6	0.89	1.85	1.74	0.98	0.55	0.61
***BRCA2* carrier**	**97 plasma**	1.16	0.7	0.9	1.69	1.6	0.74	0.54	0.45
***BRCA1* affected**	**82 plasma**	0.19	0.94	0.71	3.42	1.24	0.53	0.23	0.31
***BRCA1* affected**	**80 plasma**	0.43	1.14	1.3	1.4	1.48	0.93	0.59	0.39
***BRCA1* affected**	**78 plasma**	2.04	1.23	2.62	2.45	2.89	1	0.66	0.45
***BRCA1* affected**	**16 plasma**	1.73	1.08	1.61	1.88	2.71	0.63	0.6	0.52
***BRCA1* affected**	**48 plasma**	1.59	0.99	1.24	2.1	1.97	0.97	0.43	0.35
***BRCA1* affected**	**62 plasma**	1.6	0.97	1.63	2.45	2.24	1.15	0.52	0.36
***BRCA1* affected**	**67 plasma**	2.41	1.37	1.08	1.82	1.86	0.92	0.62	0.38
***BRCA1* affected**	**85 plasma**	2.41	1.49	1.2	2.68	1.53	1.06	0.48	0.33
***BRCA1* affected**	**86 plasma**	0.99	0.74	0.95	0.52	1.11	0.5	0.58	0.45
***BRCA2* affected**	**99 plasma**	1.26	1.14	1.25	1.49	2.1	0.73	0.76	0.55
***BRCA2* affected**	**18 plasma**	1.5	1.04	0.97	1.86	1.2	0.67	0.53	0.43
***BRCA2* affected**	**54 plasma**	1.42	0.83	0.9	2.18	1.44	0.89	0.96	0.94
***BRCA2* affected**	**60 plasma**	1.63	1.04	1.01	1.76	1.78	0.99	0.61	0.58
***BRCA2* affected**	**90 plasma**	1.26	0.65	1.07	1.08	1.42	0.74	0.47	0.41
***BRCA2* affected**	**101 plasma**	1.51	0.77	0.94	1.31	1.12	0.73	0.99	0.58

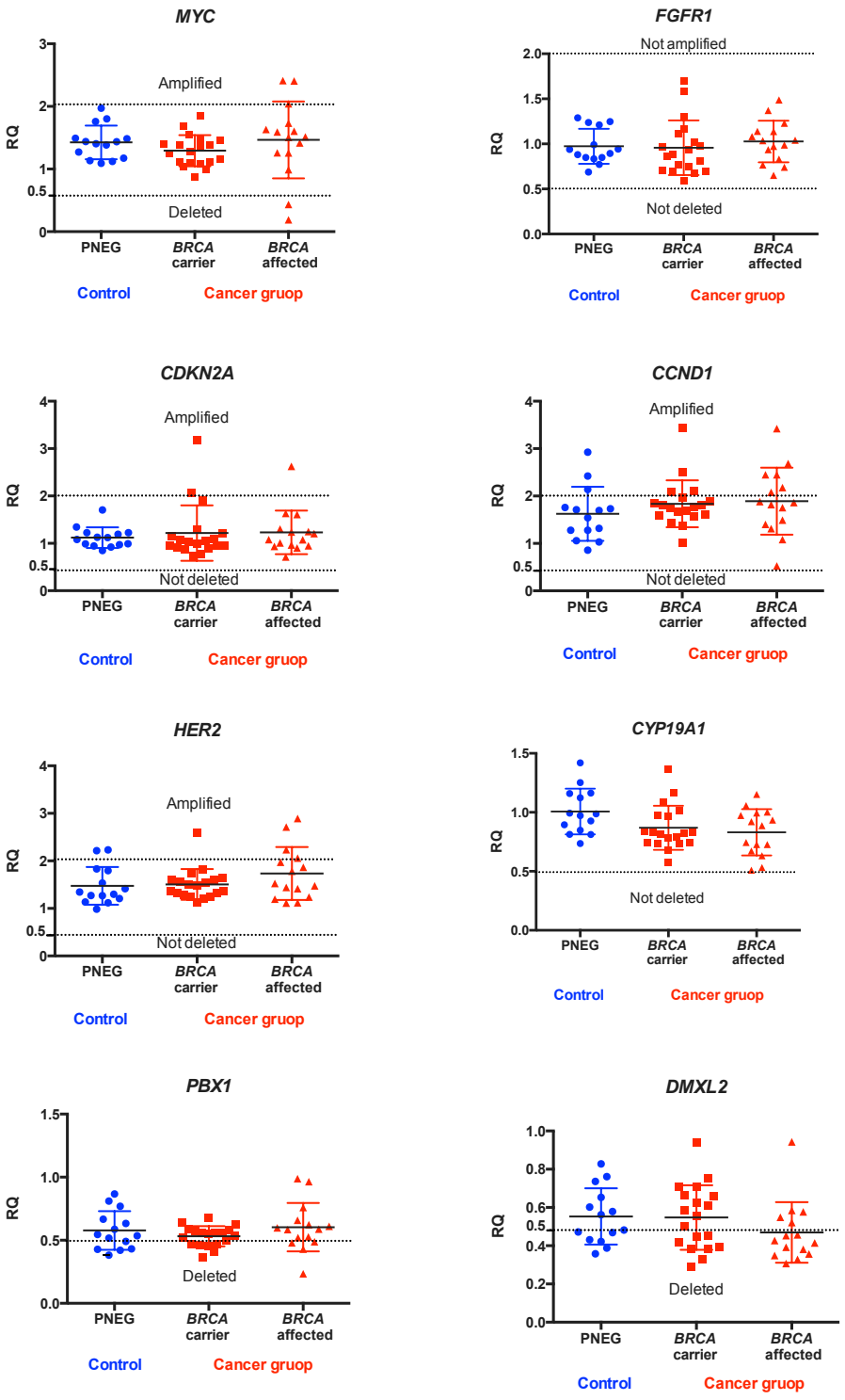
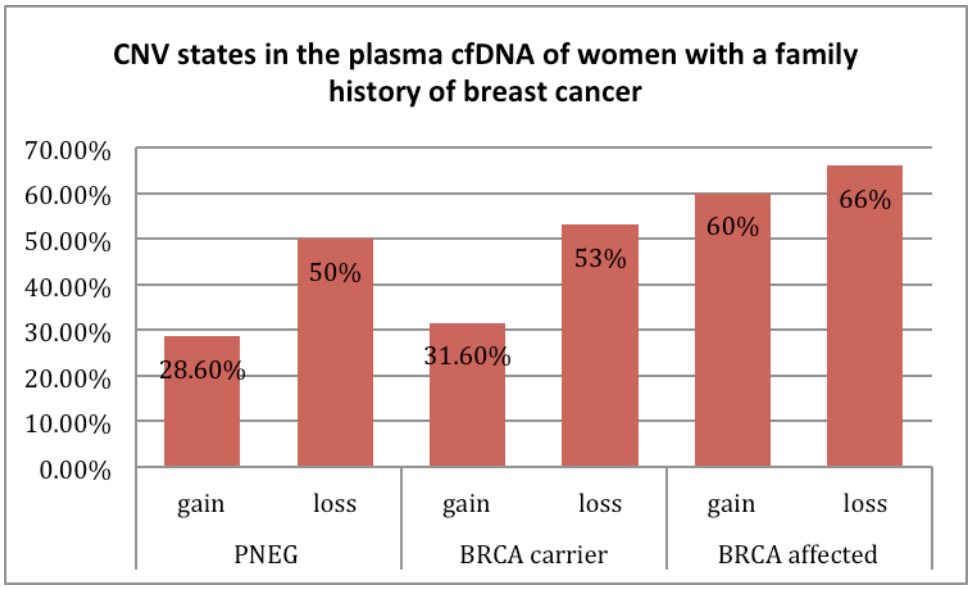


Figure 4.13 CNV states of 8 targets discriminate between control and cancers in 10-cycle preamplified plasma

CN alteration detection in the plasma cfDNA of women with a family history of breast cancer (PNEG (n=14), *BRCA* carrier (n=19), and *BRCA* affected (n=15)). The plasma level was analysed using Dunn's multiple comparison test. No significant difference was found across three groups. ΔCT values were normalised to the average of *KDELC2*, *CNTNAP1*, and *RPPH1* and used to obtain ΔΔCT. Normal lymphocytes used to calculate ΔΔCT to give RQ values. CNV state is based on the RQ values. Thresholds were ≥2.1 for amplification and <0.5 for deletion (horizontal dotted lines). Error bars represent the mean with SD.

A



B

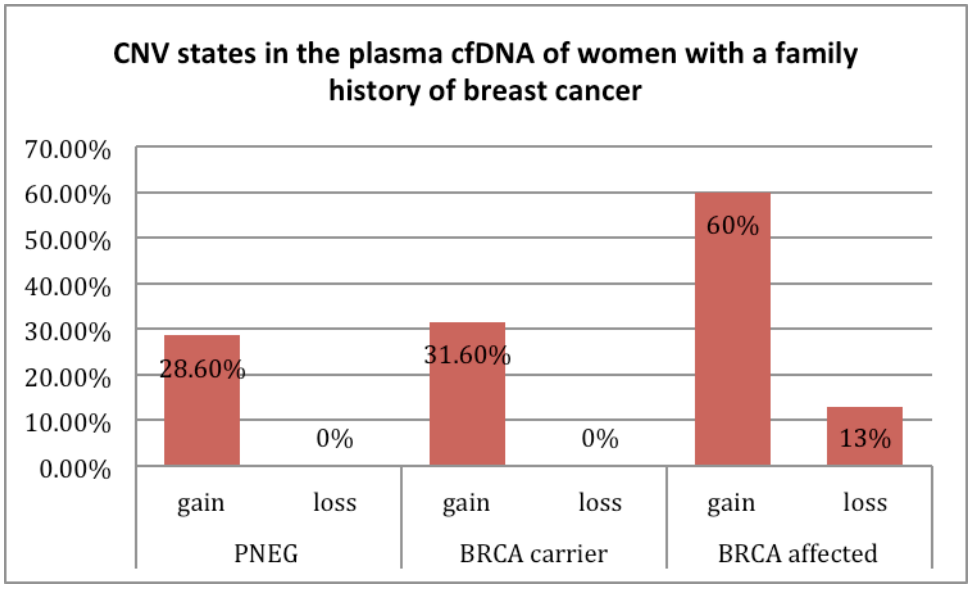


Figure 4.14 percentages of CN gains and losses in women with a *BRCA* mutation (*BRCA* carrier (no cancer) and *BRCA* affected group (have cancer)) compared to PNEG group (healthy female)

A: Percentages of gains and losses for *MYC*, *CDKN2A*, *CCND1*, *HER2*, *PBX1*, and *DMXL2*. B: Percentages of gains and losses when *PBX1* and *DMXL2* data were excluded.

4.2.4 ddPCR CNV assay validation

In this study, ddPCR technology was applied to analyse CNV status, focusing on several clinically important loci including: *MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2* to validate results achieved by qPCR. Target CNV FAM-labeled assays were estimated using duplex assays with *RPPH1* as the VIC-labeled reference gene. Samples with the two genes in equal proportion were considered as a normal CN state (diploid genome). HGDNA was used as a control and was expected to have a CN value of ~2, whereas the target to the reference ratio across CN assays was expected to be ~1. NTC were performed using water as a negative control. A threshold of CN>2.2 indicated amplification and CN≤1.2 indicated deletion.

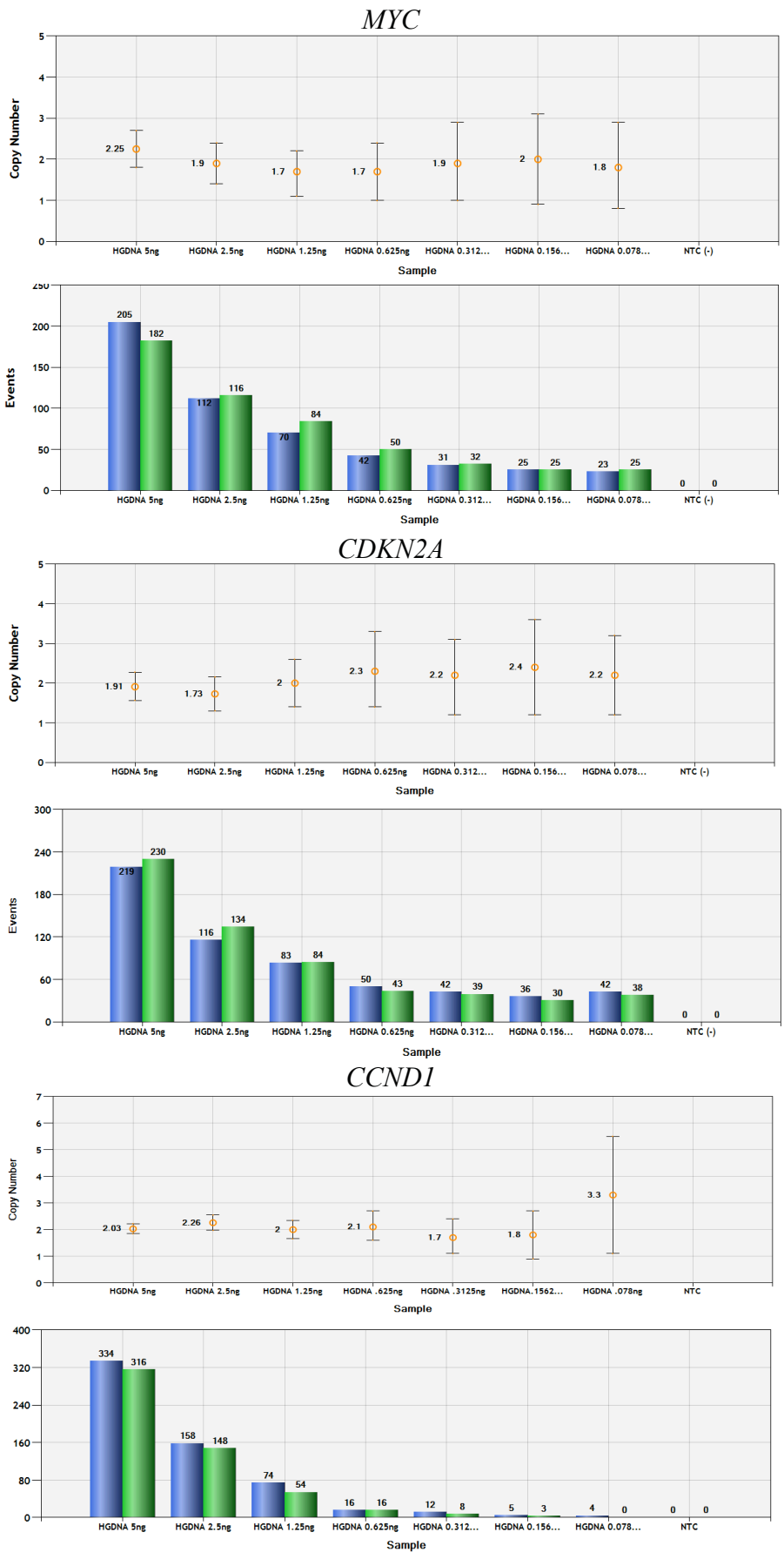
ddPCR experiments were used to measure CN in HGDNA, 6 breast cancer cell lines including spike-in to dilute the cell line DNA, and plasma of high-risk breast cancer families. Plasma cfDNAs from 19 healthy *BRCA* mutation carrier, 15 post-surgical *BRCA* affected women, and 14 *BRCA* mutation negative controls were compared against their matched lymphocyte DNA. Since plasma cfDNA levels are typically very low, 10 cycles pre-amplification was applied as for qPCR starting with 10 µl template cfDNA.

4.2.4.1 CNV analysis in HGDNA

Five ddPCR duplex assays (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2)* with known CNV status were evaluated independently via ddPCR using a 7-point, 1:2 serial dilution of HGDNA. Dilution series ranging between 5 ng-0.078 ng were performed to measure the lowest DNA concentration that could detect CNV in plasma cfDNA. The lowest concentration with 100 positive events was considered an accurate threshold for detection of CNV, as determined by Prof. Shaw's research group.

A diploid CN of ~2 was obtained across a 7-point serial dilution for *MYC* and *CDKN2A* (excluding 5 ng and (0.625 and 0.156 ng) respectively). At the time, the *CCND1* gene showed normal CN, which was up to 0.1562 ng DNA. However, *DMXL2* and *HER2* dropped out below 0.3125 ng DNA (Figure 4.15). Total events were between 10496 and 18707, indicating reliable results across the five target genes (see Appendix 2). As 100 positive events were detected between 2.5 ng/µl and 1.25 ng/µl templates, this indicated that the minimum acceptable amount to be used for plasma cfDNA analysis was 1.25 ng.

A


MYC
Copy Number
2.25
1.9
1.7
1.7
1.9
2
1.8
HGDNA 5ng
HGDNA 2.5ng
HGDNA 1.25ng
HGDNA 0.625ng
HGDNA 0.312...
HGDNA 0.156...
HGDNA 0.078...
NTC (-)
Sample
Events
205
182
112
116
70
84
42
50
31
32
25
25
23
25
0
0
CDKN2A
1.91
1.73
2
2.3
2.2
2.4
2.2
219
230
116
134
83
84
50
43
42
39
36
30
42
38
CCND1
2.03
2.26
2
2.1
1.7
1.8
3.3
HGDNA .625ng
HGDNA .3125ng
HGDNA.1562...
HGDNA .078ng
NTC
334
316
158
148
74
54
16
16
12
8
5
3
4
0

