Constitutional Tumour Suppressor Gene Methylation in Cancer Predisposition

Submitted by

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Thesis Abstract

Introduction

Cancer is commonly recognised as a genetic disease that is driven by mutations. However, aberrant DNA methylation is increasingly being accepted as vital in cancer development and progression. DNA methylation of tumour suppressor genes has been identified as a recurrent change in cancer patients. In particular, DNA methylation of the promoter is strongly associated with silencing gene expression, thus suppressing the activity level of that gene. Interestingly, tumour suppressor gene methylation is not limited to the tumour, and has also been observed in disease-free tissue.

Constitutional methylation is an aberration in DNA methylation that is detectable in normal tissue, either in a complete or mosaic fashion. This phenomenon has been reported in various cancer types, and has been implicated in breast cancer predisposition. Constitutional methylation of the *BRCA1* promoter was first reported in peripheral blood of certain sporadic breast cancer patients who presented with early onset disease. Aberrant *BRCA1* promoter methylation has been strongly associated with reduced *BRCA1* protein expression, implicating promoter methylation as a likely cause of transcriptional inactivation of *BRCA1*, and as a mediator of breast carcinogenesis.

DNA methylation of tumour suppressor genes has also identified in various cancer types including melanoma. Methylation of *RASSF1A* and *RARβ* have been observed in primary and metastatic melanoma lesions, and methylation of *RARβ* in particular has been recognized as crucial prognostic factors in early stage melanoma. However, constitutional methylation of these loci in the context of melanoma has seldom been explored.

In light of these findings, this thesis aimed to further understand the role of constitutional methylation of tumour suppressor genes as a predisposing factor in breast cancer and melanoma.

Main findings

This thesis assessed constitutional methylation in the context of two independent tumour streams: breast cancer and melanoma.

Firstly, the *BRCA1* methylation frequencies were established in a case-control cohort using peripheral blood and tumour samples obtained from women who were enrolled in the LifePool project. This was a population study assessing constitutional *BRCA1* methylation in peripheral blood of healthy women and women with breast cancer, and also in tumour DNA from case women. Surprisingly, constitutional *BRCA1* methylation was detected in peripheral blood of cases and controls at near identical frequencies; however, the level of constitutional *BRCA1* methylation detected in cases was significantly higher compared to controls. The importance of the level of detectable *BRCA1* methylation in peripheral blood was highlighted when methylation above 4% was only present in women with breast cancer. Additionally, an age-association was also identified, with results revealing significantly higher rates of constitutional *BRCA1* methylation in women under 40 years of age compared to women above 40. Once corresponding tumour samples were analysed for case women, *BRCA1* methylation was detected at significantly higher levels compared to peripheral blood. These finding suggest that low levels of constitutional *BRCA1* methylation can indicate a *BRCA1*-methylated tumour.

Secondly, constitutional *BRCA1* methylation was assessed in a cohort of monozygotic twin pairs at birth and 6 years. *BRCA1* methylation was present in buccal mucosa and white blood cell DNA of newborns and 6-year-old twins in a highly discordant fashion, within twin pairs and across tissue types. Interestingly, methylation observed at birth was not sustained in the same individuals at 6 years old. The observed discordance highlights the lack of genetic influence on constitutional *BRCA1* methylation. This data also suggests that

constitutional *BRCA1* methylation can be erratic, and occurs early during embryonic development.

Finally, constitutional methylation of *RASSF1A* and *RARβ* was assessed in peripheral blood of patients with primary melanoma, as well as in their tumours. Constitutional methylation of both loci was not detected, indicating that peripheral blood methylation of these *RASSF1A* and *RARβ* is not a useful predictor of melanoma predisposition. Once tumours were assessed, *RARβ* methylation was detected at significantly higher frequencies compared to *RASSF1A*. Although constitutional methylation was not identified, it is possible that peripheral blood methylation of *RASSF1A* and *RARβ* may be occurring as a rare epigenetic event.

Conclusions

The results of this thesis challenge the notion that constitutional *BRCA1* methylation is present at low frequencies in healthy individuals. Constitutional methylation of the *BRCA1* gene appears to predispose younger women to developing breast cancer. The case-control study relating to breast cancer did not reveal methylation in every corresponding patient tumour, despite the presence of constitutional methylation. Unexpectedly, most patients with constitutional methylation had hormone-receptor positive tumours suggesting that this epigenetic event is unlikely associated with the presence of constitutional methylation. The twin study revealed that constitutional methylation was detected in newborns, but is not hereditary and is unstable overtime, suggesting that healthy tissue methylation of the *BRCA1* gene most likely occurs as a sporadic embryonic event.

Assessment of *RARβ* and *RASSF1A* methylation in peripheral blood and tumours of melanoma patients revealed that both genes are unlikely to be drivers of melanoma, and that constitutional methylation of these loci is not an adequate tool in identifying individuals who may be predisposed to the development of the disease.

Collectively, interesting insights were obtained into constitutional methylation of tumour suppressor genes in breast cancer and melanoma; however, this phenomenon remains complex and requires additional investigation into its impact on cancer predisposition.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

All laboratory experiments and analyses were carried in the Translational Genomics and Epigenomics Laboratory at the Olivia Newton-John Cancer Research Institute in Melbourne.