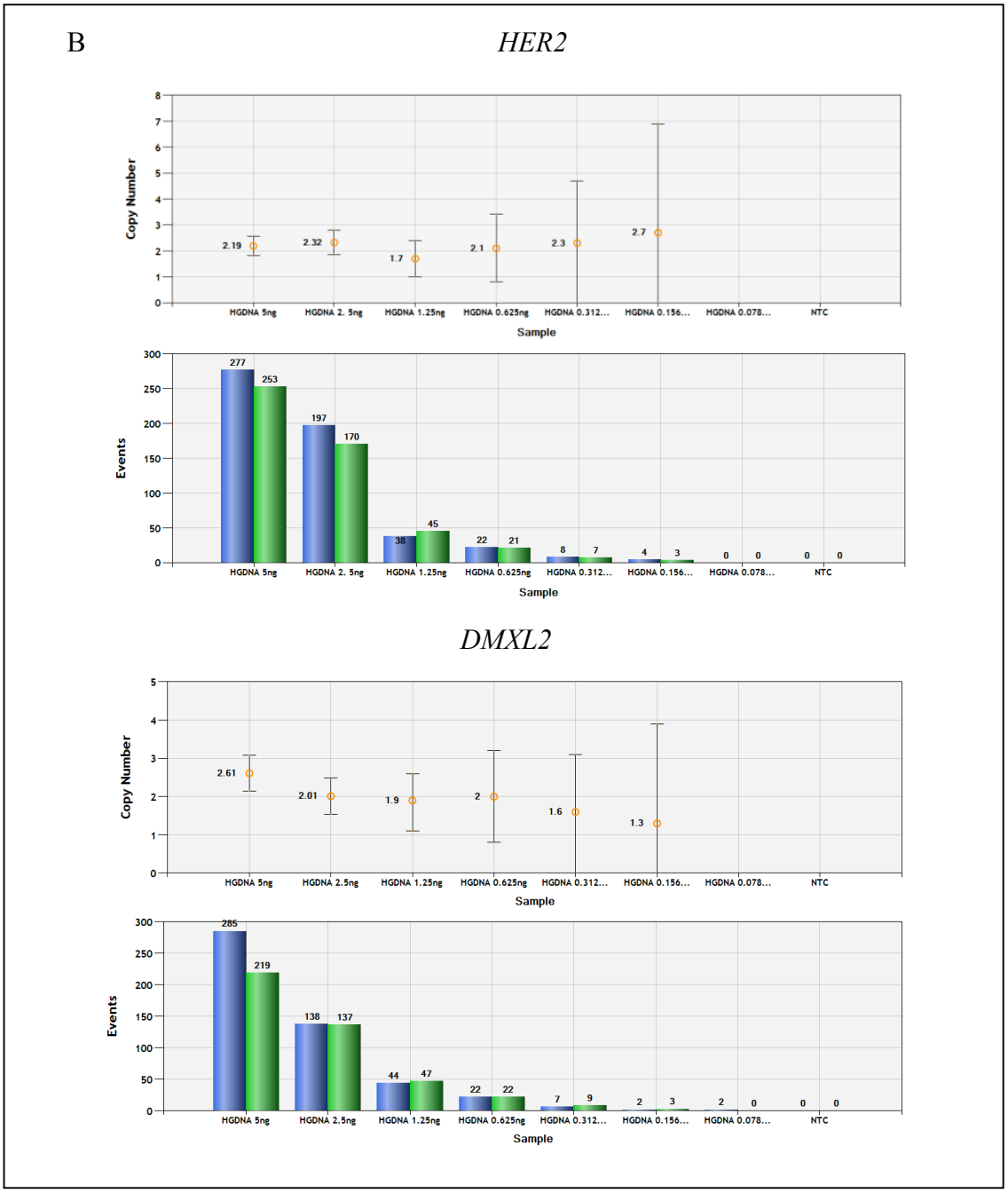


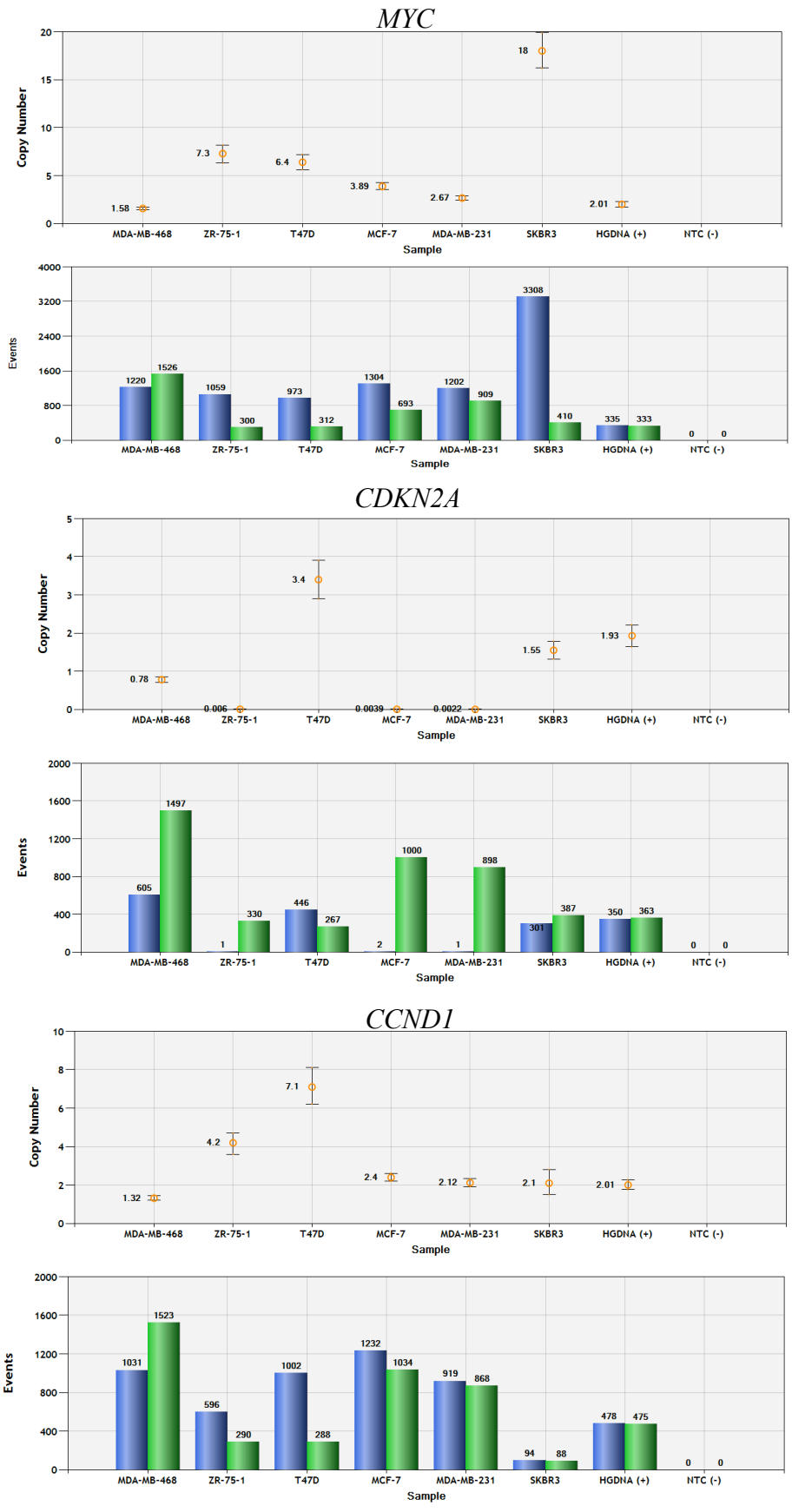
Figure 4.15 Assessing accurate detection of CNV at 5 genes using 7-point serial dilution of HGDNA

Illustrates normal CN values (~2) and ratios (~1) of HGDNA (control) across different dilutions detected by ddPCR. Positive events for the duplex assays (target gene (blue bar)/reference gene (green bar)) were reported for each target. A: validation of CNV of *MYC*, *CDKN2A*, and *CCND1*. B: validation of CNV of *HER2* and *DMXL2*. Poisson error bars are represent the 95% confidence interval. NTC: no template control.

4.2.4.2 Detection of CNV in the cell line models

Six breast cancer cell lines (SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D) were chosen as the second line of investigation for CNV analysis of 5 genes (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2*) by ddPCR (Figure 4.16). This showed a CN gain of *MYC* in SK-BR-3, MDA-MB-231, MCF-7, ZR-75-1, and T-47D cells with CN values of 18, 2.67, 3.89, 7.3, and 6.4 respectively. Clear deletion (CN<1) was detected in *CDKN2A* in MDA-MB-231, MDA-MB-468, MCF-7, and ZR-75-1 cell lines, and T-47D was the only cell line where amplification was observed. *CCND1* was amplified with CN values of 7.1, 2.4, and 4.2 in T-47D, MCF-7, and ZR-75-1, respectively. High-level DNA amplification was detected with a CN of 19.9 at the *HER2* locus in SK-BR-3 DNA. Additionally, amplification was detected in ZR-75-1 (CN=2.45) and T-47D (CN=5.7) cell lines, whereas deletions were seen in MDA-MB-468 with a CN value of 0.74. ZR-75-1 had high-levels of *DMXL2* amplification (19.9); MCF-7 and T-47D had low-levels of amplification (CN values of 3.82 and 5.2, respectively), whereas MDA-MB-468 showed deletion with a CN value of 0.7 (Figure 4.16). The number of total events fluctuated between 10286 and 17890, reflecting reliable results (see Appendix 2).

A


MYC
Copy Number
Sample
MDA-MB-468
ZR-75-1
T47D
MCF-7
MDA-MB-231
SKBR3
HGDNA (+)
NTC (-)
1.58
7.3
6.4
3.89
2.67
18
2.01
Events
1220
1526
1059
300
973
312
1304
693
1202
909
3308
410
335
333
0
0
CDKN2A
0.78
0.006
3.4
0.0039
0.0022
1.55
1.93
605
1497
1
330
446
267
2
1000
1
898
301
387
350
363
CCND1
1.32
4.2
7.1
2.4
2.12
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1232
1034
919
868
94
88
478
475

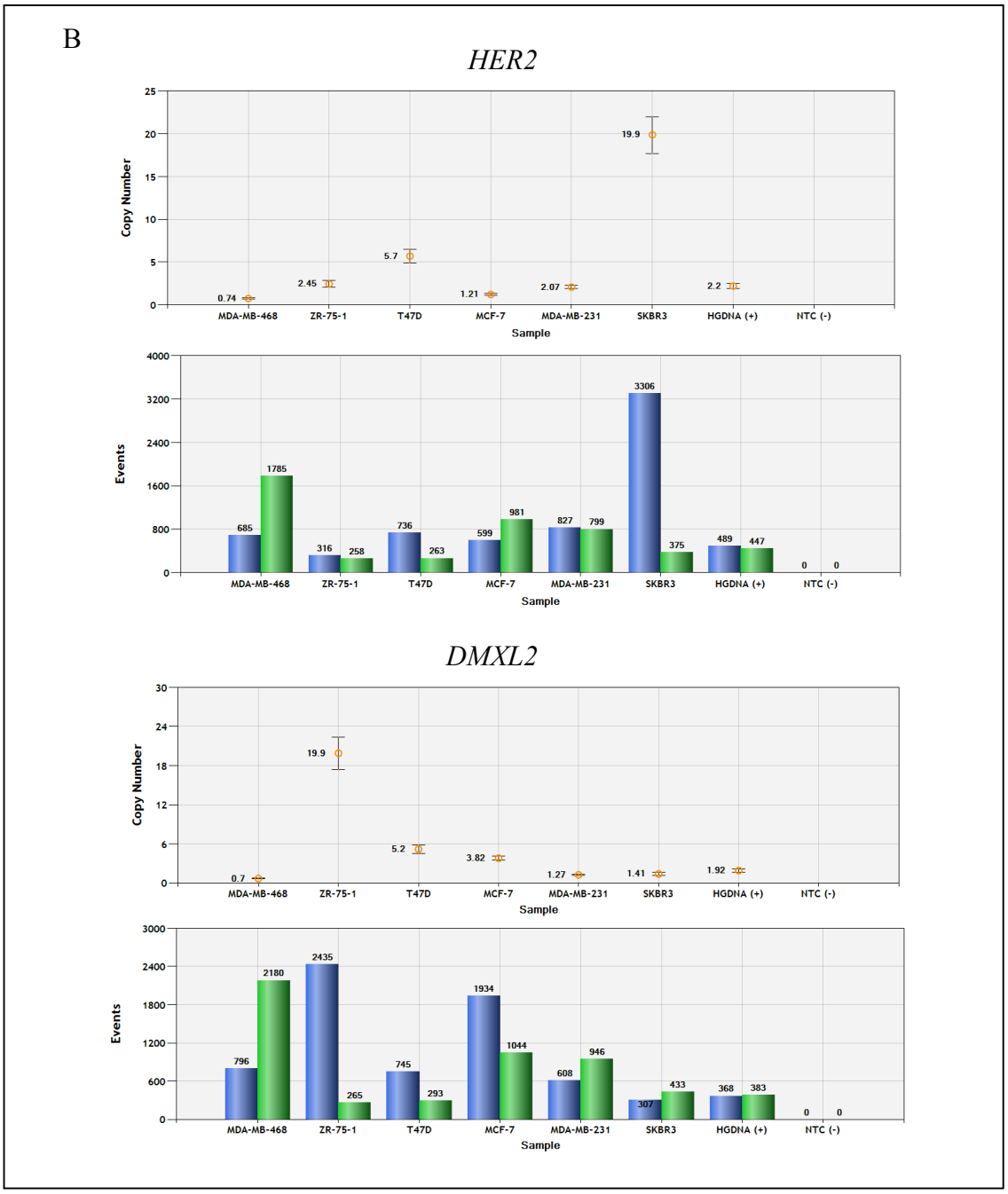


Figure 4.16 ddPCR detection of CNV states of 5 target genes in 6 breast cancer cell lines

A: shows CNV states of *MYC*, *CDKN2A*, and *CCND1* genes in SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D cell lines. B: represents CNV statuses of *HER2* and *DMXL2* genes in the same cell lines. Bars show the targets (blue) and the reference (green) positive events. CN>2.2 amplification; CN≤1.2 deletion; and ratios of ~1 were considered normal. HGDNA (positive control); NTC (negative control); Poisson error bars are represent the 95% confidence interval.

4.2.4.3 Spiked-in experiment for analysing CNV

As additional validation, cell line DNA was spiked into HGDNA at 7 fixed concentrations to generate a theoretical range for each target (HGDNA: cell line DNA) (0%-100%, 25%-75%, 50%-50%, 75%-25%, 90%-10%, 95%-5%, and 100%-0%) in order to survey CN detection on dilution of cell line DNA into wild type DNA. As pre-amplification was needed to generate a sufficient amount of cfDNA from plasma as mentioned earlier, spike-in experiments were performed for both unamplified and preamplified samples.

4.2.4.3.1 *MYC* amplification

Previous data indicated a high-level gain at the *MYC* locus in SK-BR-3, so this was consequently spiked into HGDNA. Evidence of amplification were detected between 0% HGDNA-100% SK-BR-3 (CN=16.3) and 95% HGDNA-5% SK-BR-3 (CN=2.53) in unamplified DNA and between similar ratios with CN=17.9 and 2.65 in the preamplified DNA. In addition, total events were between 18256 and 10460 indicating acceptable results. Thus, the limit of detection for CN gain using this cell line was 5% SK-BR-3 DNA (Table 4.6; Figure 4.17-A).

Table 4.6 ddPCR validation of *MYC* amplification across 7 ratios of HGDNA: SK-BR-3

H: HGDNA; S: SK-BR-3; FAM-positives: droplets containing *MYC* target; VIC-positives: droplets containing *RPPH1* reference; CN: copy number; CN>2.2 amplification; ratios of ~1 were considered normal.

***MYC* gain - unamplified SK-BR-3**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%S	**2526**	**349**	**10460**	**16.3**	**8.1**
25%H- 75%S	**3022**	**435**	**16881**	**15.1**	**7.6**
50%H- 50%S	**2473**	**500**	**18256**	**10.5**	**5.2**
75%H- 25%S	**806**	**260**	**13381**	**6.3**	**3.17**
90%H- 10%S	**371**	**239**	**11506**	**3.1**	**1.56**
95%H- 5%S	**266**	**211**	**11594**	**2.53**	**1.26**
100%H- 0%S	**224**	**188**	**11723**	**2.1**	**1.05**
***MYC* gain - preamplified SK-BR-3**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%S	**14572**	**4677**	**15121**	**17.9**	**8.96**
25%H- 75%S	**10806**	**2577**	**13594**	**13.9**	**6.96**
50%H- 50%S	**11940**	**3888**	**15336**	**9.1**	**4.57**
75%H- 25%S	**4159**	**1818**	**14135**	**5.06**	**2.53**
90%H- 10%S	**4589**	**3012**	**13880**	**3.28**	**1.64**
95%H- 5%S	**2878**	**2233**	**14339**	**2.65**	**1.32**
100%H- 0%S	**4005**	**4027**	**14251**	**1.99**	**0.994**

4.2.4.3.2 *CDKN2A* deletion

The resulting loss of *CDKN2A* in MCF-7 (CN=0.0022) was further examined in 7 different dilutions. CN deletion could still be detected in the mixture containing 25% of cell line DNA. The CN deletion dropped off steeply with decreasing concentrations of the cancer DNA and was not detected at 100%, 0.27 at 75%, 0.46 at 50%, and 1.17 at 25% in unamplified DNA, whereas in the preamplified DNA the CN values were 0.0005 at 100%, 0.24 at 75%, 0.60 at 50%, and 1.15 at 25%. 10% and 5% of the MCF-7 DNA showed normal CN (Table 4.7; Figure 4.17-A). So the limit of detection for CN loss was 25% cell line DNA.

Table 4.7 ddPCR validation of *CDKN2A* deletion across 7 ratios of HGDNA: MCF-7

H: HGDNA; M: MCF-7; FAM-positives: droplets containing *CDKN2A* target; VIC-positives: droplets containing *RPPH1* reference; CN: copy number; CN≤1.2 deletion; ratios of ~1 were considered normal.

***CDKN2A* deletion - unamplified MCF-7**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%M	**0**	**1216**	**14105**	**0**	**0**
25%H- 75%M	**153**	**1087**	**15012**	**0.273**	**0.136**
50%H- 50%M	**254**	**772**	**13221**	**0.64**	**0.322**
75%H- 25%M	**393**	**664**	**13799**	**1.17**	**0.59**
90%H- 10%M	**295**	**336**	**13964**	**1.75**	**0.88**
95%H- 5%M	**357**	**400**	**14852**	**1.78**	**0.89**
100%H- 0%M	**303**	**340**	**11029**	**1.78**	**0.89**
***CDKN2A* deletion - preamplified MCF-7**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%M	**3**	**7322**	**12409**	**0.0005**	**0.00027**
25%H- 75%M	**1352**	**7887**	**13601**	**0.241**	**0.121**
50%H- 50%M	**1950**	**5334**	**12361**	**0.608**	**0.304**
75%H- 25%M	**2115**	**3340**	**13799**	**1.15**	**0.574**
90%H- 10%M	**1708**	**2129**	**10523**	**1.56**	**0.78**
95%H- 5%M	**357**	**400**	**14852**	**1.55**	**0.78**
100%H- 0%M	**2810**	**2917**	**12085**	**1.9**	**0.95**

4.2.4.3.3 *CCND1* amplification

The ZR-75-1 genome with evidence of *CCND1* amplification (section 4.2.4.2) was also surveyed for CN evaluation using ratios of 100%, 75%, 50%, 25%, 10%, and 5% of ZR-75-1 DNA diluted in HGDNA. Amplification of *CCND1* was observed at 100% (CN=4.09), 75% (CN=3.72), 50% (CN=2.84), 25% (CN=2.5), and 10% (CN=2.43) of the cancer DNA in unamplified state. However, preamplified ZR-75-1 showed unexpected results, across different dilutions as the CN went down when amplified samples were diluted, indicating a potential bias between the target and the reference gene. As the total events were >10000, this suggests that pre-amplification biased the results here (Table 4.8; Figure 4.17-A).

Table 4.8 ddPCR validation of *CCND1* amplification across 7 ratios of HGDNA: ZR-75-1

H: HGDNA; Z: ZR-75-1; FAM-positives: droplets containing *CCND1* target; VIC-positives: droplets containing *RPPH1* reference; CN: copy number; CN>2.2 amplification; ratios of ~1 were considered normal.

***CCND1* gain - unamplified ZR-75-1**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%Z	**1058**	**526**	**15762**	**4.09**	**2.05**
25%H- 75%Z	**882**	**481**	**14798**	**3.72**	**1.86**
50%H- 50%Z	**719**	**511**	**12975**	**2.84**	**1.42**
75%H- 25%Z	**618**	**505**	**14553**	**2.46**	**1.23**
90%H- 10%Z	**545**	**450**	**13737**	**2.43**	**1.22**
95%H- 5%Z	**471**	**468**	**14067**	**2.01**	**1.01**
100%H- 0%Z	**747**	**687**	**14248**	**2**	**1**
***CCND1* gain - preamplified ZR-75-1**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%Z	**4145**	**3768**	**12285**	**2.26**	**1.13**
25%H- 75%Z	**3926**	**4100**	**12768**	**1.91**	**0.953**
50%H- 50%Z	**4267**	**5148**	**15437**	**1.6**	**0.801**
75%H- 25%Z	**3386**	**4381**	**14165**	**1.49**	**0.743**
90%H- 10%Z	**2758**	**4062**	**12030**	**1.27**	**0.637**
95%H- 5%Z	**2315**	**3570**	**11582**	**1.22**	**0.608**
100%H- 0%Z	**2607**	**3942**	**10586**	**1.2**	**0.602**

4.2.4.3.4 *HER2* amplification

The SK-BR-3 genome was used as a model of *HER2* gene amplification (identified earlier) and was diluted into HGDNA at 7 different percentages. In unamplified and preamplified DNA, high-level gains were observed at 100%, 75%, and 50%. Low-level gains were detected at 25%, 10%, and 5% cell line DNA. Total events ranged between 17496 and 10144, indicating agreeable results. The limit of detection for this high-level gain was 5% (Table 4.9; Figure 4.17-B).

Table 4.9 ddPCR validation of *HER2* amplification across 7 ratios of HGDNA: SK-BR-3

H: HGDNA; S: SK-BR-3; FAM-positives: droplets containing *HER2* target; VIC-positives: droplets containing *RPPH1* reference; CN: copy number; CN>2.2 amplification; ratios of ~1 were considered normal.

***HER2* gain - unamplified SK-BR-3**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%S	**4905**	**600**	**17496**	**18.8**	**9.4**
25%H- 75%S	**2333**	**288**	**12329**	**17.8**	**8.9**
50%H- 50%S	**2260**	**432**	**15471**	**11.1**	**5.6**
75%H- 25%S	**689**	**237**	**11842**	**5.9**	**2.95**
90%H- 10%S	**364**	**177**	**10144**	**4.1**	**2.04**
95%H- 5%S	**374**	**217**	**13166**	**3.5**	**1.73**
100%H- 0%S	**191**	**169**	**11889**	**2.07**	**1.03**
***HER2* gain - preamplified SK-BR-3**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%S	**12139**	**3959**	**12517**	**18.4**	**9.21**
25%H- 75%S	**10806**	**2577**	**13594**	**15.1**	**7.53**
50%H- 50%S	**11940**	**3888**	**15336**	**10.31**	**5.15**
75%H- 25%S	**4052**	**1622**	**11968**	**5.67**	**2.84**
90%H- 10%S	**3907**	**2382**	**11968**	**3.56**	**1.78**
95%H- 5%S	**2754**	**1972**	**13356**	**2.89**	**1.44**
100%H- 0%S	**2989**	**3018**	**10626**	**1.97**	**0.99**

4.2.4.3.5 *DMXL2* amplification

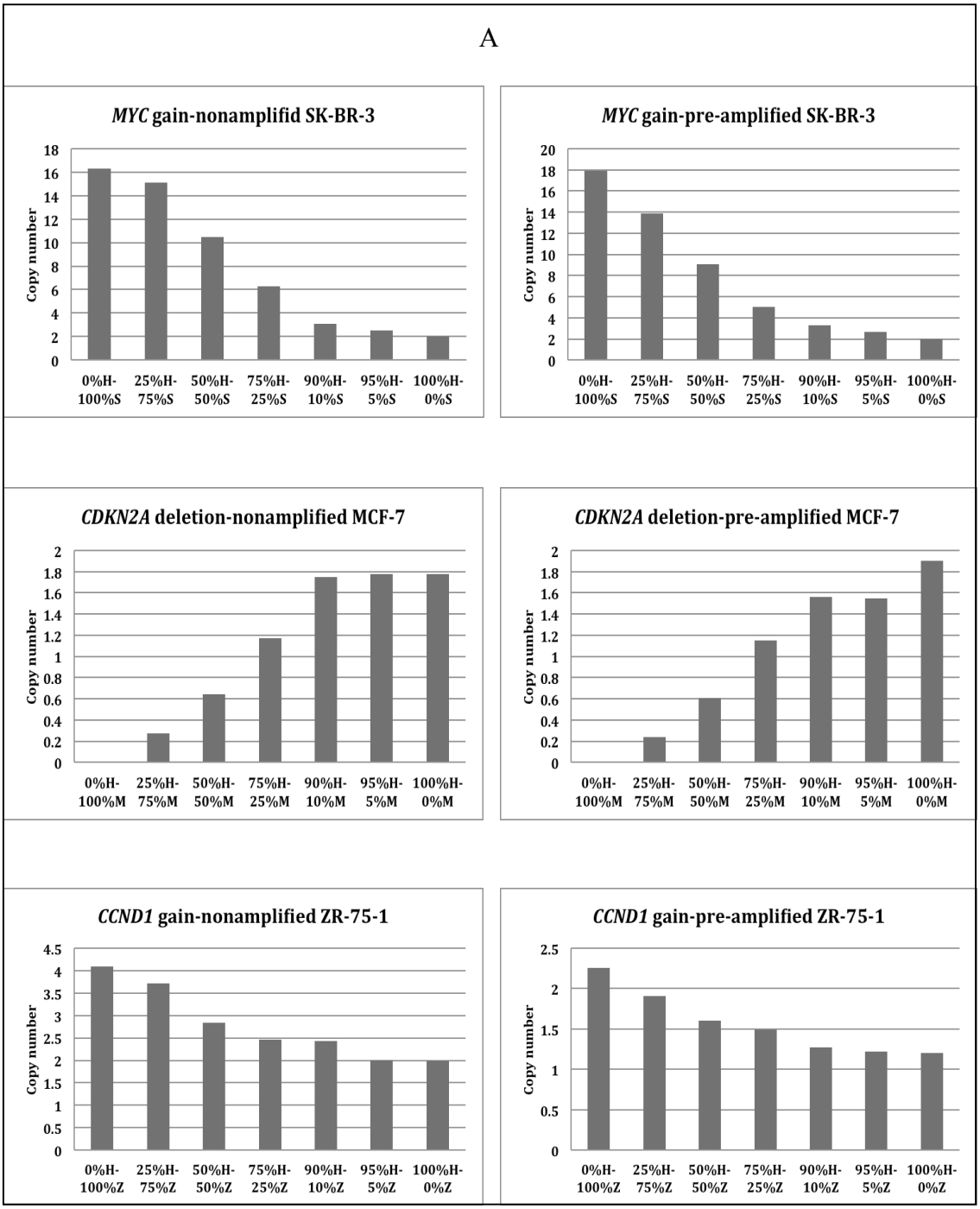
DMXL2 gene amplification was further evaluated by spiking MCF-7 into normal control (HGDNA) at 7 ratios after detection of amplification as proved earlier. CN values ranged between 3.74 and 2.77 at ratios from 100% to 10% of unamplified MCF-7. CN values ranged between 3.73 and 2.3 at ratios from 100% to 10% in the preamplified state. Total events fluctuated between 18267 and 14787, and 15435 and 13951, in unamplified and preamplified conditions, respectively, demonstrating reliable outcomes. Therefore, the limit of detection for CN gain was 10% (Table 4.10; Figure 4.17-B).

Table 4.10 ddPCR validation of *DMXL2* amplification across 7 ratios of HGDNA: MCF-7

H: HGDNA; M: MCF-7; FAM-positives: droplets containing *DMXL2* target; VIC-positives: droplets containing *RPPH1* reference; CN: copy number; CN>2.2 amplification; ratios of ~1 were considered normal.

***DMXL2* gain - unamplified MCF-7**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%M	**2616**	**1454**	**17118**	**3.74**	**1.87**
25%H- 75%M	**2015**	**1124**	**14787**	**3.71**	**1.85**
50%H- 50%M	**1619**	**987**	**16155**	**3.35**	**1.68**
75%H- 25%M	**1235**	**885**	**17900**	**2.82**	**1.41**
90%H- 10%M	**567**	**411**	**15246**	**2.77**	**1.39**
95%H- 5%M	**452**	**427**	**15725**	**2.12**	**1.06**
100%H- 0%M	**514**	**504**	**18267**	**2.04**	**1.02**
***DMXL2* gain - preamplified MCF-7**					
Sample Ratios	**FAM-positives**	**VIC-positives**	**Total events**	**CN**	**Ratio**
0%H- 100%M	**11858**	**8901**	**13951**	**3.73**	**1.87**
25%H- 75%M	**11049**	**7747**	**14773**	**3.71**	**1.85**
50%H- 50%M	**9124**	**6660**	**14136**	**3.26**	**1.63**
75%H- 25%M	**8104**	**6593**	**15435**	**2.67**	**1.336**
90%H- 10%M	**4342**	**3853**	**14529**	**2.3**	**1.15**
95%H- 5%M	**4665**	**4862**	**15048**	**1.9**	**0.951**
100%H- 0%M	**4844**	**4824**	**14483**	**2.01**	**1.005**

A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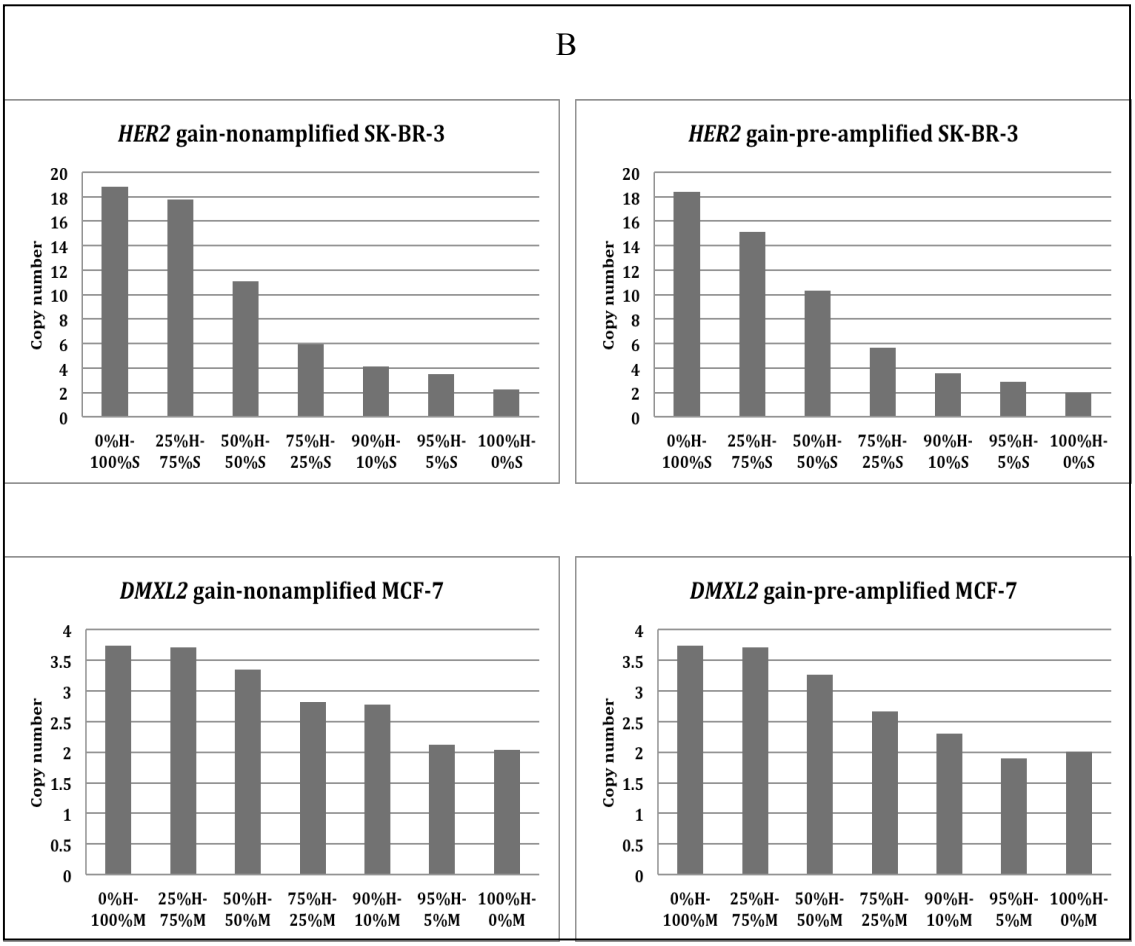


Figure 4.17 ddPCR CNV discovery across 7 ratios of HGDNA: breast cell line DNA in the preamplified and unamplified conditions

Bars show the correlation between the CN values and the ratios to estimate the limit of detection for five genes. Consistent results between preamplified and unamplified DNA, except for *CCND1* gene. A: represents *MYC* gain in SK-BR-3, *CDKN2A* loss in MCF-7, and *CCND1* gain in ZR-75-1. B: shows *HER2* gain in SK-BR-3 and *DMXL2* gain in MCF-7. CN: copy number; CN>2.2 indicates amplification; CN≤1.2 indicates deletion; and normal CN were ~2.

4.2.4.4 ddPCR detection of CNV status in *BRCA* patient plasma samples

Populations of individuals with a family history of breast cancer were profiled with ddPCR for CN measurement of 5 genes of interest (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2*), in order to confirm amplifications and/or deletions of genes/markers within CNVs identified previously by qPCR. 10 cycles preamplified selected cfDNA plasma samples were analysed and compared with matched unamplified healthy lymphocyte DNA (reference sample). Plasma samples with evidence of amplification and deletion (with RQ values >2 and ≤0.35 respectively) were selected and surveyed. Each ddPCR reaction was run in a duplex assay, i.e. target and (*RPPH1*) reference gene. A control experiment was performed using HGDNA with two copies of each gene per diploid genome expected to achieve in each run. No template controls were performed in all cases, to rule out possible contamination. CN values of >2.2 and ≤1.2 were used as thresholds for gain and loss, respectively, while CNs of ~2 reflected a normal diploid number. In each experiment, a number of events were reported (Figures 4.18; 4.19; 4.20).

4.2.4.4.1 *MYC* amplification

Plasma DNA samples 82, 78, 67, and 85 from a *BRCA* affected group where amplification was detected in *MYC*, as CNs obtained were 2.36, 2.51, 2.88, and 2.88, respectively. There was no evidence of amplification in matched lymphocyte controls, with CNs of ~2: 1.79, 1.91, 1.64, and 1.7 respectively (Figure 4.18).

4.2.4.4.2 *CDKN2A* amplification

3 plasma samples were surveyed for amplification of *CDKN2A* using ddPCR and compared with matched lymphocyte controls. Plasma DNA samples 35, 23 (*BRCA* carrier), and 78 (*BRCA* affected) showed CN gain with values of 4.33, 2.75, and 3.15 respectively, while matched lymphocyte controls showed CNs of ~2. Results were matched with qPCR data (Figure 4.18).

4.2.4.4.3 *CCND1* amplification

CfDNA samples from 3 independent groups PNEG (n=3), *BRCA* carrier (n=4), and *BRCA* affected (n=6) were screened via ddPCR after pre-amplification. *CCND1* gave unexpected results for preamplified templates, as CN values ranged between 1 and 2 (Figure 4.19) (with the exception of 35 and 21 plasma), whereas qPCR results showed amplification.

4.2.4.4.4 *HER2* amplification

HER2 amplification was measured in a cohort of 7 patients from PNEG (♯109 and ♯94), *BRCA* carrier (♯42), and *BRCA* affected (♯78, ♯16, ♯62, and ♯99) after 10 cycles of pre-amplification. Gains were observed for the following patients: 109, 94, 42, 78, 16, 62, and 99 with CN values 3.8, 2.78, 6.4, 2.92, 2.21, 2.82, and 3.33, respectively, compared to the matched lymphocyte samples, where CN obtained were 2.04, 1.87, 1.95, 1.94, 1.94, 1.96, and 2.03 respectively (Figure 4.20-A).

4.2.4.4.5 *DMXL2* deletion

ddPCR CNV analysis in the plasma cfDNA of 3 patients from *BRCA* carrier (♯114 and ♯17), and *BRCA* affected (♯85) showed a single *DMXL2* allele deletion with CN values ≤1.2 when compared to the matched lymphocyte samples as normal CN reported (~2) (Figure 4.20-B).

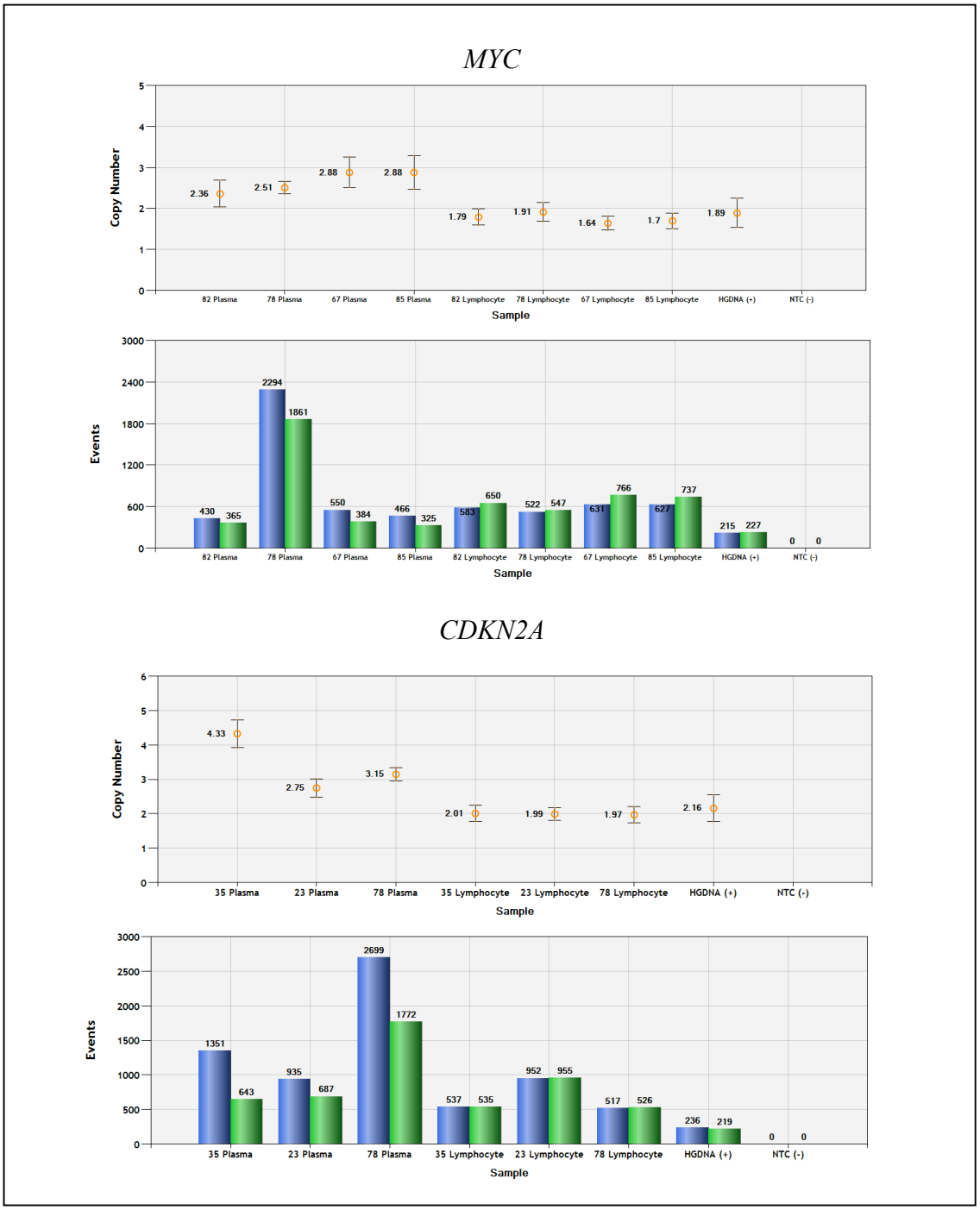
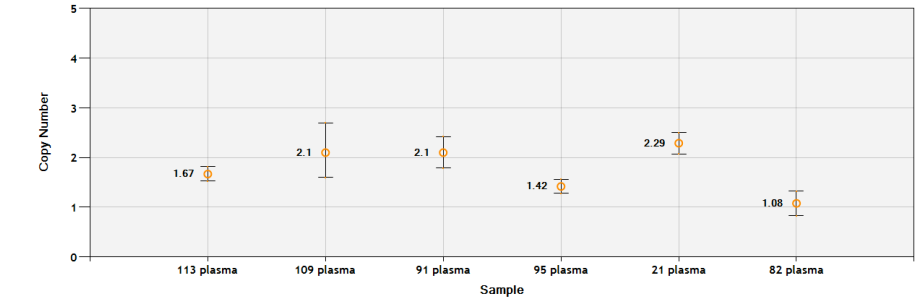


Figure 4.18 Detection of *MYC* and *CDKN2A* gene amplification in clinical samples by ddPCR

Illustrates CN values of *MYC* and *CDKN2A* genes in preamplified plasma cfDNA and matched lymphocyte DNA; normal CN of ~2 was detected in lymphocyte DNA; plasma cfDNA exhibited amplification in both genes (CN>2.2). Positive events for each target (blue column) and reference (green column) were represented. HGDNA (positive control) displayed normal diploid genome CN; NTC (negative control) indicated no contamination. Poisson error bars are represent the 95% confidence interval.