Chapter 3:

- The fully methylated cell line control (WEHICS62) was provided by Professor Clare Scott at the Walter and Eliza Hall Institute of Medical Research.
- Ms Basant Ebaid performed the optimization experiments for the *BRCA1* ddPCR methylation assay.
- Associate Professor Alexander Dobrovic and Dr Hongdo Do provided overall technical guidance and data review.

Chapter 4:

- Genomic DNA from peripheral blood and tumour samples were provided by Ms Lisa Devereux in collaboration with the LifePool Project.
- Ms Giada Zapparoli assisted with bisulfite modification of genomic DNA samples obtained from peripheral blood of patients.
- Ms Basant Ebaid performed bisulfite modification of tumour DNA and carried out all *BRCA1* ddPCR experiments and data analysis.
- Mr Mark Tacey performed all biostatistical analysis.

- Associate Professor Alexander Dobrovic provided overall study direction and assisted with data interpretation.

Chapter 5:

- Associate Professor Jeffrey Craig and Dr Jane Loke at the Murdoch Children's Research Institute kindly provided genomic DNA for all identical twin samples analysed throughout this study.
- Ms Basant Ebaid performed bisulfite modification, ddPCR experiments and data analysis.

Chapter 6:

- FFPE tumours and blood samples obtained from melanoma patients were provided by Ms Sonia Mailer from Melanoma Research Victoria.
- FFPE tissue blocks were sectioned by Mr Basisth Dahal-Mohun and Ms Basant Ebaid.
- Pathology review of tumour samples was performed by Dr Louise Jackett at Austin Health.
- Ms Basant Ebaid performed staining, DNA extraction of FFPE histopathology tissue slides, bisulfite modification of all genomic DNA samples, and all MS-HRM experiments and data analysis.

Basant Ebaid

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A PhD is a once-in-a-lifetime journey that I have had the privilege to undertake. This thesis is a culmination of my PhD journey, which, akin to climbing a mountain, was accompanied with encouragement, hardship, and frustration. When I reached the top and experienced the feeling of fulfilment, I realised that although only my name appears on the cover of this thesis, a great number of people including my colleagues, collaborators, family and friends have contributed to the successful completion of my candidature. No words can express my appreciation for the people in my life who helped make this journey possible.

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To my Mother in particular, words cannot express how much I cherish and love you for all the things you are, and have been to me. Thank you for the unconditional love and support you have provided, and for being my mentor and closest friend.

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List of Abbreviations

ABC	Apocrine breast carcinoma
ACC	Adenoid cystic carcinoma
AIHW	Australian Institute of Health and Welfare
AutoDG	Automated Droplet Generator
BRAF	B-Raf Proto-Oncogene
BRCA1/2	Breast cancer type 1/2
BWS	Beckwith-Wiedemann syndrome
CDKN2A	Cyclin dependent kinase inhibitor 2A
CLDN1	Claudin 1
COL2A1	Collagen Type II Alpha 1 Chain
CpG	Cytosine-phosphate-Guanine
CTCAE	Common Terminology Criteria for Adverse Events
ctDNA	Circulating tumour DNA
DCIS	Ductal carcinoma *in situ*
ddPCR	Droplet digital PCR
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DOK7	Docking protein 7
DZ	Dizygotic
EBCTCG	Early Breast Cancer Trialists' Collaborative Group

ER	Estrogen Receptor
FFPE	Formalin-Fixed Paraffin Embedded tissue
HER2	Human epidermal growth factor receptor 2
HR	Hormone Receptor
HRM	High Resolution Melting
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma *in situ*
LDH	Lactate dehydrogenase
LUMA	Luminometric based Assay
MAPK	Mitogen-activated protein kinase
MBC	Metastatic breast carcinoma
MedBC	Medullary breast carcinoma
MEK1/2	Mitogen-activated protein kinase kinase 1/2
MGB	Minor groove binder
MGMT	O-6-Methylguanine-DNA Methyltransferase
MLH1	MutL Homolog 1 gene
MS-HRM	Methylation-Sensitive High Resolution Melting
mRNA	Messenger RNA
MRV	Melanoma Research Victoria

MZ	Monozygotic
NBCF	National Breast Cancer Foundation
NCDB	National Cancer Center Database
NEC	No extraction control
NGS	Next Generation Sequencing
NR	No response
NRAS	Neuroblastoma Ras oncogene
NST	No special type
ORR	Objective response rate
OS	Overall survival
PARPi	Poly (ADP-ribose) polymerase inhibitor
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDX	Patient derived xenograft
PFS	Progression free survival
PR	Progesterone Receptor
PTEN	Phosphatase and tensin homolog
RARβ	Retinoic Acid Receptor Beta
RASSF1A	Ras association domain family 1 isoform A
RB	Retinoblastoma gene

RT-PCR	Real time PCR
SNP	Single nucleotide polymorphism
TNBC	Triple-negative breast cancer
UV	Ultraviolet
WBC	White blood cell
WGBS	Whole genome bisulfite sequencing
WHO	World Health Organization

CHAPTER 1.

LITERATURE REVIEW

1.0. Overview of Literature Review

This thesis is organised into four main chapters based on constitutional DNA methylation in two main tumour streams. Each chapter was written to be primarily independent and complete. Chapter 1 will review the literature on breast cancer in Section 1.1, while melanoma will be reviewed in section 1.2. Constitutional DNA methylation will be addressed relative to tumour suppressor genes associated with either breast cancer or melanoma.