A

CCND1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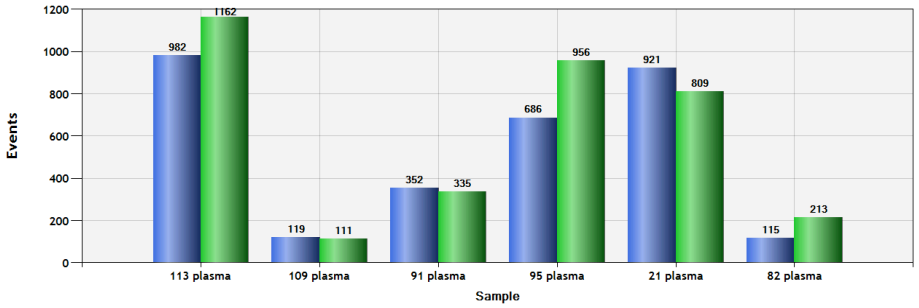
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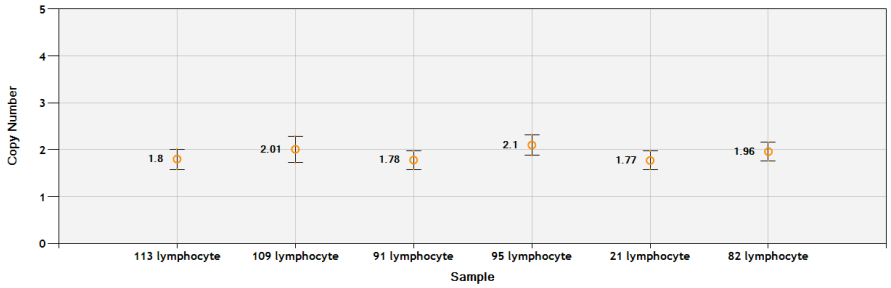
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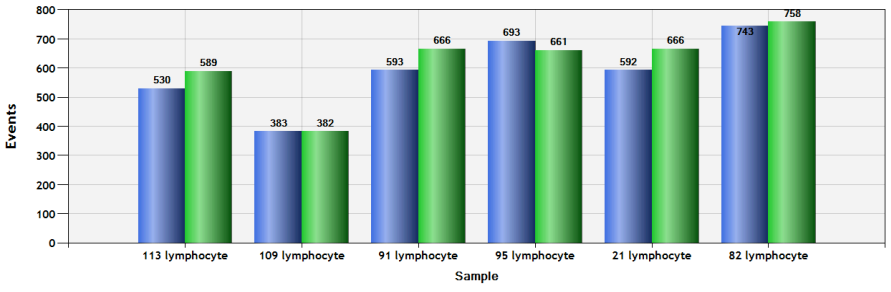
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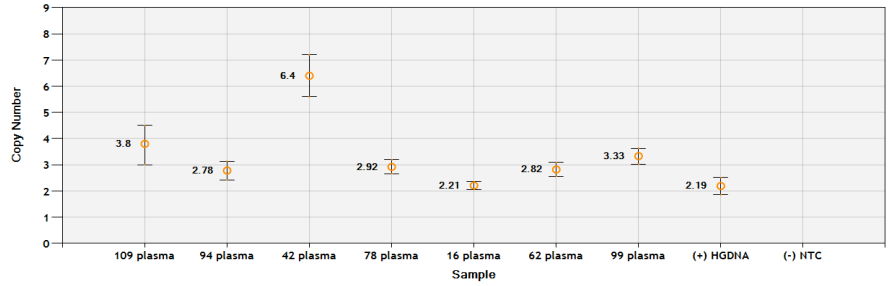
95 lymphocyte
21 lymphocyte
82 lymphocyte
Sample

Figure 4.19 ddPCR validation of *CCND1* amplification in paired plasma and matched lymphocyte samples

Shows normal CN (~2) in lymphocyte DNA; *CCND1* amplification (CN>2.2). A: shows CNV states in sample numbers 113, 109, 91, 95, 21, and 82. B: shows CNV states in sample numbers 35, 37, 78, 48, 62, 85, and 54. Positive events for *CCND1* (blue bars) and *RPPH1* (green bars) were represented. Positive (HGDNA) and negative (NTC) controls were run on each plate. Poisson error bars are represent the 95% confidence interval.

A

HER2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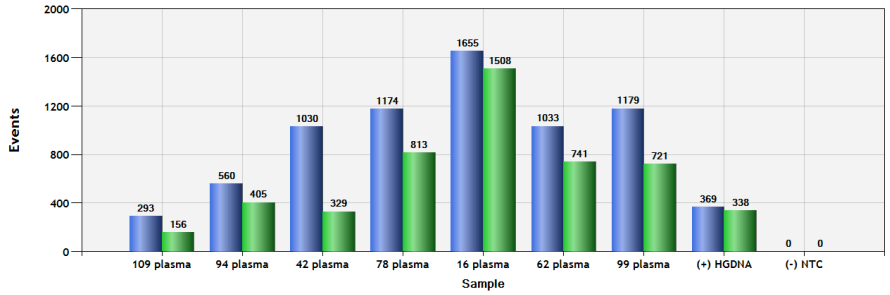
(-) NTC
Sample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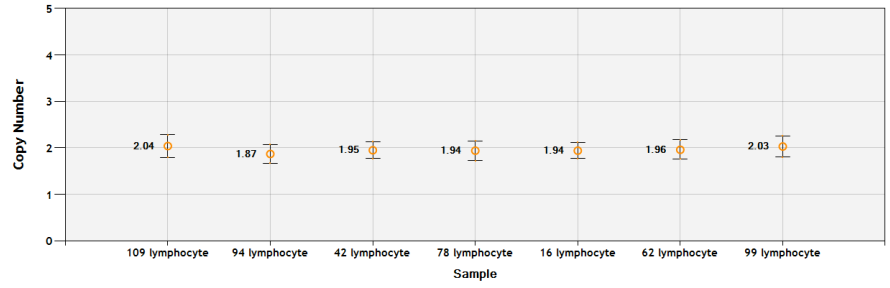
(-) NTC
Sample


Copy Number
2.04
1.87
1.95
1.94
1.94
1.96
2.03
109 lymphocyte
94 lymphocyte
42 lymphocyte
78 lymphocyte
16 lymphocyte
62 lymphocyte
99 lymphocyte
Sample

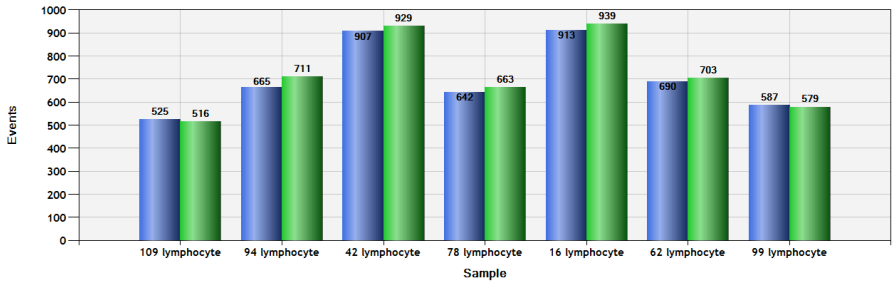

Events
525
516
665
711
907
929
642
663
913
939
690
703
587
579
109 lymphocyte
94 lymphocyte
42 lymphocyte
78 lymphocyte
16 lymphocyte
62 lymphocyte
99 lymphocyte
Sample

Figure 4.20 ddPCR detection of *HER2* amplification and *DMXL2* deletion in plasma cfDNA of breast cancer patients

Matched lymphocytes were used as reference samples (CN~2); CNV data of preamplified plasma samples with (A) *HER2* amplification (CN>2.2) and (B) *DMXL2* deletion (CN≤1.2) were presented. Positive events at which droplets having a copy of target gene (blue bars) or reference gene (green bars) were exhibited for each gene. Positive (HGDNA) and negative (NTC) controls were run for each gene. Poisson error bars are represent the 95% confidence interval.

4.2.5 Comparison between qPCR and ddPCR

While qPCR and ddPCR were reliable and reproducible for measuring CN alteration, there were some differences in the CNV data obtained by the two techniques but generally most of the results showed similar CNV statuses. Preamplified plasma cfDNA samples with alterations were selected after qPCR analysis, then validated by ddPCR. In cell line analysis, about 77% of CNV data were matched in both approaches and approximately 60% of CNV results in both methods were matched with the other studies (Table 4.11). Statistical analysis was performed to test for the differences in the CN using the Unpaired t-test, and no significant differences were found (Figure 4.21-A). Pearson correlation was carried out as CN measured by the two methods was highly correlated ($r=0.8076$; P value<0.0001) (Figure 4.21-B). Overall, ddPCR values were generally lower than qPCR.

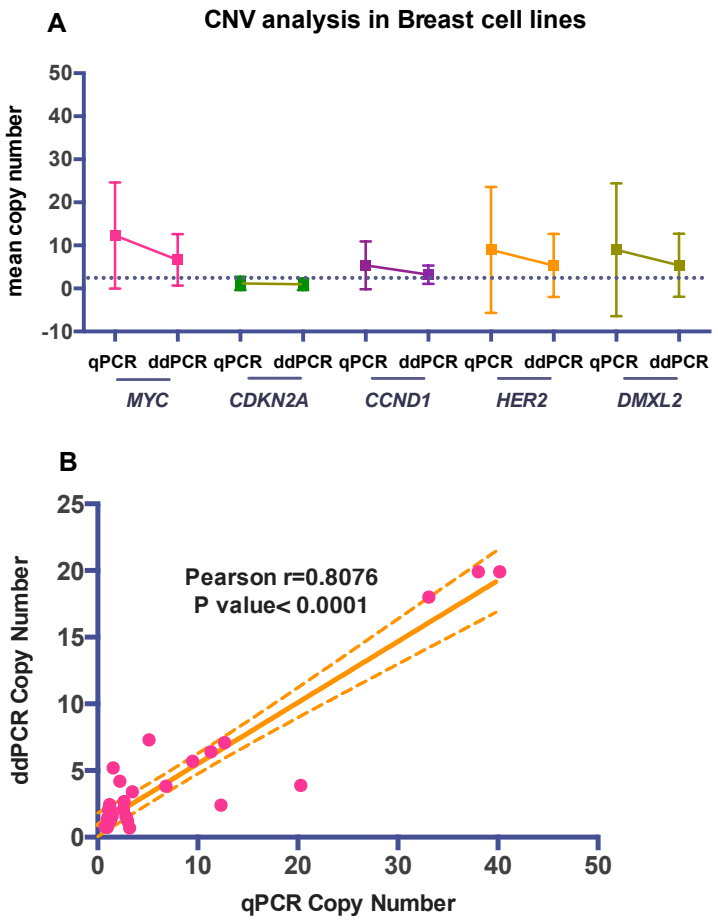


Figure 4.21 Comparison of CN detected in 6 breast cancer cell lines using ddPCR and qPCR

A: Student's t-test was performed to compare the mean copy number identified by quantitative and digital PCR of 5 targets (did not differ significantly). Error bars represent the mean with SD. B: Correlation (Pearson's) between CN values determined by ddPCR and qPCR (statistically significant correlated; Pearson's r and P value were presented). The line represents a linear regression fit. Dashed lines represent the 95% confidence interval.

Table 4.11 CNV analysis of 5 targets measured across 6 cell lines by qPCR and ddPCR compared to other studies

Amp: amplified CN>2.2 (red); Del: deleted CN≤1.2 (green); and normal CN of ~2.

Cell line name	Gene Name	qPCR CNV data	ddPCR CNV data	Published data	References
SK-BR-3	*MYC*	Amp	Amp	Amp	Savinainen et al., 2004; Kozbor and Croce 1984; Shadeo and Lam 2006; Cosmic
MDA-MB-231		Amp	Amp	Amp	Shadeo and Lam 2006
MDA-MB-468		Normal	Normal	Normal	Cosmic
MCF-7		Amp	Amp	Amp	Cosmic; Shadeo and Lam (2006); Kozbor and Croce, 1984
ZR-75-1		Amp	Amp	Normal	Cosmic
T-47D		Amp	Amp	Amp	Shadeo and Lam 2006; Cosmic
SK-BR-3	*CDKN2A*	Amp	Normal	-	-
MDA-MB-231		Del	Del	Del	Berns et al., 1995;CellMiner
MDA-MB-468		Del	Del	Del	Cosmic
MCF-7		Del	Del	Del	Berns et al., 1995; CellMiner
ZR-75-1		Del	Del	-	-
T-47D		Amp	Amp	Normal/ Del	CellMiner; Cosmic
SK-BR-3	*CCND1*	Amp	Normal	-	-
MDA-MB-231		Normal	Normal	Normal/Amp	Neve et al., 2006; CellMiner
MDA-MB-468		Normal	Normal	Amp	Cosmic
MCF-7		Amp	Amp	Amp	CellMiner
ZR-75-1		Amp	Amp	Amp	Neve et al., 2006
T-47D		Amp	Amp	Amp	CellMiner; Cosmic
SK-BR-3	*HER2*	Amp	Amp	Amp	Shadeo and Lam 2006; Neve et al., 2006; Lacroix and Leclercq 2004; Kallioniemi etal., 1992; Alexandra et al., 2012
MDA-MB-231		Normal	Normal	Normal	Neve et al., 2006
MDA-MB-468		Del	Del	Normal/Del	Neve et al., 2006; Cosmic
MCF-7		Amp	Normal	Normal/Amp	Neve et al., 2006; Alexandra et al., 2012
ZR-75-1		Normal	Amp	Normal/Amp	Neve et al., 2006; Whale et al., 2012
T-47D		Amp	Amp	Normal/Amp	Neve et al., 2006; Alexandra et al., 2012; Whale et al., 2012; Cosmic
SK-BR-3	*DMXL2*	Del	Normal	-	-
MDA-MB-231		Normal	Normal	Normal	CellMiner
MDA-MB-468		Amp	Del	-	-
MCF-7		Amp	Amp	Amp	CellMiner
ZR-75-1		Amp	Amp	-	-
T-47D		Normal	Amp	Normal	CellMiner

The approaches were then applied to clinical samples from 3 independent groups (*PNEG* (n=4), *BRCA* carrier (n=8), and *BRCA* affected (n=9)) and the results suggest that ddPCR obtained lower values for amplification than qPCR. Conversely, deletions were lower in qPCR (Table 4.12). In order to validate how comparable are the CNV data obtained by the two approaches, qPCR data of 5 intervals were normalised against the average of the three references (*KDELC2*, *CNTNAP1*, and *RPPH1*), which was validated earlier and against *RPPH1* reference alone then converted to CN by multiplying RQ values by 2 and compared to the ddPCR data (run in a duplex assay target/*RPPH1* reference). Statistical comparisons to establish CNV measurement were performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. No significant differences were found between CN of qPCR relative to the average of the three references and CN of ddPCR (*CCND1* results were excluded because there was no evidence of CN alteration found in ddPCR, while qPCR data showed amplification). Significant differences were found between qPCR CN values relative to *RPPH1* and ddPCR CN values (Figure 4.22-A). Despite these small differences in the CN values, qPCR and ddPCR were positively correlated. A Spearman correlation of 0.5517 (P value<0.0095) was obtained between CN values of qPCR relative to the average of three references and CN values of ddPCR; and of 0.7005 (P value<0.0004) between CN values of qPCR relative to *RPPH1* and CN values of ddPCR (Figure 4.22-B&C). Overall, ddPCR was less variable and more accurate for detection of CN alteration.

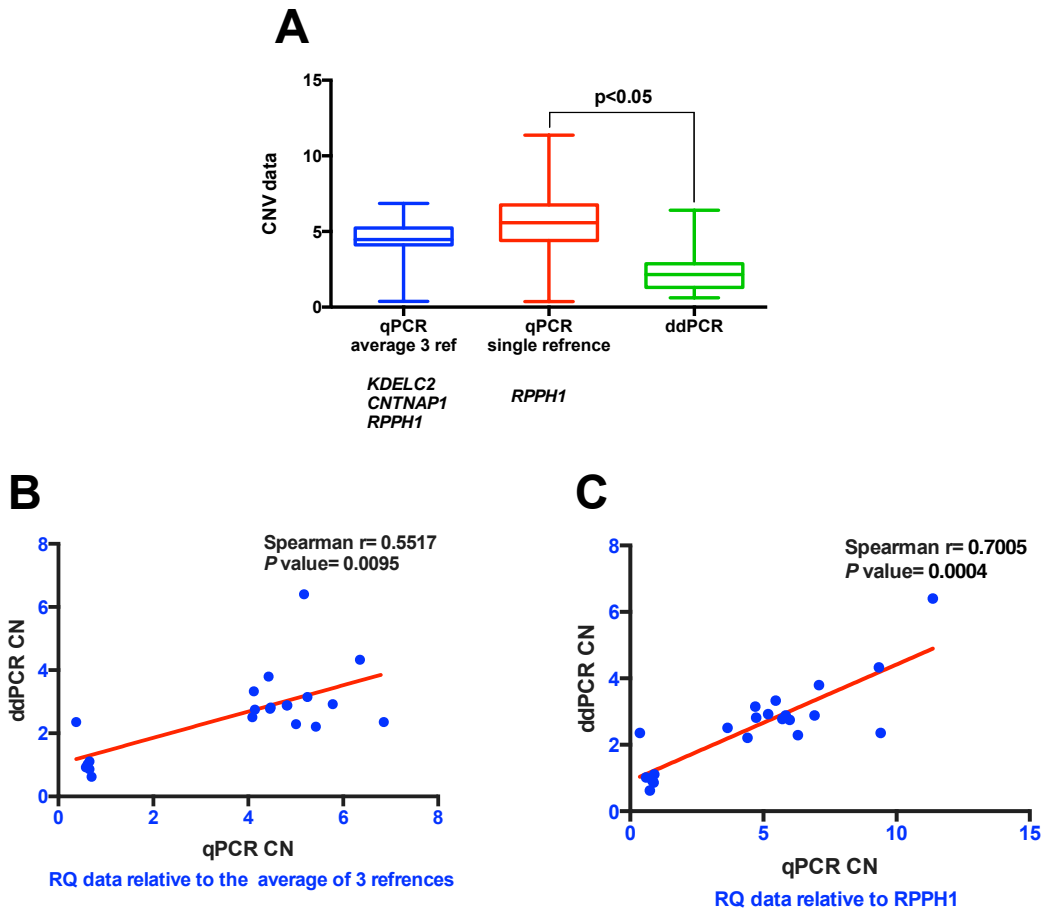


Figure 4.22 Comparison of CN detected in plasma cfDNA by qPCR and ddPCR

CN of 5 target genes (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2*) were assessed by two approaches and analysed. A. Box and whisker plot showing ANOVA multiple comparison test to compare the CN obtained by qPCR, which normalised to 3 references and single reference separately, and ddPCR. Whiskers of the box-plot represent minimum and maximum values. B. Correlation analysis between CNs of qPCR (3 references) and ddPCR. C. Correlation analysis between CNs obtained by qPCR (*RPPH1*) and ddPCR. The lines represent a linear regression fit, Spearman r and P values are indicated in each graph (a high correlation achieved in B and C).

Table 4.12 CNV profiling of qPCR and ddPCR in plasma of women with family history of breast cancer

CN>2.2 gain; CN≤1.2 loss.

Group name	Plasma Sample No.	Gene Name	qPCR		ddPCR
			CN (RQ*2)		CN
			Average of 3 references	Single reference	*RPPH1*
BRCA affected	82	*MYC*	0.38	0.36	2.36
BRCA affected	78		4.08	3.66	2.51
BRCA affected	67		4.81	5.84	2.88
BRCA affected	85		4.82	6.92	2.88
BRCA carrier	23	*CDKN2A*	4.14	5.99	2.75
BRCA carrier	35		6.36	9.34	4.33
BRCA affected	78		5.25	4.70	3.15
BRCA Predictive negative	113	*CCND1*	4.84	6.80	1.67
BRCA Predictive negative	109		5.85	9.59	2.1
BRCA Predictive negative	91		4.28	6.63	2.1
BRCA carrier	35		6.86	9.41	2.62
BRCA carrier	37		4.20	6.56	1.87
BRCA carrier	95		4.19	5.17	1.42
BRCA carrier	21		5.01	6.30	2.29
BRCA affected	82		6.84	6.60	1.08
BRCA affected	78		4.89	5.43	1.45
BRCA affected	48		4.16	4.41	1.45
BRCA affected	62		4.90	5.18	1.5
BRCA affected	85		5.37	7.17	1.79
BRCA affected	54		4.36	6.48	1.68
BRCA Predictive negative	109	*HER2*	4.43	7.09	3.8
BRCA Predictive negative	94		4.46	5.71	2.78
BRCA carrier	42		5.17	11.37	6.4
BRCA affected	78		5.78	5.18	2.92
BRCA affected	16		5.43	4.40	2.21
BRCA affected	62		4.47	4.73	2.82
BRCA affected	99		4.12	5.46	3.33
BRCA carrier	114	*DMXL2*	0.58	0.83	0.76
BRCA carrier	17		0.66	0.90	1.05
BRCA affected	82		0.61	0.59	1.52
BRCA affected	48		0.70	0.74	1.36
BRCA affected	85		0.66	0.88	1.06

4.2.6 Sequencing of cfDNA from plasma samples

After CNV profiling using qPCR and ddPCR techniques, further analysis was carried out using the Ion Torrent PGM to screen for known hotspot mutations and CNV in cfDNA. Nine cfDNA samples from the *BRCA* carrier (95, 42, 17, and 21) and *BRCA* affected (78, 16, 48, 62, and 99) groups with evidence of amplification and/or deletion (indicative of circulating tumour derived DNA) were selected. Higher frequencies of CNV were found in the plasma of post-surgical breast cancer patients on follow-up, who are disease free (*BRCA* affected) compared to the PNEG and *BRCA* carrier groups (Figure 4.14) using qPCR and ddPCR. For DNA sequencing, cfDNA was re-extracted from plasma using the QIAamp Circulating Nucleic Acid Kit, then quantified using ddPCR. A total of 4.1, 7, 5.6, 3.8, 5.5, 7.6, 7.5, 4.3, and 4.5 ng DNA was sequenced from the 9 samples. The analysis was performed using the Ion Torrent™ – Torrent Suite™ software. All variants were individually analysed using the Integrative Genomics Viewer (IGV) software and reads were aligned against the human genome (hg19).