1.1. Breast Cancer

1.1.1. Breast cancer incidence

Breast cancer is the most frequently diagnosed cancer in women worldwide, and is the leading cause of cancer-related deaths among women (Global Health Estimates 2016). Although the mortality rate of breast cancer is decreasing in the developed world, the incidence of breast cancer continues to rise. In 2011, 1.7 million breast cancer cases were reported worldwide, with 508,000 deaths (Bray *et al.,* 2018). According to GLOBOCAN (2018), the incidence of breast cancer has risen to 2.1 million in 2018 and a mortality rate of 627,000. The 5-year survival rate is over 80% in western countries, which is double that of developing countries.

Approximately 28% of all cancers diagnosed in Australian women in 2017 were attributed to breast cancer (AIHW 2017). Breast cancer is the second leading cause of cancer deaths in Australia, accounting for more than 14% of all cancer related deaths. The number of new breast cancer cases reported by the Australian Institute of Health and Welfare (AIHW) has

more than doubled from 1982 to 2008, and the incidence of breast cancer is steadily increasing. Predictive models by AIHW (2017) estimate the number of new breast cancer cases to increase to more than 17,000 by 2020 - an increase of 20% since 2011. Although the incidence of breast cancer is increasing, Australia has one of the highest breast cancer survival rates in the world, with a 5-year survival rate of 90%, and a 10-year survival rate of 83% (AIHW 2017). The improved survival rates are attributed to early detection through regular mammographic screening, advances in medical treatment and enhanced treatment outcomes.

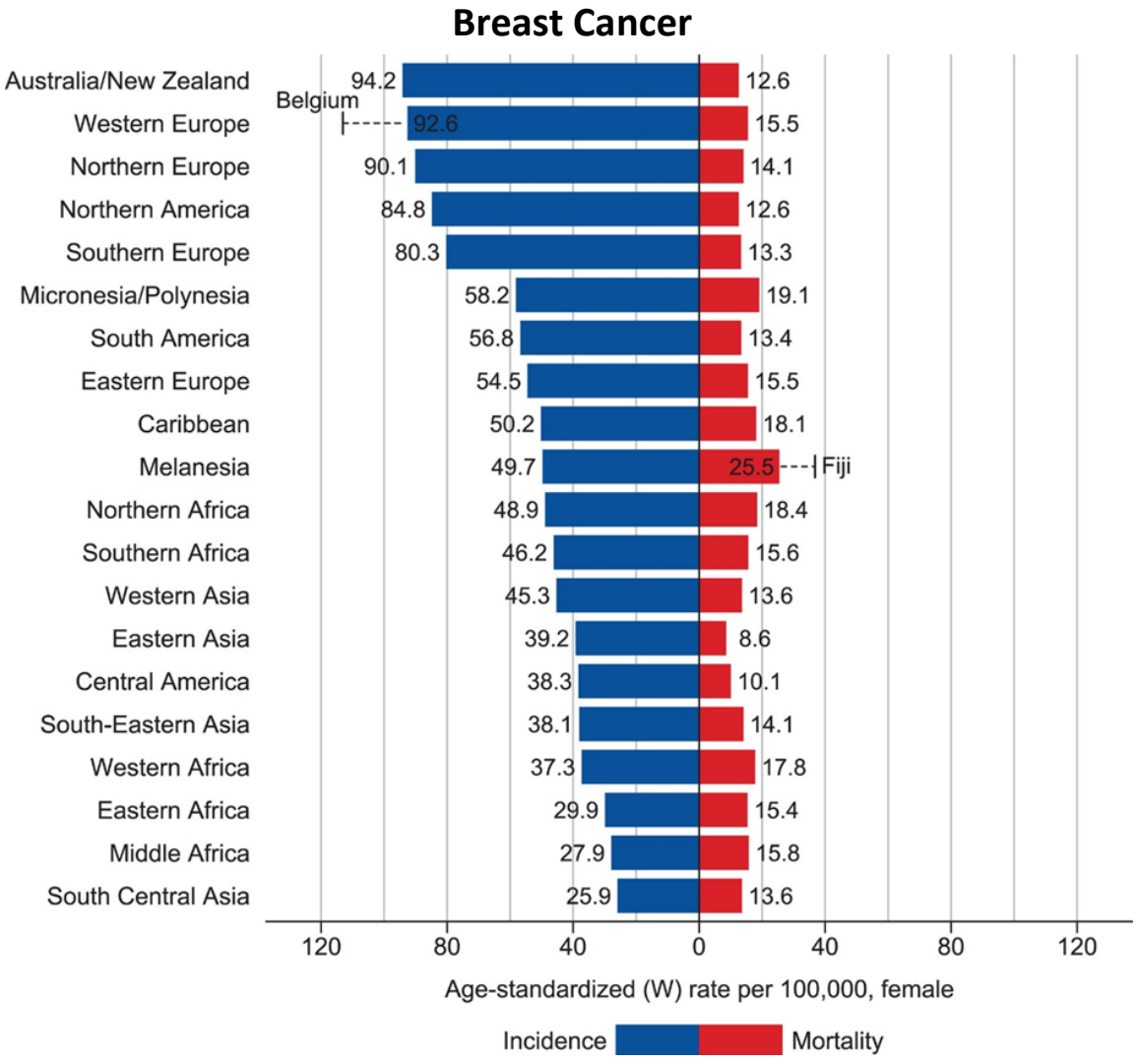


Figure 1-1. Region-specific and mortality age-standardised rates for female breast cancer in 2018. Breast cancer rates are presented in descending order of the world (W) age-standardised rate. The highest national age-standardised rates for breast cancer incidence is outlined in blue, and mortality in red. (Figure adopted from GLOBOCAN 2018).