Mutation hotspots and CNV were evaluated in 16 genes using targeted NGS (*ERBB2*, *MYC*, *CCND1*, *FGFR1*, *FGFR2*, *KDELC2*, *STK11*, *LMTK3*, *NOMO2*, *DUB3*, *CNTNAP1*, *TP53*, *PIK3CA*, *GATA3*, *ESR1*, and *NR3C3**). Normal lymphocyte samples were used as germline controls for each plasma sample. The Ion Torrent PGM NGS was applied to sample numbers 95, 42, 17, 21, 78, 16, 48, 62, and 99, working alongside by Dr. Karen Page in the group. Unfortunately, the cfDNA sequencing failed for sample numbers 95, 42, 17, 21, 78, 16, 48, and 62, and results were only obtained for a single sample, 99 (Table 4.13). Across 16 genes, no somatic mutations and CNVs were detected by NGS, although qPCR and ddPCR had showed evidence of CNV in the same sample (*HER2* amplification). Overall, there was good agreement between results obtained for the cfDNA and the matched white blood cells and the analysis showed reliable data for plasma and matched lymphocyte samples according to the frequencies and qualities obtained, which were around 50 and 100, and above 1000, respectively (Table 4.13).

Table 4.13 Ion PGM seqencing results in plasma cfDNA and matched lymphocyte DNA of patient 99

Chr	Position	Gene ID	Ref	Variant	Lymphocyte		Plasma	
					Frequency	Quality	Frequency	Quality
chr4	69417570	*UGT2B17*	A	G	100	2197.76	100	1103.46
chr4	69512847	*UGT2B17*	T	G	50.8	7466.46	48.5	3151.87
chr6	152129077	*ESR1*	T	C	47.9	9509.56	45.6	13112.8
chr8	11995274	*USP17L2*	G	A	100	16826.3	99.1	11731
chr10	123243197	*FGFR2*	G	A	100	18325.7	100	10273.2
chr10	123298158	*FGFR2*	T	C	100	31947.7	100	41762.8
chr17	7578645	*TP53*	C	T	100	32079.3	100	23991.7
chr17	7579472	*TP53*	G	C	96.9	23335.1	93.2	9857.88
chr17	37884037	*ERBB2*	C	G	100	17931.7	99.2	7491.17
chr17	40835922	*CNTNAP1*	A	C	100	6627.15	99.4	3347.25
chr17	40843392	*CNTNAP1*	G	T	49.8	9889.17	50.3	10109
chr17	40849842	*CNTNAP1*	A	G	50.1	4625.65	50.4	2865.34
chr19	1219274	*STK11_4*	G	A	50.8	10408.7	49.3	13132.4

Chr: chromosome; Ref: reference

4.3 Discussion

In this study, CNV was investigated in cfDNA by qPCR, ddPCR, and the Ion Torrent PGM.

4.3.1 qPCR CNV reference and target assay optimisation

A standard curve was generated for determining the initial quantity of the target template in experimental samples or for assessing the reaction efficiency. Typically, the PCR reaction efficiency (E) should be 100%, meaning the amount of template doubles after every cycle during exponential amplification. Overall, experimental factors may influence the efficiency, such secondary structure, the length, and GC content of the amplicon. Further factors that can have an impact on the efficiency are the dynamics of the reaction itself, enzyme quality, and the use of non-optimal reagent concentrations that could produce efficiencies less than 90%. The existence of PCR inhibitors in one or more of the reagents result in efficiencies of more than 110% (Real-time PCR handbook, 2012). To ensure optimal performance, each PCR ready assay should meet the following criteria: high amplification efficiency between 90% and 110%, which corresponds to a slope of between −3.58 and −3.10 (Real-time PCR handbook, 2012); linear standard curve (R2>0.980); and consistency across replicate reactions (Dietrich et al., 2011; Applied Biosystems, 2008; Bio-Rad, 2006). The equation of the linear regression line, R^2, and PCR efficiencies revealed an optimised qPCR assay (well fitted linear data) (Table 4.1). Since the qPCR was performed in triplicate and showed low variation, this is possibly the most accurate and reliable data.

4.3.2 qPCR CNV reference assay validation

qPCR is a sensitive and accurate technique for measuring target gene levels (Radonic et al., 2003). A key step of this technique is normalisation of the results to a reference gene in order to correct results that may have been skewed by differing amounts of the input nucleic acid template. Ideally, target levels should be normalised to an internal reference gene that shows minimal variation between all samples. Recently, it was

proposed that the use of multiple reference genes might be necessary for accurate quantification (Vandesompele et al., 2002; Hellemans et al., 2007; Devonshire et al., 2014).

In this study, four reference genes *GAPDH*, *CNTNAP1*, *RPPH1*, and *KDELC2* were compared for subsequent target gene analysis. On the basis of non-normalised levels (based on CT values), the one-way ANOVA analysis reported that *GAPDH* was found to have a higher variability across samples and accordingly was unsuitable for subsequent analysis (Figure 4.2); this result was agreed with several publications (Schek et al., 1988; Revillion et al., 2000; Schmittgen and Zakrajsek, 2000; Hamalainen et al., 2001; Glare et al., 2002; Valenti et al., 2006; Ruan and Lai, 2007; Sikand et al., 2012).

By contrast, *CNTNAP1*, *RPPH1*, and *KDELC2* exhibited similar levels across all samples (Figure 4.2). Therefore, a variety of potential reference combinations were used to improve the accuracy of the cfDNA load quantification by comparing measurements assessed by single reference, mean of two references, and the mean of all three references. At the same time, we assessed pre-amplification reaction for qPCR analysis in cancer cell lines and reference samples, and used the method in plasma cfDNA samples as they presented at low concentration, as low amount of DNA molecules is one of the limiting factors that could hamper the result, as suggested previously (Bettegowda et al., 2014; Pantel and Alix-Panabieres, 2013; Del Gaudio et al., 2013; Li et al., 2008).

Several parameters can influence the efficiency, sensitivity, specificity, and reproducibility of targeted pre-amplification including: the concentration of the template used (0.278 ng/µl), primer concentration (200-100 pmol/µl), number of assays (12), the number of pre-amplification cycles at least 5 for precise sensitivity (10 cycles), and annealing time and annealing temperature (60°C for 4 min), which was achieved in this study (Andersson et al., 2015). In order to maximise the use of template DNA, a

pre-amplification approach was taken using TaqMan PreAmp Master Mix. After pre-amplification, samples were diluted (10 fold) to deactivate the enzymatic activity that remained in the reaction mix as approved by Andersson et al., (2015). This was also used to maintain concentration within the range of unamplified DNA to enable direct comparison, in order to achieve the main aim of analysing limiting concentrations of DNA (Sanders et al., 2011). Preamplified HGDNA was used as a positive control (as a threshold) to determine if the preamplified product was below (<0.5 loss) or above (≥2.1 gain) the threshold. To ensure that quantification was not affected by assays that would amplify genomic DNA or by primer-dimer formation, water (NTC) was included in the pre-amplification reaction as a negative control (Korenková et al., 2015). Different CNV intervals were determined (*MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) in order to compare the RQ results obtained by unamplified and preamplified products and to investigate measurement discrepancies of the mean of multiple references compared to single references. Despite the fact that there were some differences observed in some samples, the overall trend was similar as when CN alteration observed in unamplified samples; the same trend occurred in the preamplified samples. Therefore, it can be concluded that CNV measurements of an average value based on three reference assays minimised biases due to assay or genome location, as agreed with previous publications (Hellemans et al., 2007; Devonshire et al., 2014).

Although the pre-amplification method resulted in higher RQ values compared with the unamplified results in some samples, it also decreased in other samples. For example, in the measurement relative to single references, there were markedly higher levels detected in the preamplified MCF-7 and T-47D at *PBX1* and *DMXL2* loci than unamplified DNA (RQ relative to *CNTNAP1* and *RPPH1* separately (Figures 4.3 and 4.4) and to the average of both (Figure 4.6)). In contrast, CNV data relative to *KDELC2* exhibited significantly lower levels by preamplified DNAs compared to the unamplified DNAs as showed for the *MYC* and *CCND1* genes in MCF-7 and T-47D, besides *FGFR1* and *CDKN2A* in T-47D (Figure 4.5). The observed differences of preamplified versus unamplified products in some samples could be explained with the following explanations; one probability can be that primer cross-reactivity might occur in the

pooled assays throughout the pre-amplification reaction. Additionally, an amplification bias for high-abundancy targets could be present (Del Gaudio et al., 2009). Comparisons of preamplified vs. unamplified DNAs showed a high correlation (between 0.6 and 0.8) for various control genes used, suggesting that the 10-cycle pre-amplification step provides linear amplification (Figure 4.8). Preamplified DNA was compliant with unamplified DNA since no significant differences were found using the ANOVA test and Unpaired Student's t-test, and they were highly correlated (Figure 4.9). In conclusion, the pre-amplification procedure can improve the sensitivity of qPCR, especially for low-abundancy targets and could subsequently expand the number of analysable target genes; it is a balance between obtaining results for several genes and biasing this by pre-amplification.

4.3.3 CNV profiles of breast cancer

Multiple genetic alterations are a significant feature of breast cancer, which associate with its complicated biology and clinical behaviour (Nessling et al., 2005). Cell lines have been used as model systems for breast cancer cell biology to identify regions of segmental DNA loss and gain. qPCR was used to evaluate gene CNVs present in breast cell lines that have been widely used (SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D), (Shadeo and Lam, 2005; Lacroix and Leclercq, 2004). In this study, the results of eight target genes *MYC*, *FGFR1*, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2* were comparable to previous reports. CNVs of *HER2* and *MYC* genes have been widely recognised in breast disease and are found in model cell lines (Nessling et al., 2005; Watanabe et al., 1992; Jarvinen et al., 2000; Kauraniemi et al., 2001; Neve et al., 2006; Shadeo and Lam, 2006; Lacroix and Leclercq, 2004; Kallioniemi et al., 1992). Savinainen et al., (2004) reported 21 copies of *MYC* in SK-BR-3, while Shadeo and Lam (2006) showed that the *HER2* locus is highly amplified in the same cell line, which were also exhibited in the present study. Other cell lines such as MDA-MB-231, MDA-MB-468, MCF-7, and ZR-75-1 showed a single copy number at 17q12 locus; these results were supported by Watanabe et al., (1992) and Neve et al., (2006). Chromosome 8q24.21 gain was recognised in MCF-7 and T-47D, in agreement with published data by Shadeo and Lam (2006) and Escot et al.,

(1986). Other analysis showed the presence of alteration (gain) in T-47D and ZR-75-1 at 8p11.2-p12 (Rummukainen et al., 2001; CellMiner database), whereas, no evidence of CNVs was identified to SK-BR-3, MDA-MB-231, MDA-MB-468, and MCF-7 DNAs was additionally reported by Neve et al., (2006) and in CellMiner database. Chromosome arm 9p21 has been reported to be deleted in MDA-MB-231 and MCF-7 in agreement with this study (Berns et al., 1995; CellMiner database). Likewise, amplification was seen at 11q13 in MCF-7 and T-47D (data was compatible with CellMiner database) in addition to normal copy number in MDA-MB-231 (Neve et al., 2006). The detection of amplification at 15q21.1 and 15q21.2 loci in MCF-7 was consistent with previous report (CellMiner database). Another CNV feature identified through this analysis was the amplification of 1q23.3 locus within T47-D (CellMiner database). Results generally agreed with other previously published data. Cell lines can change in culture so this is very encouraging data. In summary, most of the copy number analysis, which was based on the mean of three references in preamplified and unamplified breast cell lines, was compatible with earlier studies. Thus, the findings based on cell lines data clarify that the averaging of the three references provides reasonable data and could be applicable to clinical samples.

4.3.4 CNV analysis in plasma cfDNA of women with a family history of breast cancer and matched lymphocyte DNA

We used a novel TaqMan PreAmp method that we found to be a sufficient solution to lower CT values, and in particular to create qPCR products from limited quantities of template DNA that were recognised in cfDNA. Hence, levels of 8 intervals were measured in 17 lymphocyte controls to optimise the pre-amplification conditions between five and ten cycles, which were used as a reference for plasma cfDNA analysis. The results of ten cycles' reaction showed more variation than five cycles' reaction, but the CNV state was preserved after pre-amplification demonstrating normal copy number (Figure 4.10). A possible interpretation for these differences was that, as cycle numbers increased, the probability of success decreased accordingly because high concentration targets will cause consumption of reagents and primers from pre-amplification reaction, so they will reduce pre-amplification achievement (Korenková et

al., 2015; Andersson et al., 2015). However, unlike the 10 preamplified samples, for the 5 preamplified samples, there was no significant difference in the mean RQ values between the two settings, when the Unpaired two-tailed t-test was used. For all 8 candidates, the mean RQ in both settings showed high correlation between samples amplified for five cycles versus ten cycles (Figure 4.11). These signified the optimisation of 10 preamplified lymphocyte specimens, which could be considered as an ideal reference for plasma analysis.

In the plasma cfDNAs of women with a family history of breast cancer, amplifications and deletions were detected relative to the matched lymphocyte DNAs. Chromosomal regions 8q24, 11q13, and 17q12 were amplified (Tanner et al., 1996 and Gelsi-Boyer et al., 2005). *HER2* amplification was detected in 7 out of 48 (15%) patients, which was also previously found in 15% to 25% of breast cancers. *HER2* amplification is a marker of poor prognosis; the *HER2* gene encodes a tyrosine kinase receptor that is the target of trastuzumab (Herceptin) (Slamon et al., 1987; Vogel et al., 2002). Amplifications at 8q24 and 11q13 also have potential clinical interest as prognostic markers and/or therapeutic targets (Letessier et al., 2006). *MYC* is localised in 8q24 and encodes a nuclear protein that plays a role in cell cycle progression. Amplification of 8q24 occurs in up to 20% of breast cancers and is associated with a poor clinical outcome, but was detected in 2/48 (4%) cases in this cohort of patients (Bonilla et al., 1988; Aulmann et al., 2006). *CCND1* localised in 11q13 encodes cyclin D1, which is active during the G1 phase of the cell cycle. Amplification of *CCND1* occurs in 10% to 30% of breast cancers and was also seen in 13 out of 48 (27%) cases (Cuny et al., 2000; Seshadri et al., 1996; Naidu et al., 2002), whereas, *CDKN2A* is homozygously deleted in 60-65% of primary breast tumours and cell lines (Foulkes et al., 1997; Okamoto et al., 1994; Cairns et al., 1995; Nobori, 1994), however, in this study this gene showed amplification in 3 out of 48 (6.25%) cases. Within the published data by Chen et al., (1989), the incidence of deletion of 5 markers at Chr1q23-32 was about 25% of breast cancer. Additionally, Monica et al., (1991) confirmed mapping of *PBX1* gene at 1q23 locus. In this study, the *PBX1* gene was deleted in 16/48 (33%) of cases. The *DMXL2* at 15q21.2 locus showed deletion in 24/48 (50%) patients; previously Mao et al., (2005) found allelic loss at 15q21.2 (12%).

Through the analysis of cfDNA in 48 cases, it can be concluded that *BRCA* affected women after treatment in the follow-up of primary breast cancer acquired the most frequent aberrations with 60% gain and 66% loss than PNEG control and *BRCA* carrier healthy females (Figure 4.14-A). This result confirms previous study (Shaw et al., 2012) where it was shown that cfDNA with CNV at specific regions could be detected at higher levels in breast cancer patients than in healthy female controls. However, loss of *PBX1* and *DMXL2* target genes occurred frequently within these patients. The reason was arguably due to the fact that those amplicons in the pre-amplification reaction did not work properly across all samples. Consequently, these genes were excluded from the analysis as they skewed the loss and made it higher (Figure 4.14-B).

4.3.5 ddPCR CNV assay validation

ddPCR has been employed for quantification of germline copy number alteration (Hindson et al., 2011; Pinheiro et al., 2011). This technique enables large-scale partitioning, which result in better sensitivity and precision (Hindson et al., 2011; Pinheiro et al., 2011, Henrich et al., 2012). Partitioning strategy in picoliter droplets might allow ddPCR to contain relatively abundant quantities of template DNA with little interference from PCR inhibitors. ddPCR could be used to enhance measurement of smaller fold change such as a tumour associated CNV in the cfDNA segment of patient blood plasma (Whale et al., 2012). This study established five CNV assays (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2*) that have been investigated formerly by qPCR.

To investigate the precision of CNVs detection in low amount template by ddPCR, we applied 7 points serial dilutions of HGDNA for five CNV assays independently. As a result, a diploid CNs were achieved across those assays in different ranges (Figure 4.15). Thus, reflecting the detectable concentration for CNV analysis with total events exceeding 10000 as an indication of reliable results according to the Bio-Rad guidelines. Whale et al., (2012) suggests that ddPCR could identify small CN changes (1.25-fold difference) (Weaver et al., 2010), as its accuracy is directly assessed by both

the template amounts and the number of replicate measurements; we also used a method to examine the CNV assays.

It was estimated that a typical DNA concentration that can provide the most accurate concentration measurements for 20,000 partitions is 1.6 CPD. This concentration equals to ~100 ng of HGDNA in a 20 µl ddPCR reaction for a single copy gene. However, when the target gene CN is greater than approximately eight copies per diploid genome, it is necessary to reduce the total input template per well in order to preserve a sufficient number of negative droplets so that a precise concentration and CNV estimate can be obtained (Hindson et al., 2011; Karlin-Neumann et al., 2012).

In this project, we have also investigated CNV conditions in six BC cell lines comprising of: SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T-47D (Figure 4.16). The analysis showed that DNA from three different cell lines revealed different levels of *HER2* amplification, such as high-level gain at SK-BR-3 (19.9) and low-level gain at ZR-75-1 and T-47D (2.45 and 5.7); this was also approved previously by Whale et al., (2012). Moreover, MDA-MB-468 showed *HER2* deletion, which has been reported in COSMIC database. Different levels of *MYC* amplification were observed across four cell lines, as higher-level obtained to SK-BR-3, whereas, MDA-MB-231, MCF-7, ZR-75-1, and T-47D showed lower-level amplifications. Higher-level of *MYC* gain was reported in SK-BR-3 in contrast to other cell lines such as MCF-7 (Kozbor and Croce, 1984). In addition, the analysis indicated *CCND1* amplification in MCF-7, ZR-75-1, and T-47D cell lines, which agreed with previous reports (Neve et al., 2006; CellMiner database). Of the six breast cancer cell lines studied, MDA-MB-231, MDA-MB-468, MCF-7, and ZR-75-1 showed deletion of the *CDKN2A* gene, whereas, only deletions in MCF-7 and MDA-MB-231 have been described by Berns et al., (1995). A recent study showed low-level *DMXL2* gain to MCF-7 DNA, and evidence of this has been reported previously (CellMiner database) (Figure 4.16).

To measure the smallest ratios of altered genes, we used the BC gene aberration model to be spiked into HGDNA with 7 ratios. Another aspect is to identify pre-amplification strategy in a small amount of DNA when considering cfDNA as a template. This because in cancer patients its concentration can range from 180 to 600 ng/ml (Leon et al., 1977; Levenson, 2007; Jung et al., 2010; Fournie et al., 1986; Diehl et al., 2005).

Linear correlation was observed through different ratios of SK-BR-3 as when the ratio was decreasing, the number of copies was declining for *MYC* and *HER2* amplification (limit of detection was 5% of SK-BR-3 genome) in both conditions (Tables 4.6; 4.9). As *CDKN2A* showed deletion in MCF-7 cell line (limit of detection was 25% of the cell line), therefore, the CN was increasing gradually by decreasing the breast cell line concentration (Table 4.7). The amplification of *DMXL2* gene was detected in up to 10% of MCF-7 DNA as the CN dropped off with decreasing percentages of the cell line DNA (Table 4.10). For *MYC*, *CDKN2A*, *HER2*, and *DMXL2* genes, the CN values were consistent between pre-amplification and unamplification settings as a linear correlation between CN and sample ratios were obtained (Figure 4.17); the number of droplet reaction in all ddPCR experiments were exceeding 10000 and all NTC were negative. Pinheiro et al., (2011) has proved that the number of target DNA fragments in replicate ddPCR assays varies considerably when the copy number concentration of a template decreases.