1.1.2. Breast cancer subtypes

The understanding of the molecular and biological basis of breast cancer has advanced over the past decade, thus exposing the diversity of breast cancer and various pathways to breast cancer development. Establishing breast cancer subtypes has both biological and clinical implications, and considering factors like tumour grade, hormone receptor status and HER2 status enables the categorisation of breast cancers by both biology and therapeutic options.

As technology advances and genomic data becomes incorporated into analysis, breast cancer subtypes can become complex. Data from Curtis and colleagues (2012) analysed over 2,000 breast cancer samples and found over 10 biologically distinct subtypes of breast cancer correlating to treatment outcome. These findings suggest that molecular classification of breast cancer continues to evolve.

Breast cancers can be classified into certain subtypes based on the genes expressed in the breast cancer. Assays that classify breast cancers into certain subtypes, particularly the PAM-50 assay, have become commercially available (Nielson *et al.,* 2014). Deciphering breast cancer subtypes is crucial, and plays an important role in informing clinical treatment decisions for breast cancer patients.

1.1.3. Histological characteristics

Breast carcinomas are normally classified by their histological appearance, and the histological features viewed under a microscope can vary depending on tumour grade

(Weigelt *et al*., 2008). Indeed, these distinct characteristics influence the treatment course and outcome of a patient, and the histological diversity of breast cancers has relevant prognostic implications (Leong and Zhuang 2011). Breast cancers are histologically diverse. According to the World Health Organisation (WHO), there are up to 21 distinct histological subtypes of breast cancer based on morphology, growth, and architectural patterns (Eble *et al.,* 2003).

Breast cancers can be broadly categorised into invasive carcinoma and *in situ* carcinoma. The latter is further divided into two sub-categories; ductal and lobular carcinoma (Makki 2015). Ductal carcinoma *in situ* (DCIS) occurs much more frequently than lobular carcinoma *in situ* (LCIS), with about 1200 women diagnosed with DCIS in Australia each year (Tavassoli 2003). However, DCIS is non-invasive. Studies have also shown that DCIS is more abundant in women over 50 years of age, compared to LCIS which is normally diagnosed in pre-menopausal women under 50 years old (Williams *et al.,* 2019).

Invasive ductal carcinoma (IDC) is the most common type of breast cancer, accounting for approximately 80% of all breast cancers (AIHW 2006). IDC is highly heterogeneous, and displays a vast array of morphological variation. IDC can be categorised into numerous histological subtypes depending on various and distinct features that are expressed (Mills *et al.,* 2018). Assessing tumour histology can provide valuable prognostic information, however most breast cancers (60% to 75%) lack distinct features and therefore cannot be categorised into an existing subtype of breast cancer (Badowska-Kozakiewicz *et al.,* 2017). These breast cancers are labelled as IDC of ‘no special type’ – or NST. As a result, clinical decision-making and patient management on the basis of histological type alone is unsatisfactory.

1.1.4. Molecular characteristics

The relationship between molecular breast cancer subtypes and the prognostic relevance to survival is well recognised (Fallahpour *et al.,* 2017). Five main molecular subtypes of breast cancer have been identified based on the hormone receptors expressed on the tumour, and the presence of human epidermal growth factor receptor 2 (HER2). These subtypes (often referred to as intrinsic subtypes) are known as luminal A, luminal B, HER2-related, basal-like (or triple-negative) breast cancers and unclassified ("normal-like") (Perou *et al.,* 2000; Larsen *et al.,* 2014).

Gene expression studies have found that breast cancer risk factors may vary depending on molecular subtypes (Yang *et al.,* 2007). In 2015, a collaborative study reported on population-based cancer trends and breast cancer incidence by molecular subtype. Data was collated from years 1975 to 2011 based on the North American population (Kohler *et al.,* 2015). Their findings show that the incidence of breast cancer is stable among adults, with a decrease in mortality. However, racial and ethnic variation was observed within these trends. Hormone receptor positive (HR+) and HER2 negative (HER2-) breast cancers are the subtype of breast cancer with the best prognosis, and were found to be the most frequently diagnosed in non-Hispanic white women of all races and ethnicities. Triple-negative breast cancers (TNBC) (or basal-like breast cancers) are the breast cancer subtype with the poorest prognosis. Interestingly, incidence rates for TNBC were highest among non-Hispanic black women.

Perou and colleagues (2000) characterised variations in gene expression patterns in 65 human breast tumour specimens from 42 women, using DNA microarrays representing 8,102 human genes. Some tumours were sampled twice (before and after doxorubicin chemotherapy), two of which were paired with a lymph node metastasis from the same patient. Perou *et al.,* (2000) found that tumours from the same patients pre- and post-

chemotherapy treatment expressed similar gene expression patterns than those from other patients. This was also observed for the two primary patient tumour samples with matched metastatic lesions, showing similar gene expression patterns to each other than to samples from other individuals.

Breast tumours are histologically complex (Weigelt *et al.,* 2010). Interestingly, Perou *et al.,* (2000) identified eight independent gene clusters representing eight distinct cell types within the breast tumours, including endothelial cells, stromal cells, adipose-enriched cells, B cells, T cells, macrophages, luminal cells and basal epithelial cells. These findings support the heterogeneity and distinct histological and biological complexity of breast cancers.

1.1.5. Hormone receptor status

Breast cancers differ in the hormones and protein receptors that are expressed on the tumour surface, and can be broken up into five broad categories (outlined in section 1.1.4) based on their molecular signature (Perou *et al.,* 2000; Onitilo *et al.,* 2009). The hormone receptor status of each of these subtypes is outlined in Table 1-1.

Table 1-1. Hormone receptor status for each molecular subtype of breast cancer.

Molecular Subtype	Estrogen Receptor (ER)	Progesterone Receptor (PR)	HER2-enriched
Luminal A	+	+	-
Luminal B	+/-	+/-	+/-
Triple-negative/basal-like	-	-	-
HER2-enriched	-	-	+
Normal-like	+	+	-

The most commonly studied hormonal markers in breast cancer are estrogen receptor (ER) and progesterone receptor (PR) (Bauer *et al.,* 2007). Breast cancers that are ER+/PR+ often have a favourable prognosis and show a strong clinical response to endocrine therapy when compared to breast cancers that are hormone-receptor negative (EBCTCG 1998). In

addition, age-related risk has been associated with breast cancers that are of ER+/PR+ subtype, with a rising incidence in women as they age (Yasui & Potter, 1999). These findings were supported by Anderson *et al.,* (2002), who found that hormone-receptor positive breast cancers were correlated with post-menopausal women, compared to hormone-receptor negative breast cancers that were associated with pre-menopausal women.

In 2015, Bae and colleagues examined the clinical and biological characteristics of 6,980 women with breast cancer. These women were stratified into three groups according to their tumour ER and PR expression, as double HR+ (ER + PR+), single HR+ (ER+ PR- and ER- PR+) and double HR-negative (ER- PR-). Interestingly, their findings demonstrate that women with tumours that were positive for single-hormone expression and lacked HER2 expression (i.e. HER2-) had comparable poor survival to women with triple-negative (i.e. ER- PR- HER2-) breast cancer (Bae *et al.,* 2015).

Triple-negative breast cancer (TNBC) is more aggressive than hormone-positive breast cancers, and is often diagnosed in pre-menopausal women (De Laurentiis *et al.,* 2010). TNBC lacks targeted therapeutic options and has poor prognosis, and chemotherapy remains the standard treatment approach for patients with all stages of the disease (Rakha *et al.,* 2007). The histologic heterogeneity of TNBC poses treatment challenges, therefore highlighting the unmet medical need for more personalised therapeutic options. More recently, Mills *et al.,* (2018) analysed data from the National Cancer Center Database (NCDB) of over 89,000 TNBC patient tumours, to establish the prognostic value of TNBC histology. They found that metaplastic breast carcinoma (MBC), medullary breast carcinoma (MedBC), adenoid cystic carcinoma (ACC), invasive lobular carcinoma (ILC) and apocrine breast carcinoma (ABC) had significantly different proportions of triple negative features when compared to invasive ductal carcinoma (IDC) ($p < 0.001$).

Additionally, reduced poorer overall survival was observed in IDC and ILC (Mills *et al.,* 2018). These findings demonstrate the impact of TNBC heterogeneity on patient outcome.

1.2. Melanoma

1.2.1. Melanoma incidence and mortality

Malignant melanoma is the least common form of skin cancer, accounting for approximately 1-2% of all skin cancer cases (Cancer Council Australia 2018). The most common melanoma subtype is cutaneous melanoma, which originates in the pigment-producing cells (melanocytes) of the skin (Ali *et al.,* 2013), and accounts for more than 90% of all melanoma cases. However, melanoma is the deadliest type of skin cancer and is responsible for most skin cancer-related deaths (AIHW, 2016). A large number of studies indicate the risk of melanoma correlates with genetic characteristics as well as an individual's exposure to ultraviolet (UV) radiation (Sample and He., 2018; Schadendorf *et al.,* 2015). In Australia, melanoma is the third most commonly diagnosed cancer in males (following prostate and bowel cancer) and in females (following breast and bowel cancer) (AIHW, 2014). According to AIHW (2016), the 5-year survival rate of melanoma in Australia from 2007 to 2011 was 90%, and was greater for woman than men.

Interestingly, gender differences have been observed in relation to the incidence of melanoma. In 2016, there were 14,485 newly diagnosed melanoma cases in Australia (8,455 males [58%] and 6,030 females [42%]) (AIHW 2020). In 2018, 1,429 deaths were attributed to melanoma, with higher mortality rates observed in males compared to females (965 and 464 respectively) (AIHW 2020). The AIHW has predicted a slight reduction in mortality rates in 2020, with an anticipated 891 melanoma deaths in males and 484 deaths in females. Due to extensive skin cancer screening programs available in Australia as well as extensive skin cancer prevention campaigns, there has been a decrease in average tumour depth at the time of diagnosis, indicative of early detection, and hence improved prognosis.

Australia has the world's highest incidence of melanoma (Cancer Council Australia 2018). In 2016, more than 1,200 Australians died from melanoma, and the incidence of this disease is increasing (Cancer Council Australia 2018). According to a 2016 report by AIHW, the incidence of melanoma has increased by 181% since 1982 to 2016, from 27 cases per 100,000 individuals to approximately 49 cases per 100,000 people. Whether the rise in melanoma incidence is a consequence of enhanced methods of detection, or if the underlying disease is in fact truly increasing, remains unknown.