In contrast, the results for *CCND1* gene was unexpected for the preamplified DNAs as the amplification was not detected within samples and the CN values were declining with decreasing target levels (from 2.26 (100%) to 1.33 (5%) of ZR-75-1 DNA), when unamplified DNAs were showing amplification with linear correlation between CNs and ratios (CN values ranged from 4.09 (100%) to 2.46 (25%) of ZR-75-1 DNA) (Table 4.8). Accordingly, the CN values amongst preamplified and unamplified DNA was inconsistent, although, the number of droplet reaction above 10,000 and NTC were negative, which indicated the pre-amplification process could not work properly with this gene. Overall, several factors could influence the reliability of ddPCR results including: the number of partitions analysed and the number of target fragments in the

assay (Bhat et al., 2009). For a 20,000 partitioned droplet, the dynamic range for absolute quantitation lengths from a single copy up to 100,000 copies, which is corresponding to a maximum of ~5 copies per droplet according to the manufacturer's recommendation (Hindson et al., 2011; Pinheiro et al., 2011). A study by Gevensleben et al., (2013) suggests at least 400 positive droplets for accurate assessment. However, our lab group has shown that 100 positive droplets are generally sufficient for accurate quantification of CNV. Though it was approved that accuracy of CN measurement can be succeeded with no other sources of variability when the number of reactions is more than 10,000 (Pinheiro et al., 2011; Whale et al., 2013).

Following ddPCR, the variation in template CN concentration would be influenced significantly by variation in the size of droplets generated from different wells either within or between cartridges (Pinheiro et al., 2011; Corbisier et al., 2015). With low amount sample, it might be better to apply pre-amplification to increase the sample concentration and so precision of ddPCR, but at the risk of introducing bias correlated with this further process. For instance, in regards to duplex PCR, bias could be introduced including existence of inhibitors in the sample that influences one assay more than the other or a preferential amplification of one target upon the other. In addition to this, template type that could have an effect on technical performance should be considered (Whale et al., 2013; Hindson et al., 2011; Huggett et al., 2008). Variability in the measurement in ddPCR may also occur due to degradation of the template because of long periods of heating (Bhat et al., 2011; Pienaar et al., 2006). Overall, these findings inform us that ddPCR could offer a practical solution to measure precise estimates of a small amount of cfDNA CN but with high precaution to avoid any technical error.

4.3.6 ddPCR detection of CNV intervals in plasma cfDNA

Through the analysis of the 5 genes (*MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2*) in women with a family history of breast cancer, amplification was seen in *MYC* and *HER2* loci (Figure 4.18; 4.20-A) and this was supported by several previous studies. For

example, *MYC* gene has been reported to be commonly amplified in breast cancer (Rummukainen et al., 2001). Grushko et al., (2004) observed that about 57% tumours from *BRCA1* mutation carrier had *MYC* amplification. Those results are in contrast to the results achieved by Adem et al., (2004), who found a high incidence of *MYC* amplification (57%) but with lower frequency of amplification (8%) between invasive carcinomas from *BRCA1/2* mutation carrier. *CCND1* and *HER2* genes are also most frequently amplified in breast cancers (Hicks et al., 2006; Page et al., 2011). Regarding *HER2* amplification, limited reports have analysed gene situation by FISH in hereditary *BRCA1/2* associated cancer (Palacios et al., 2003; Grushko et al., 2002). Grushko et al., (2002) approved that around 19% of *BRCA1* tumours had a *HER2*. Results of *CCND1* gene were discounted since preamplified plasma did not show any sign of amplification, which gives almost equal results to the lymphocyte DNAs and this was approved earlier in the cell lines (Figure 4.19). *CDKN2A* locus was frequently deleted in breast cancers according to Hicks et al., (2006); however, despite this study, our data showed amplification in this gene in the *BRCA* carrier and *BRCA* affected group (Figure 4.18). Deletion was seen at *DMXL2* locus in patients from *BRCA* carrier and *BRCA* affected group (Figure 4.20-B), and was also deleted in a study by Mao et al., (2005).

4.3.7 Analysis comparison between qPCR and ddPCR

In this analysis, the measurements of CN alterations in six breast cancer cell lines and cfDNAs of patients' blood plasma by ddPCR were compared with the conventional qPCR. Analysis in cell lines showed lower CN levels by ddPCR compared to qPCR for *MYC*, *CDKN2A*, *CCND1*, *HER2*, and *DMXL2* (Figure 4.21-A). This study showed that about 77% of the cases were matched by the two approaches, and around 60% were consistent with other studies (Table 4.11). However, the results were consistent among qPCR and ddPCR data, since a very high correlation ($r=0.8076$, P value<0.0001) accomplished between both measurements. In addition, no significant differences were documented using Unpaired Student t-test (Figure 4.21).

In clinical samples, the two methods showed the same statuses of CN alterations for the

gene targets investigated with the exception of patient 82 at *MYC* locus and *CCND1* gene (section 4.2.4.4.3) (Table 4.12). However, the level of the amplification at *MYC*, *CDKN2A*, and *HER2* loci was greater using qPCR compared to ddPCR measurements, while *DMXL2* deletion was lower in qPCR. There was a good agreement between the qPCR measurements either normalised to the average or to the single reference and ddPCR measurements of CNs, when the results of the two analyses were highly correlated as the fold differences were not as large (Figure 4.22-B&C). Using Unpaired Student t-test, there was no significant difference in the estimated measurements between qPCR relative to the average and ddPCR, while significant difference ($P<0.05$) were found between ddPCR and qPCR data when normalised to a single reference (Figure 4.22-A). Overall, the results from ddPCR were arguably more reliable than the results obtained from the qPCR, and accordingly most likely to provide a more accurate data about CNV measurements of all target genes analysed in breast cancers in young women. Analysis in cell lines and plasma samples showed greater CN values by qPCR than ddPCR, which is in agreement with some studies (Devonshire et al., 2014; Henrich et al., 2012). Sanders et al., (2011) suggests that the factor for increasing the measurement level with qPCR over ddPCR, which is binding some of the primers and, therefore, delaying amplification. This combines with the single copy partitioning intrinsic to ddPCR.

While some groups have performed qPCR with success, there are still two main reasons that may cause measurement variability. Firstly, variability in the quality or amount of sample added, affected by all upstream processes needed for collection, extraction and preparation of the sample and reaction. Secondly, the major technical variability of the qPCR, expressed as the Cq value, while, ddPCR is uninfluenced by typical variation in the Cq, which may explain why it is more accurate than qPCR (Whale et al., 2013). Besides, variation between the individuals could be biological. Current studies have demonstrated that ddPCR has developed quantitation accuracy over qPCR and it has been also established that ddPCR precision might be enhanced further through the use of duplex reactions (Whale et al., 2012, Whale et al., 2013, Qin et al., 2008, Sanders et al., 2011; White et al., 2009). Additionally, Whale et al., (2012) approved that ddPCR can analyse a smaller CNV than qPCR, since ddPCR accuracy is directly supported by

both the template concentration and the number of replicate measurements. Another significant advantage of ddPCR compared to qPCR is that this technique requires no calibration, standard curve, or information concerning the template fragments molecular weight (White et al., 2009).

On the other hand, it is also probable that ddPCR was less effective under one set of PCR situations. Larger segments might not have been packaged effectively into picoliter wells and this could lead to a lower estimation of copies by ddPCR (Henrich et al., 2012). In contrast, qPCR measurement most likely could not be influenced by differences in size in gDNA strands once all genetic material is involved in each reaction without partitioning or packaging (Henrich et al., 2012). Additional restriction of ddPCR comparing to qPCR is a necessity for diluting samples with greater than 75,000 copies of the target DNA, since overloading the picoliter droplets leads to a considerable influence on linearity at higher CNs. Whereas, this problem has less effect on quantitation at lower CNs (Richman et al., 2009; Siliciano et al., 2003) as is generally the case for cfDNA.

Hayden et al., (2012) compared the performance of ddPCR versus qPCR, which showed slightly higher sensitivity than ddPCR. A high-level of linearity and quantitative correlation for standards and clinical samples were agreed by both approaches across their detectable ranges. For higher concentrations, qPCR exhibited more variability than ddPCR. By contrast, ddPCR exhibited more variability and less sensitivity than qPCR in clinical samples. ddPCR might offer an opportunity to reduce the quantitative variability recently seen by qPCR, however, methods require further optimisation to reach the sensitivity of qPCR.

This finding confirmed previous research in our group and show that CNVs in particular chromosomal intervals (*MYC*, FGFR2, *CDKN2A*, *CCND1*, *HER2*, *CYP19A1*, *PBX1*, and *DMXL2*) were detectable in cfDNA and could serve as a source of

minimally invasive material for breast cancer detection in plasma of women with a family history of breast cancer and can discriminate between patients with breast cancer and healthy female controls (Shaw et al., 2012).

4.3.8 Sequencing of cfDNA from plasma samples

In this study, the Ion Torrent PGM sequencer was used to confirm CNV analysis based on qPCR and ddPCR methods and to detect further mutations by investigating 16 genes/regions (*ERBB2*, *MYC*, *CCND1*, *FGFR1*, *FGFR2*, *KDELC2*, *STK11*, *LMTK3*, *NOMO2*, *DUB3*, *CNTNAP1*, *TP53*, *PIK3CA*, *GATA3*, *ESR1*, and *NR3C3**) in plasma cfDNA of 9 patients with *BRCA* mutations (95, 42, 17, 21, 78, 16, 48, 62, and 99). Unfortunately, the result was only obtained for a single patient and no genetic mutations and CNVs were detected when matched plasma and lymphocyte samples were compared. The possible reasons that could explain why alterations were not detected in the plasma of this patient could be due to the following: the DNA sample quality and quantity; the alterations were absent; or the allele frequencies of mutations were below the detection limit of this method. In general, several factors can influence sequencing experiments, for example, the correct amplicon size is crucial for subsequent sequencing success, as amplicons with shorter length could enhance the sequencing performances, particularly for highly fragmented DNA such as cfDNA (Vanni et al., 2015). Additionally, accurate experimental design is critical to avoid a failed or poor read quality. Furthermore, the right library preparation approach is essential to avoid amplification artifacts, GC bias, uninformative reads, unevenness of coverage, and poor mapping (Genohub, 2015). Moreover, using more than one method for analysis is sufficient to reduce false negatives and false positives (Lin et al., 2014). Generally, multiple pre-analytic factors come into play in determining the success of NGS testing including: the input DNA quantity, the DNA quality, and the specimen type (Chen et al., 2015). To conclude, the technique is sufficiently robust for cancer research, provided that one appreciates the limitations and the principles of the method.

Chapter 5

Conclusions and Future Direction

This study focuses on miRNA and CNV profiling in circulating free nucleic acids of women from families at high-risk of breast cancer due to an inherited *BRCA* mutation in comparison with healthy female controls. The results of this study support previous research by Prof. Shaw's group targeting specific CNVs (identified by single nucleotide polymorphism (SNP 6.0) arrays) in cfDNA of patients with breast cancer as well as circulating miRNAs. Those nucleic acids are increasingly recognised as biomarkers for early detection and monitoring of cancers (Gahan, 2010; Shaw et al., 2012; De Maio et al., 2014).

This study provides experimental evidence that circulating biomarkers in blood plasma, derived from either RNA or DNA, could have potential in the clinical setting. The assessment of 384 miRNAs using TaqMan microfluidic cards led to identification of five miRNAs (mir-26a, mir-27b, mir-130b, mir-324-3p, and mir-181a) that showed a significant difference in expression between the PNEG control group and women who have *BRCA* mutation in the follow-up after surgery (*BRCA* affected). We screened plasma RNA samples from patients and controls for corresponding changes in expression of selected miRNAs using qPCR. These miRNAs were identified in several studies as being involved in breast cancer and could have roles as tumour suppressors or oncogenes in addition to targets for novel therapeutics (Tang et al., 2012; Gartel and Kandel, 2008). Our results also highlighted issues with miRNA stability in plasma (−80°C) after time in storage. All miRNAs were expressed at a lower level in the re-extracted plasma (at 2 years), which indicated miRNAs could be degraded in plasma with time in storage (−80°C). Generally, these data suggests a potential role of candidate miRNAs in breast cancer as non-invasive biomarkers for monitoring women with a strong family history after breast cancer surgery and treatment, as they segregates between cancers on follow-up and mutation negative healthy controls. However, results need careful interpretation as miRNA results vary with 1) assay, 2) time of storage of RNA, and 3) time of storage of plasma.

In the cfDNA CNV study, the results of the analysis based on qPCR data of 5 target genes (*MYC*, *CDKN2A*, *CCND1*, *HER2*, *PBX1*, and *DMXL2*) in women with a family history of breast cancer showed higher frequency of gain (60%) in women who carried an inherited *BRCA* mutation in follow-up after surgery than in healthy women who carried an inherited *BRCA* mutation (31.6% gain) and in the healthy controls (28.6% gain). Analysis based on ddPCR showed similar CN values, although a little variability was observed between the two approaches. *HER2* and *CCND1* genes were frequently amplified in this cohort of patients (15% and 27% respectively), consistent with previous findings (Hicks et al., 2006; Page et al., 2011; Kallioniemi et al., 1992; Berns et al., 1992). However, the *CCND1* gene was subsequently excluded from ddPCR analysis as the pre-amplification reaction appeared to bias the results. There are several studies that describe factors that may influence the pre-amplification reaction which include: template concentration, primer concentration, annealing time and annealing temperature, number of assays, and the number of pre-amplification cycles- at least 5 for precise sensitivity (Del Gaudio et al., 2009; Del Gaudio et al., 2013; Andersson et al., 2015). *HER2* is a common amplified gene in breast cancer and could potentially serve as a biomarker in cfDNA of breast cancer patients in the follow-up who are disease-free. This result supports previously published findings by Shaw et al., (2011) and Page et al., (2011) as they detected *HER2* amplification in cfDNA post-surgery and on follow-up in breast cancer patients who are disease free, with higher cfDNA levels in cancers compared to the healthy controls (Leon et al., 1977; Zhong and Holzgreve, 2009; Kamat et al., 2010).

The cfDNA CNV analysis was also carried out using the Ion Torrent PGM sequencer in 9 patients with *BRCA* mutations to confirm results obtained by qPCR and ddPCR approaches. Unfortunately, no results were obtained for 8 out of 9 patients (95, 42, 17, 21, 78, 16, 48, and 62). The two main causes, which might cause sequencing run or sample to fail, are designing sequencing experiment and library preparation (Genohub, 2015). In general, more samples could be included for further validation, taking into account other factors that could influence the DNA sequencing process, in order to avoid sample failure.

5.1 Conclusions

Overall, these data demonstrate the potential of miRNA and CNV analysis to distinguish between women with breast cancer due to inherited *BRCA* mutation on follow-up after treatment and healthy controls and might have utility for monitoring patients on follow-up in the adjuvant and post adjuvant period. The expression profiles of miRNA and gene CN values in cfDNA suggested the cancer profile persists after surgery and treatment, indicating minimal residual disease. Of note the *BRCA* carriers had all had bilateral prophylactic mastectomy to reduce their risk of developing breast cancer, which means that women with *BRCA* mutations are able to obtain a level of breast cancer risk that is either equal to or lower than that of the general population. In conclusion, this study has demonstrated that circulating nucleic acids in plasma specifically miRNAs and CNVs in cfDNA can distinguish between women with an inherited *BRCA1/BRCA2* gene mutation who have had breast cancer after surgery and treatment and healthy controls and could have potential as circulating biomarkers for monitoring women with breast cancer.

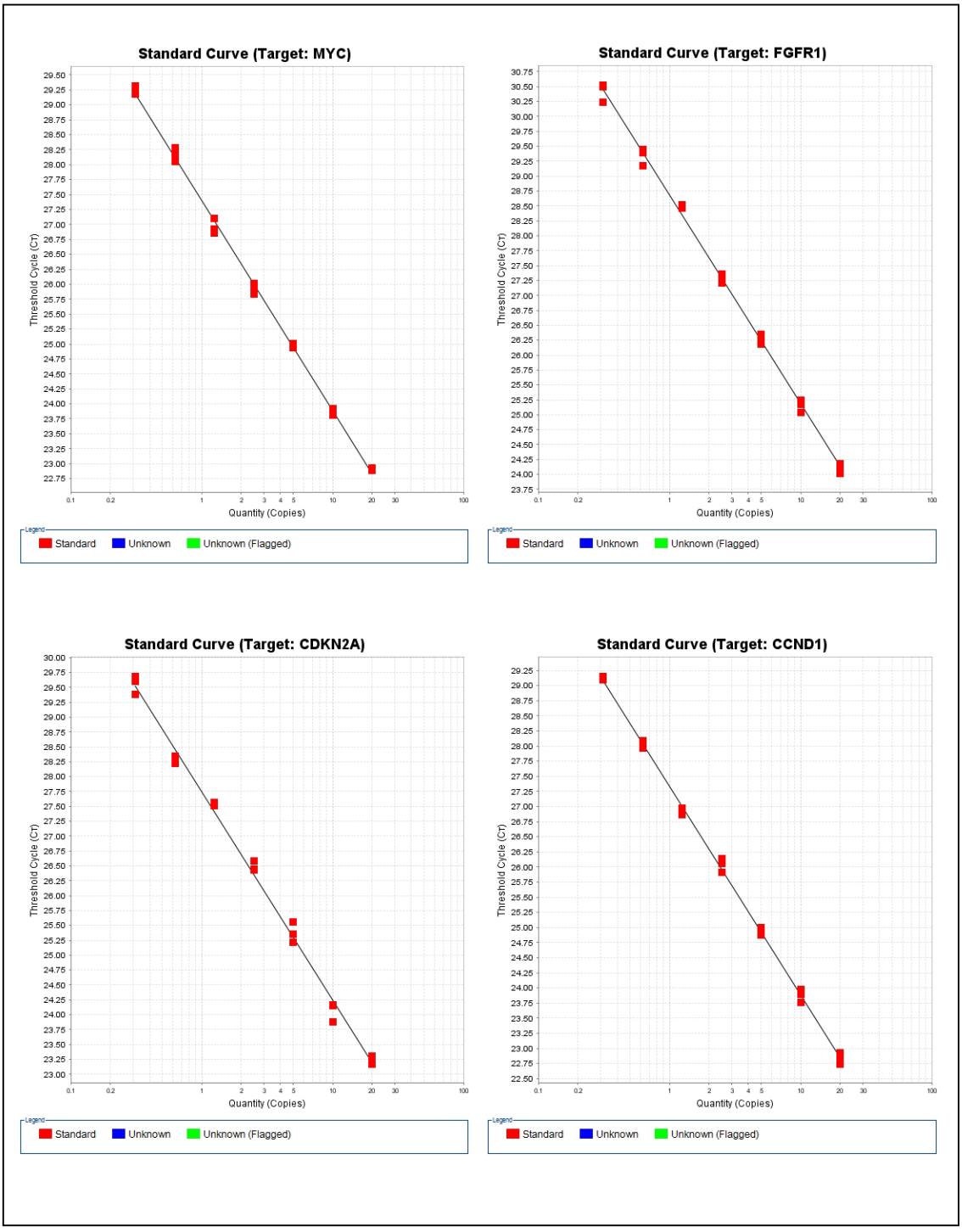
5.2 Future Direction

Thus, there is strong evidence that the analysis of miRNAs and CNVs by qPCR and ddPCR technologies in plasma may be useful for breast cancer monitoring and will enable future research to look into the possibility of using miRNAs and CNVs as early diagnostic tools in monitoring women with breast cancer due to inherited *BRCA* mutation prior to mammograms (imaging changes) and magnetic resonance imagery (MRI). If validated in a larger cohort of patients this could also support identifying other tumour-associated miRNAs/CNVs as diagnostic and prognostic biomarkers among women with breast cancer based on their family histories. In other future experiments we wish to look at different strategies to identify the origins of those miRNAs coming from breast cancer cells using pull-down method for example from microvesicles or exosomes and then profiling their miRNA cargo. By selecting key miRNAs that predict breast cancer we could carry out functional studies in cell line models (for example using gene knock-out with siRNA technology) to investigate whether this alters the cell phenotype; for example by looking at apoptosis, invasion,

migration and proliferation. Another goal of research in the future could be to use other techniques for example RNAseq, for more comprehensive miRNA analysis. Lastly, I set up a separate study, to investigate these circulating biomarkers in the blood of Saudi women who have developed breast cancer at a young age (<50 years), as an independent validation cohort, but unfortunately ran out of time to do the laboratory research.