1.2.2. Diagnostic and prognostic markers in melanoma

Over the past decade, medical advances have drastically improved the overall survival of melanoma sufferers with stage III and IV disease, particularly with the introduction of immunotherapy and mitogen-activated protein kinase (MAPK) targeted therapies (Wellbrock *et al.,* 2016). Melanomas with mutations in the MAPK pathway have the highest oncogenic and therapeutic relevance for the disease (Schreuer *et al.,* 2017). Although the 5-year survival of patients with early stage melanoma is relatively high, immunotherapies and MAPK therapies have produced favourable health outcomes in approximately 20% of patients, extending their life expectancy up to 10 years following treatment with the immune checkpoint inhibitor, ipilimumab (Hodi *et al.,* 2016).

Mutations in melanoma are common, and are often mutually exclusive. Germline mutations in *CDKN2A* and *p16* have been observed in familial melanoma cases, and are known to be associated with melanoma predisposition (Helgadottir *et al.,* 2016). Somatic mutations in *BRAF* and *NRAS* genes are also quite common, with *BRAF* mutations occurring in about 50% of melanoma patients, and *NRAS* mutations occurring in approximately 13% of patients (Thomas *et al.,* 2015). The most common *BRAF* mutation type in melanoma is V600E, accounting for up to 90% of all *BRAF* mutations. To date, *BRAF* mutation status is a crucial predictor of therapeutic outcome for melanoma patients (Ascierto *et al*., 2018).

Over recent years, multiple therapies have been deemed effective in the treatment of melanoma (Davey *et al.,* 2016). For this reason, developing diagnostic, prognostic, and predictive biomarkers that are specific to melanoma may help improve patient outcome and treatment response. The importance of biomarker detection in melanoma to predict patients most likely to benefit from certain therapies is currently evolving. This has been demonstrated in several clinical trials that have included the evaluation of blood and tissue-based biomarkers as secondary endpoints or assessments in Stage III and Stage IV melanoma patients (Table 1-2).

Table 1-2. Phase III clinical trial outcomes of systemic melanoma therapies.

ORR, objective response rate; NR, not reported; PFS, progression free survival; OS, overall survival; LDH, lactate dehydrogenase. Grade 3/4 toxicity as defined by Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Dabrafinenib and vemurafenib are BRAF V600 inhibitors. Trametinib and cobimetinib are MEK1/2 inhibitors. gp100 is a human melanoma peptide vaccine. Ipilimumab is an antibody targeting the CTLA-4 receptor. Nivolumab and pembrolizumab are anti PD-1 therapies.

Therapy	ORR	Median PFS (months); % survival (year)	Median OS (months); % survival (year)	Grade 3/4 toxicity	Biomarkers examined	Reference
Molecular Therapies						
Vemurafenib (*n* = 337)	48%	6.9; 14% (1.5 years)	13.6; 39% (1.5 years)	73%	BRAF V600 mutation and LDH	McArthur *et al.,* (2014); Chapman *et al.,* (2017)
Dabrafenib (*n* = 187)	50%	5.1; 12% (3 years)	20; 45% (2 years)	53%	BRAF V600 mutation and LDH	Hauschild *et al.,* (2014); Hauschild *et al.,* (2020)
Trametinib (*n* = 214)	22%	4.8; NR	NR; 81% (6 months)	NR	BRAF V600 mutation and LDH	Flaherty *et al.,* (2012); Robert *et al.,* (2019)
Dabrafenib + trametinib (*n* = 352)	64%	12.1; 30% (2 years); 24% (3 years)	25.6; 73% (1y); 52% (2y); 44% (3y)	52%	BRAF V600 mutation and LDH	Schadendorf *et al*., (2017); Robert *et al.,* (2019)
Vemurafenib + cobimetinib (*n* = 247)	70%	12.3; NR	22.3; 75% (1 year); 48% (2 years)	60%	Ki67, p56, MAPK, PI3K, CD8 T cells	Ascierto *et al.,* (2016)

Table 1-2. *Continued*

Therapy	ORR	Median PFS (months); % survival (year)	Median OS (months); % survival (year)	Grade 3/4 toxicity	Biomarkers examined	Reference
Immunotherapies						
gp100 (*n* = 136)	1.5%	2.8; 48.5% (12 weeks)	6.4; 25.3% (1 year), 13.7% (2 years)	11.4%	LDH	Hodi *et al.,* (2010); Schadendorf *et al.,* (2015)
Ipilimumab (*n* = 427)	13%	2.8; 14% (2 years)	16.0; 43% (2 years)	20%	LDH, blood lymphocyte count	Robert *et al.,* (2015); Schachter *et al*., (2017)
gp100 + ipilimumab (*n* = 403)	5.7%	2.8; 49.1% (12 weeks)	10; 44% (1 year), 21.6% (2 years)	17.4%	LDH	Hodi *et al.,* (2010); Schachter *et al*., (2017)
Nivolumab (*n* = 210)	40%	5.1; 44% (1 years)	NR; 73% (1 year)	11.7%	PD-L1, blood lymphocyte count	Robert *et al.,* (2015); Ascierto *et al.,* (2019)
Pembrolizumab (*n* = 277)	36%	4.1; 28% (2 years)	NR; 55% (2 years)	17%	LDH, blood count parameters	Robert *et al.,* (2015); Schachter *et al*., (2017)
Ipilimumab + nivolumab (*n* = 314)	57.6%	11.5; 49% (1 year), 39% (3 year)	NR; 64% (2 years), 58% (3 years)	55%	PD-L1, blood lymphocyte count	Larkin (2019); Larkin (2015); Wolchok *et al.,* (2017)