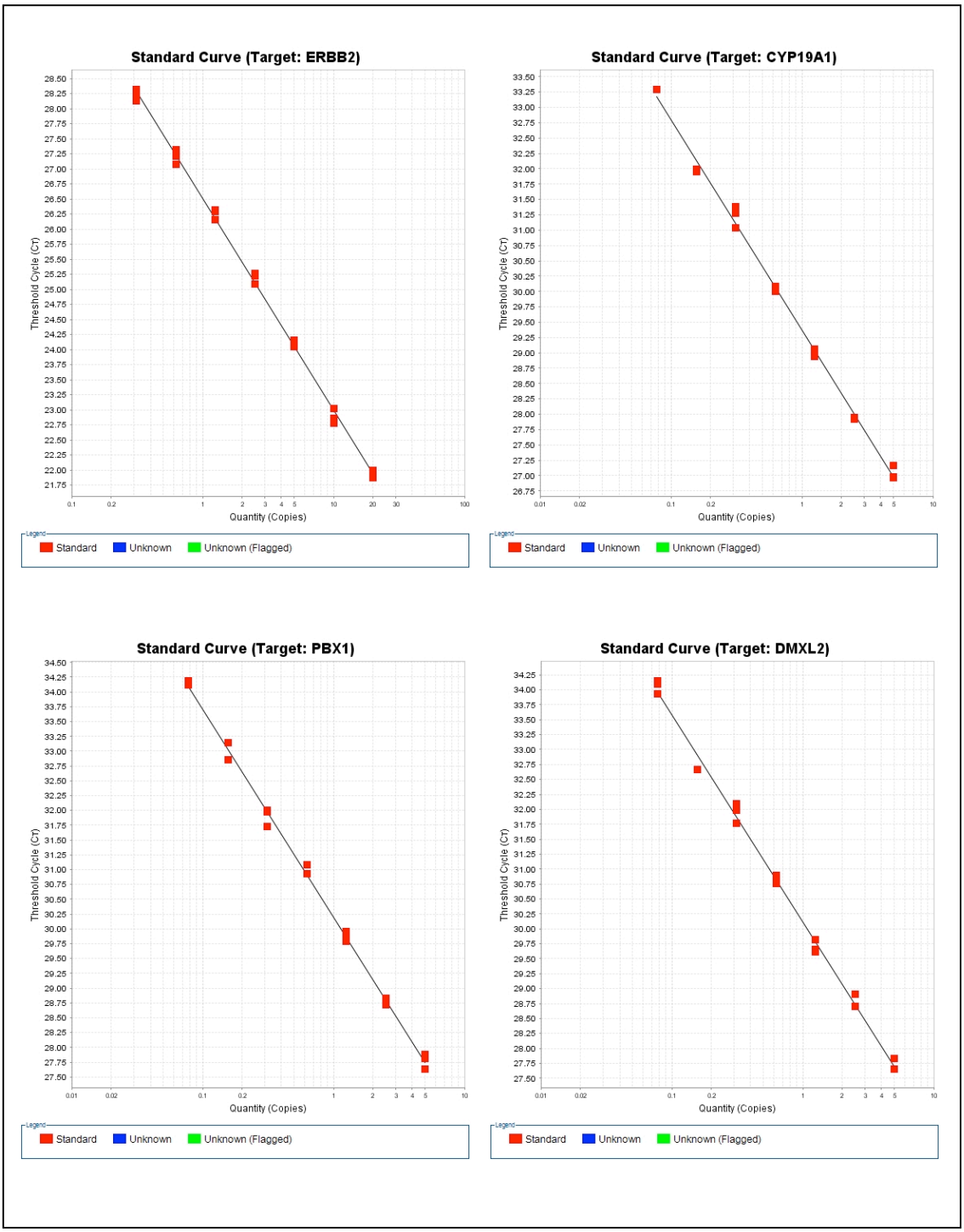
Appendix

Appendix 1 Standard curves for qPCR



Standard curves of *MYC*, *FGFR1*, *CDKN2A*, and *CCND1* CNV assays

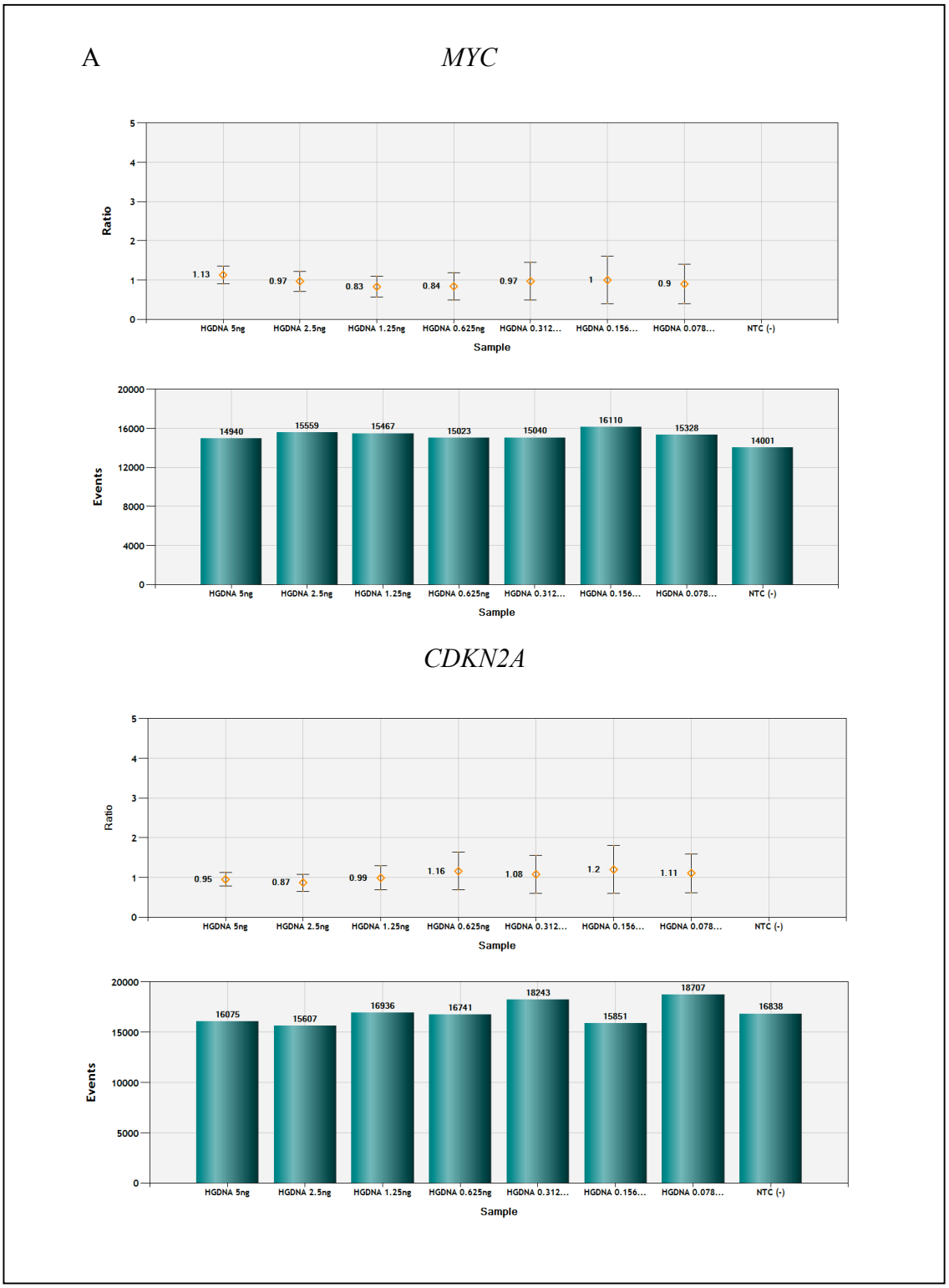
The efficiencies were 93%, 93%, 93%, and 95% respectively. The coefficient of determinations (R2) were 0.997, 0.997, 0.994, and 0.998 respectively.



Standard curves of *ERBB2* (*HER2*), *CYP19A1*, *PBX1*, and *DMXL2* CNV assays

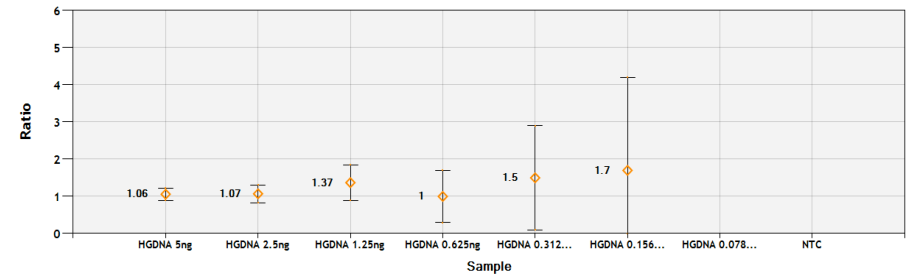
The efficiencies were 92%, 96%, 93%, and 94% respectively. The coefficient of determinations (R2) were 0.998, 0.996, 0.997, and 0.995 respectively.

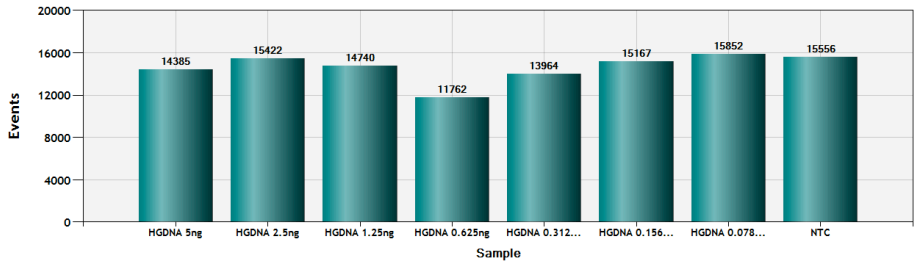
Appendix 2 Ratios and total events for ddPCR



B

CCND1


Ratio
6
5
4
3
2
1
0
1.06
1.07
1.37
1
1.5
1.7
HGDNA 5ng
HGDNA 2.5ng
HGDNA 1.25ng
HGDNA 0.625ng
HGDNA 0.312...
HGDNA 0.156...
HGDNA 0.078...
NTC
Sample


Events
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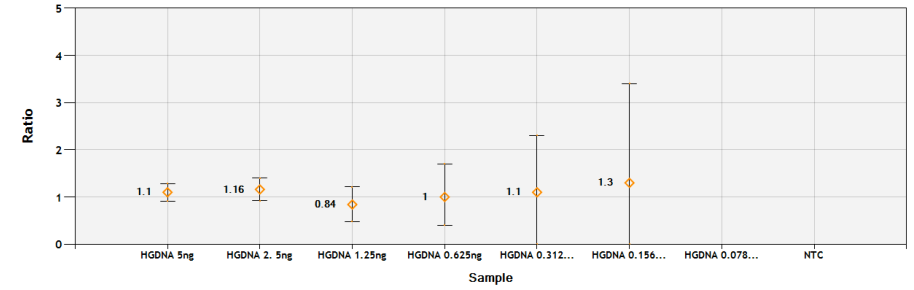
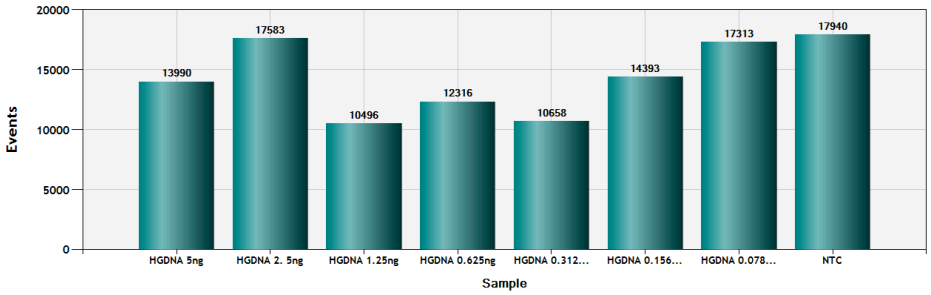
16000
12000
8000
4000
0
14385
15422
14740
11762
13964
15167
15852
15556
HGDNA 5ng
HGDNA 2.5ng
HGDNA 1.25ng
HGDNA 0.625ng
HGDNA 0.312...
HGDNA 0.156...
HGDNA 0.078...
NTC
Sample

HER2


Ratio
5
4
3
2
1
0
1.1
1.16
0.84
1
1.1
1.3
HGDNA 5ng
HGDNA 2. 5ng
HGDNA 1.25ng
HGDNA 0.625ng
HGDNA 0.312...
HGDNA 0.156...
HGDNA 0.078...
NTC
Sample


Events
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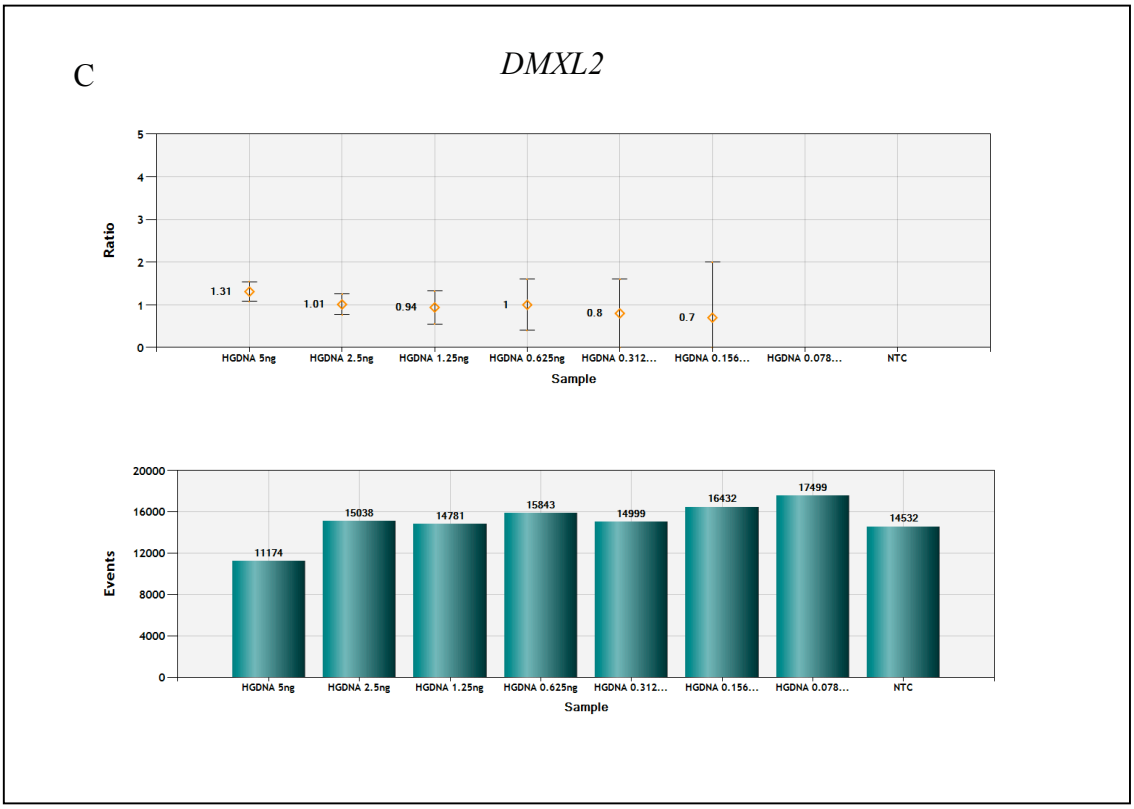
15000
10000
5000
0
13990
17583
10496
12316
10658
14393
17313
17940
HGDNA 5ng
HGDNA 2. 5ng
HGDNA 1.25ng
HGDNA 0.625ng
HGDNA 0.312...
HGDNA 0.156...
HGDNA 0.078...
NTC
Sample

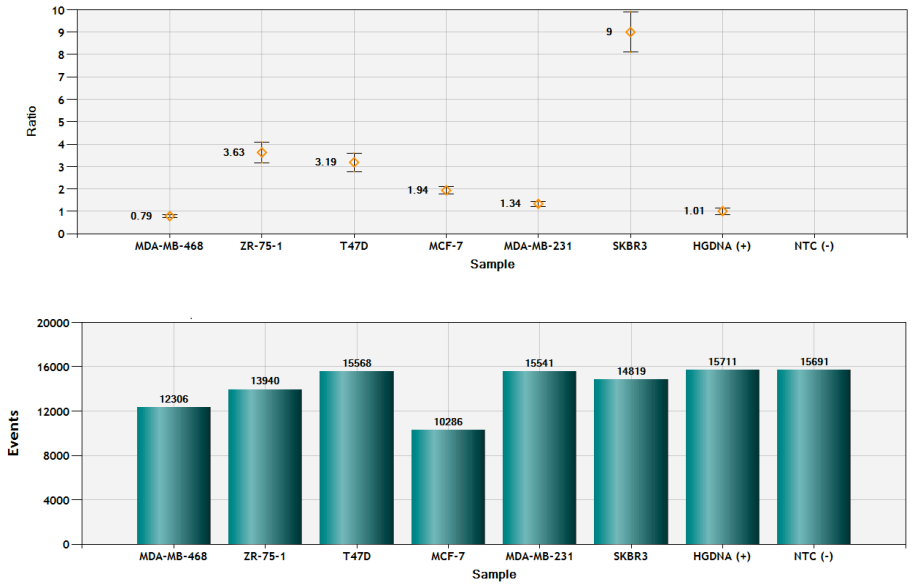


Assessment of 5 CNV genes using 7-point serial dilutions of HGDNA

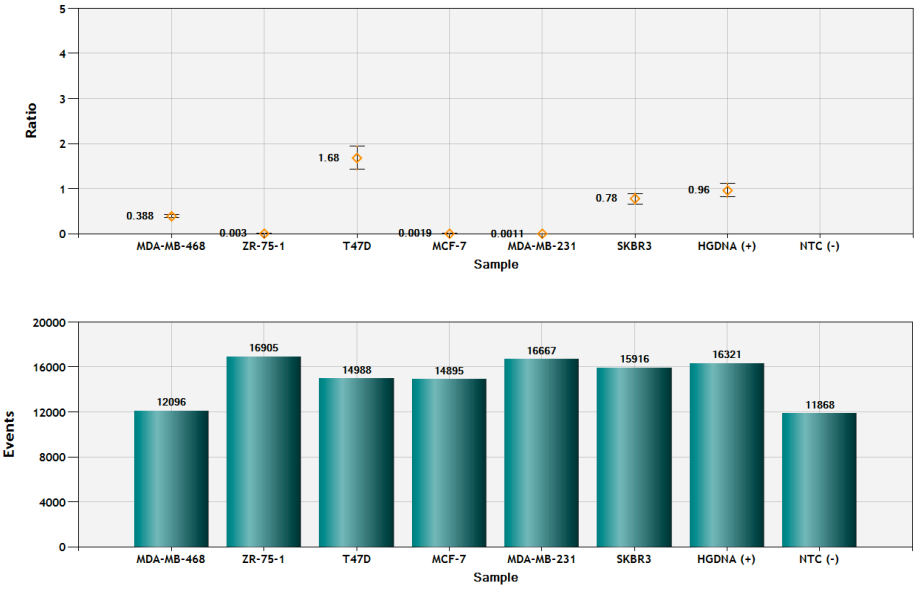
A: validation of *MYC* and *CDKN2A* genes; B: validation of *CCND1* and *HER2* genes; C: validation of *DMXL2* gene. Bars show total events for target genes exceed 10000 which indicated a reliable results with ratios of ~1 which indicate normal diploid CN. Positive (HGDNA) and negative (NTC) controls were run for each gene.