1.2.3. Liquid biomarkers in melanoma

Solid tumours often release their cellular and genetic constituents into the circulation of cancer patients (Lim *et al.,* 2018). Circulating tumour DNA (ctDNA) is fragmented DNA that is released into the bloodstream, and originates from the tumour. ctDNA serves as a surrogate to tumour biopsy for the non-invasive identification of tumour-specific biomarkers. Cancer patients have high cell turnover of tumour cells through apoptosis and necrosis, resulting in the release of ctDNA into the circulation (Calapre *et al.,* 2017). Performing liquid biopsies using the blood of melanoma patients can provide clinical insight into prognostic and predictive biomarker information that can help guide patient treatment. More importantly, liquid biopsies of ctDNA allow for routine monitoring of patient response to therapy, as well as early detection of disease relapse (Vidal *et al.,* 2017).

In 2014, Lipson *et al.* assessed whether ctDNA levels could be indicative of changes in tumour burden for patients undergoing immunotherapy with PD-1 inhibitors. Their findings demonstrate that ctDNA levels detected in the blood correspond with radiological outcomes and tumour regression. Similarly, baseline ctDNA levels in melanoma patients have been able to predict immunotherapy response, with lower levels of ctDNA significantly associated with prolonged treatment response (Gray *et al.,* 2015).

Given that ctDNA has tumour-specific origin, analysing ctDNA has proven to be valuable when monitoring disease burden in melanoma patients. A recent study compared the mutation profiles of tumour biopsies and matched plasma ctDNA from metastatic melanoma patients, to determine the level of mutational concordance across the two tissues (Calapre *et al.,* 2019). They identified somatic mutations in 20 of 24 melanoma tumour biopsies, and detected ctDNA in 16 of 20 matched patient plasma samples. Their findings highlight the use of liquid biopsy and ctDNA as an alternative to tissue biopsy for the genetic profiling and monitoring of melanoma.

Aberrant epigenetic modifications in ctDNA have also been identified in melanoma (Mori *et al.,* 2005). DNA methylation signatures are promising for biomarker discovery due to the stability of CpG island methylation marks. Methylating events are common in various cancer types and occur early in cancer development, making DNA methylation a reliable and sensitive target that is detectable in ctDNA (Micevic *et al.,* 2017; Wouters *et al.,* 2017).

1.3. DNA Methylation in Cancer Predisposition

1.3.1 Epigenetic modifications and gene silencing

Epigenetic modifications (or epimutations) are changes in the genome that alter transcription without modifying the primary DNA sequence (Esteller 2008). The regulation of transcription is under the control of several mechanisms, allowing cells to increase or decrease the amount of mRNA and hence gene product (i.e. protein). This crucial biological process is established via the interaction between regulatory proteins and specific DNA motifs within regulatory regions of the DNA sequence (i.e. promoters and enhancers) (Heintzman *et al.*, 2007). In order for cells to function normally in mammals, epigenetic processes are required. Almost all cells within one individual are comprised of identical DNA. Epigenetic marks are essential for cells within one individual to have the capacity to differentiate into distinct cell types, and for the genome to retain these cellular identities. The body contains an array of cell types and tissues, and the differences between these tissues and cell types is caused by the "switching on" (expression) or "switching off" (inhibition) of certain sets of genes (Reik *et al.*, 2001). This is known as epigenetic activation or silencing.

Tumour suppressor genes have the capacity to be silenced via CpG island methylation (Gonzalez-Zulueta *et al.*, 1995; Herman *et al.,* 1995; Merlo *et al.*, 1995). CpG islands are regions within the genome rich in CG content. DNA methylation of cytosine bases in the CpG context is an epigenetic modification that is abundant within mammalian genomes,

and is essential to their development (Jones & Takai 2001; Jaenisch *et al.,* 2003). For DNA methylation to occur, a methyl group must be added to the carbon-5 position of cytosine residues of the CpG dinucleotide (Costello *et al.,* 2000). Many genomic regions have high levels of methylation, such as centromic and pericentromic regions. However, CpG islands and promoter regions are typically unmethylated (Weber & Schubeler 2007), and many regulatory elements such as transcriptional enhancers, have low methylation (Heintzman *et al.,* 2007).

1.3.2. Molecular mechanisms for epigenetic silencing

DNA methyltransferases (DNMTs) are catalysts for CpG island promoter methylation in tumour suppressor genes. For DNA methylation to successfully take place, DNMTs must use the methylated strand from hemi-methylated DNA as a template to add a methyl group to the cytosine base on the opposite strand (Okano *et al,* 1998). DNA methylation in mammals is established via a complex interplay between at least three active DNMTs; DNMT1, DNMT3a and DNMT3b. The latter two are responsible for establishing *de novo* DNA methylation during development (Okano *et al,* 1998). DNMT1 is the most abundant methyltransferase in somatic cells. Its key role in copying methylation patterns to daughter cells during DNA replication has deemed it as the "maintenance" methyltransferase (Espada *et al.,* 2004).