A

MYC

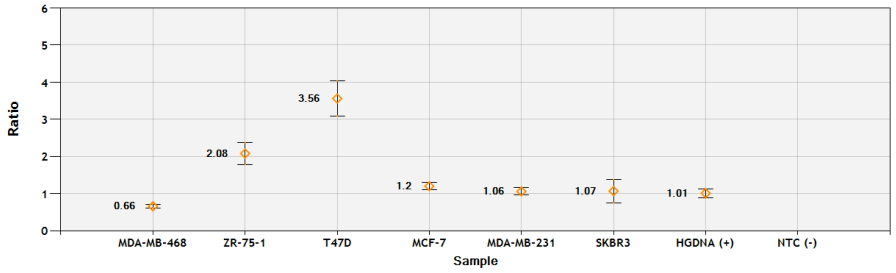

Ratio
10
9
8
7
6
5
4
3
2
1
0
0.79
3.63
3.19
1.94
1.34
9
1.01
MDA-MB-468
ZR-75-1
T47D
MCF-7
MDA-MB-231
SKBR3
HGDNA (+)
NTC (-)
Sample
Events
20000
16000
12000
8000
4000
0
12306
13940
15568
10286
15541
14819
15711
15691
MDA-MB-468
ZR-75-1
T47D
MCF-7
MDA-MB-231
SKBR3
HGDNA (+)
NTC (-)
Sample

CDKN2A


Ratio
5
4
3
2
1
0
0.388
0.003
1.68
0.0019
0.0011
0.78
0.96
MDA-MB-468
ZR-75-1
T47D
MCF-7
MDA-MB-231
SKBR3
HGDNA (+)
NTC (-)
Sample
Events
20000
16000
12000
8000
4000
0
12096
16905
14988
14895
16667
15916
16321
11868
MDA-MB-468
ZR-75-1
T47D
MCF-7
MDA-MB-231
SKBR3
HGDNA (+)
NTC (-)
Sample

B

CCND1


Ratio
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5
4
3
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1
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2.08
1.2
1.06
1.07
1.01
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T47D
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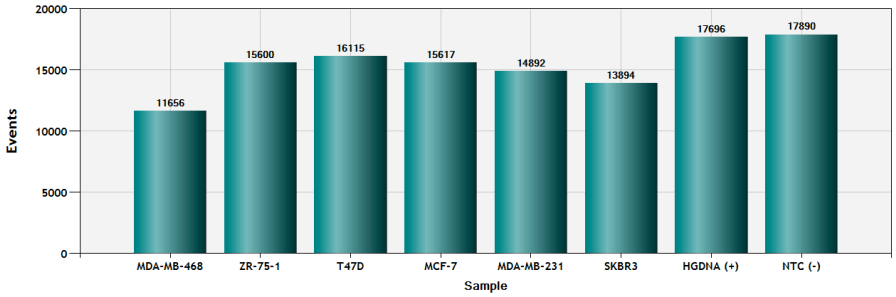
SKBR3
HGDNA (+)
NTC (-)
Sample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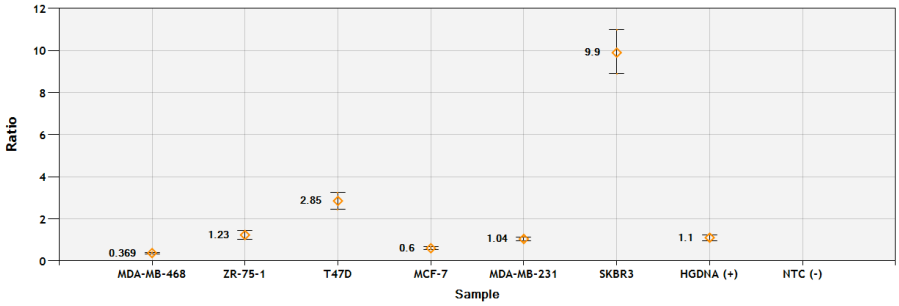
SKBR3
HGDNA (+)
NTC (-)
Sample

HER2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1.04
1.1
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MCF-7
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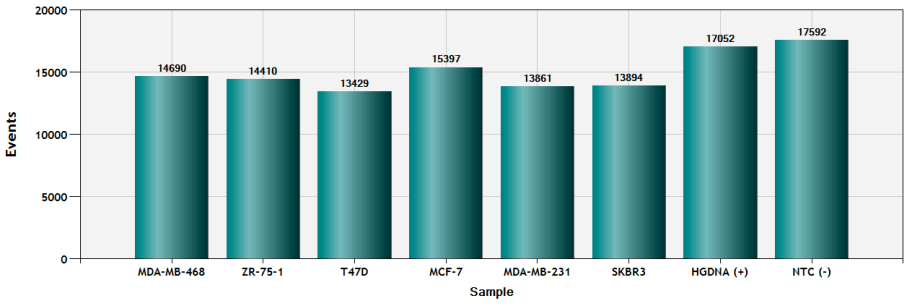
SKBR3
HGDNA (+)
NTC (-)
Sample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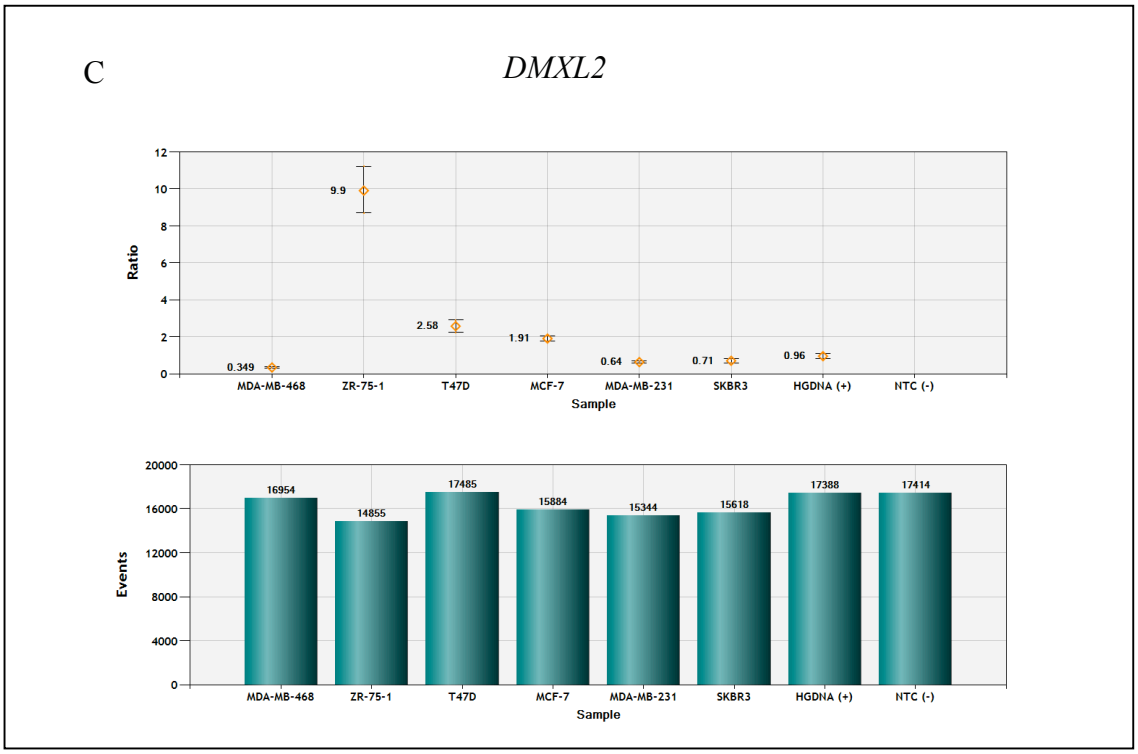
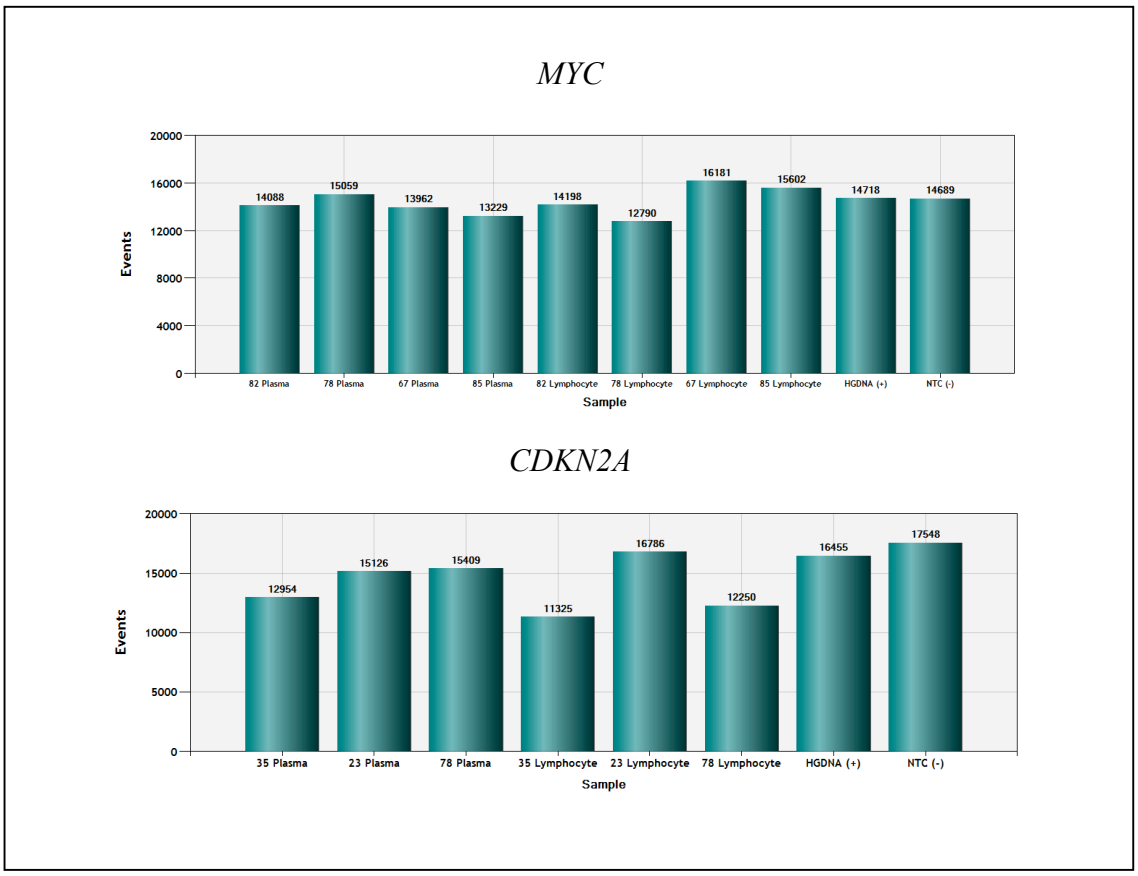
HGDNA (+)
NTC (-)
Sample



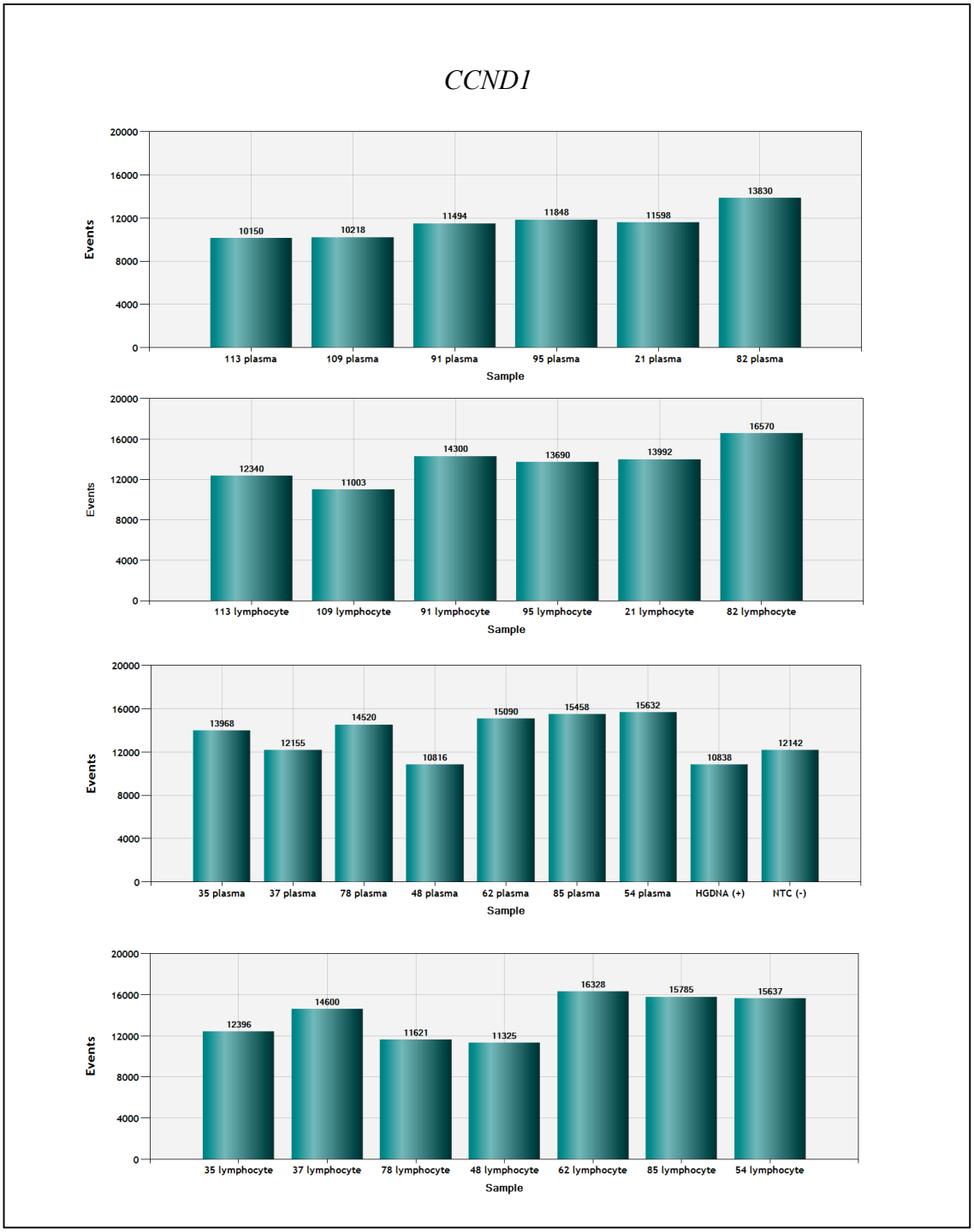
ddPCR detection of CNV states for 5 target genes in 6 breast cancer cell lines

A: shows total events and ratios of *MYC* and *CDKN2A* genes in SK-BR-3, MDA-MB-231, MCF-7, ZR-75-1, and T-47D cell lines. B: represents total events and ratios of *HER2* and *CCND1* genes; C: shows total events and ratios of *DMXL2* gene in the same cell lines. All total events above 10000 which indicated a reliable data; ratios of ~1 =normal diploid CN; ratios ≤ 0.6 deletion; ratios > 1.1 amplification. Positive (HGDNA) and negative (NTC) controls were run for each gene.



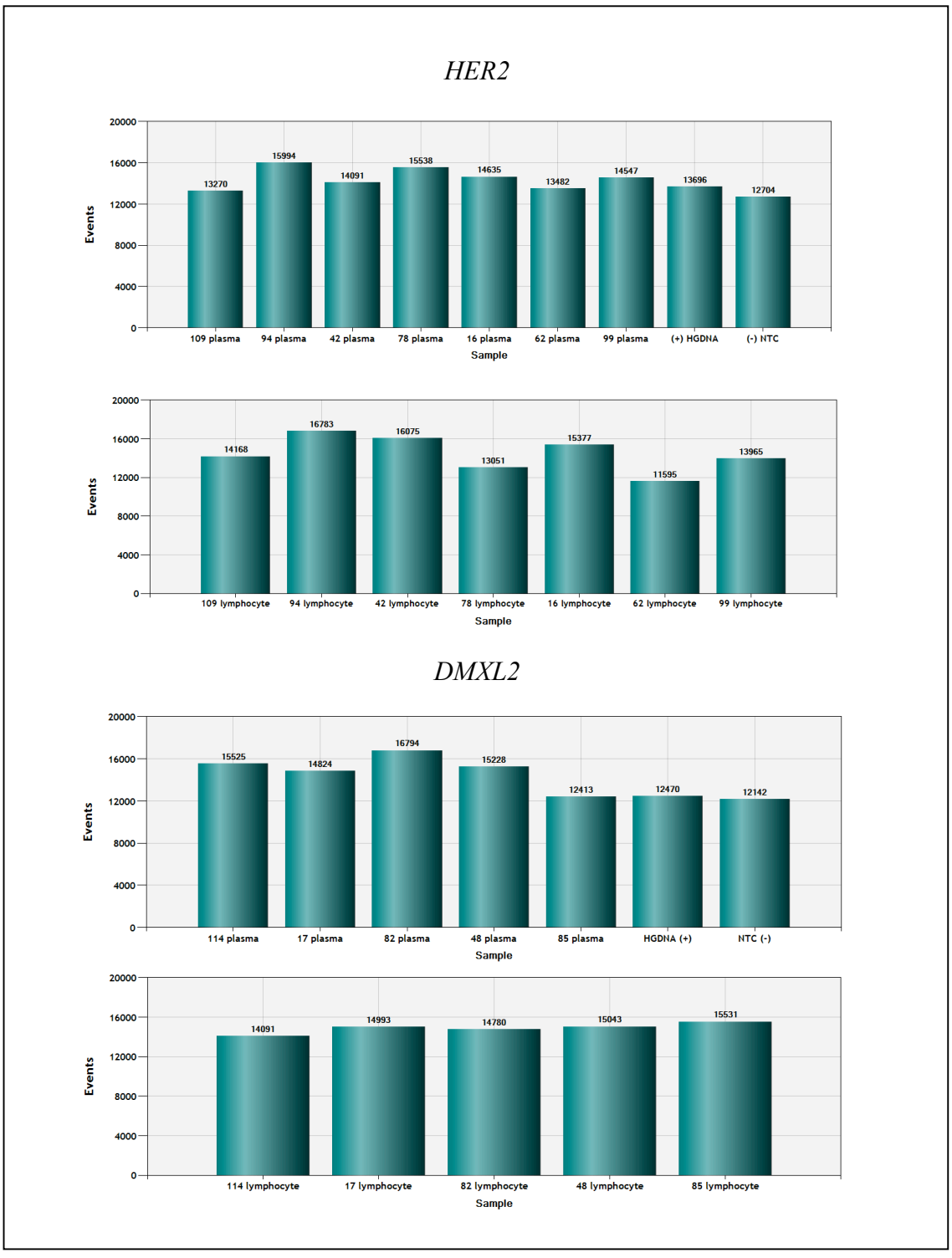
MYC and *CDKN2A* gain detection in clinical samples by ddPCR

Illustrates total events of *MYC* and *CDKN2A* gene intervals in the preamplified plasma cfDNAs and matched lymphocyte DNAs (control); total events (turquoise column) obtained (>10,000). HGDNA (positive control); NTC (negative control).



ddPCR validation of *CCND1* amplification in paired plasma and matched lymphocyte samples

Shows total events (turquoise bars); positive (HGDNA) and negative (NTC) controls were run on each plate.



ddPCR detection of *HER2* amplification and *DMXL2* deletion in plasma cfDNA of breast cancer patients

Matched lymphocytes were used as a reference samples; total events of preamplified plasma samples with *HER2* amplification and *DMXL2* deletion. Positive (HGDNA) and negative (NTC) controls were run for each gene.

Appendix 3 Copyrighted Materials

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Appendix 4 Conference abstract NCRI Liverpool November 2014

Cell-free plasma markers of breast cancer in young women and women at high risk

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Background

Circulating microRNAs (miRNAs) are under investigation as noninvasive biomarkers of cancer. Our group has been investigating these in women at risk of breast cancer who carry an inherited *BRCA* gene mutation.

Aim

The aim of this research was to identify any tumour-associated miRNAs among women with an elevated risk of developing breast cancer based on their family history (*BRCA* mutation carrier) as well as to investigate miRNA stability on storage of plasma and cDNA.

Method

In this study we measured the level of 8 candidate circulating microRNAs, identified as part of a previous study (Elshaw et al., 2013), in the plasma of women with a family history of breast disease, comprising healthy mutation negative controls (PNEG=17) and women with a known *BRCA* mutation (*BRCA1/2* carriers = 21 and *BRCA1/2* affected=15) using quantitative PCR. Plasma was stored for 12 months at -80°C before RNA extraction with the mirVana™ miRNA isolation Kit, reverse transcription to cDNAs followed by pre-amplification. CDNA was then kept in the freezer (-20°C) and Taqman miRNA assays re-run 6-months later. RNA was also re-extracted after 2 years storage from 13 selected plasma samples (PNEG (n=4), *BRCA1/2* carriers (n=5), and *BRCA1/2* affected (n=4)) and re-quantified by qPCR.

Results

There was no significant difference between miRNA levels of healthy mutation carriers and PNEG controls, although five miRs showed a significant difference between the PNEG control group and the *BRCA* affected group (mir-26a, mir-27b, mir-130b, mir-324-3p, mir-181a). There was a little variability in miR levels between replicate cDNA reactions despite 6 months storage. All miRs were expressed at a lower level in the re-extracted plasma, but expression relative to the mean of two reference miRs was unchanged.

Conclusion

The candidate miRNAs may have utility in monitoring women with breast cancer due to inherited *BRCA* mutation. However results need careful interpretation as some miRNA can be degraded with increasing time of storage of plasma.

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