The critical role of DNA methylation during development has been illustrated in mouse models. Embryos deficient in DNMT3a and DNMT3b lack *de novo* methylation, whereas deletion of *DNMT1* results in embryonic lethality, as well as activation of both X-chromosomes in females (Li *et al.,* 1992). Defects in DNA methylation patterns and aberrantly expressed DNMTs are closely associated with multiple cancer types, though the mechanisms underlying this link remain obscure.

1.3.3. Epimutations and cancer

Epimutations are aberrations in transcriptional repression (or expression) of certain genes, that occur in the absence of change in the DNA sequence (Oey & Whitelaw, 2014). Epimutations are often referred to as epigenetic hallmarks of cancer, and are major contributors to carcinogenesis (Dobrovic & Kristensen, 2009; Hitchins *et al.,* 2007). Recently, extensive evidence has emerged proposing a direct role for DNA methylation in tumorigenesis (Subramaniam *et al.,* 2014; Baylin *et al.,* 2000; Esteller *et al.,* 2001). The first case of epimutation in relation to cancer was identified in the *retinoblastoma (RB)* gene (Greger *et al.,* 1989). Greger *et al.* (1989) reported that *RB1* promoter silencing by methylation contributed to oncogenesis, and these findings were confirmed in subsequent studies (Ohtani-Fujita *et al.,* 1993). Additional oncogenes associated with various forms of sporadic cancers, including *MLH1* and *BRCA1*, have since been found to exhibit DNA methylation (Suter *et al.,* 2004; Snell *et al.*, 2008), thus prompting focus on epimutation as a mechanism for tumorigenesis.

DNA hypomethylation (i.e. undermethylation) is an epigenetic event that is prevalent across many cancer types, and is usually observed in conjunction with hypermethylation, though the two epigenetic events occur in different genomic regions (Irizarry *et al,* 2009). The genome is often subject to global hypomethylation in association with cancer. Most hypomethylation within the genome is present in repetitive DNA elements (for example, retrotransposons and endogenous retroviral elements). In contrast, cancer-associated hypermethylation (i.e. overmethylation) is generally region specific, and is a common phenomenon observed in CpG islands in the promoter of tumour suppressor genes. Numerous studies have narrowed their focus on hypermethylation of tumour suppressor genes in hope of identifying DNA methylation biomarkers of cancer (Dobrovic & Simpfendorfer 1997, Chiang *et al.,* 2006, Hansmann *et al.,* 2012). However, evidence is mounting regarding the significance of hypomethylation and cancer, as this is characteristic

of genes vital for cancer progression and metastasis (Shteper *et al.,* 2003, Pakneshan *et al.,* 2004; Szyf, 2012). The aforementioned epigenetic events are areas of growing interest in light of cancer therapy and predisposition.

1.3.4. *BRCA1* – The Human Caretaker

The *BRCA1* gene is a thoroughly annotated tumour suppressor gene essential in maintaining genomic stability, including DNA double-strand break repair. Mutations in the *BRCA1* gene can give rise to breast cancer (and ovarian cancer). Only a small proportion of breast cancers (approximately 5%) are attributed to strong family history or a known genetic mutation, whereas sporadic breast cancer represents over 90% of breast malignancies (van der Groep *et al.,* 2006). Interestingly, a large number of breast cancers diagnosed in women in the absence of *BRCA1* mutation share the same morphological features of tumours in women with germline *BRCA1* mutations. As such, these tumours are referred to as "*BRCA1*-like" (Snell *et al.,* 2008). Though mutations are frequent among familial cases of breast cancer, *BRCA1* promoter methylation is prevalent in approximately 20% of sporadic breast cancers. Such findings have sparked interest regarding alternative mechanisms of *BRCA1* inactivation and breast cancer predisposition. Methylation of the *BRCA1* gene will be addressed in more detail in the sections below.

1.3.5. The *BRCA1* pseudogene

Disparity in findings throughout some of the literature may be assay- and methodology dependent, as well as reliant on the precise target region of interest. A factor that is often overlooked when targeting the *BRCA1* gene for promoter methylation analysis is the *BRCA1* pseudogene. The *BRCA1* pseudogene is also located on chromosome 17q21. This may partly explain the differences observed in human and mouse breast cancer models, where heterozygous *BRCA1* knockout mice fail to develop breast cancer, but human carriers do (Chambers & Solomon 1996). The pseudogene is heavily hypermethylated

(Chiang *et al.,* 2006), thus unintentional targeting of this gene will result in a false positive result for methylation, irrespective of the methylation status at the *BRCA1* locus in cancer patients, or healthy individuals. For this reason, careful consideration of primer design has been taken by some groups to ensure no inadvertent amplification of the *BRCA1* pseudogene (Dobrovic & Simpfendorfer 1997; Snell *et al.,* 2008).

1.3.6. *BRCA1* methylation in breast cancer

Breast tumours in women who carry a *BRCA1* germline mutation typically have distinct morphological features, including high mitotic index, trabecular growth pattern, little to no tubule formation, malignant nuclear grade, necrosis, nuclear pleomorphism, circumscribed growth pattern, pushing margins, and lymphocytic infiltration (Eisinger *et al.,* 1996; Southey *et al.*, 2011). The heterogeneity of breast carcinomas strongly influences therapeutic response and outcome, as well as disease progression and metastasis. Research has demonstrated that inter- and intra-tumour heterogeneity involves multiple factors, including germline mutations and epigenetic modifications (Catteau *et al.,* 1999). DNA promoter methylation, an epigenetic modification that continues to be explored in relation to many cancers, is rapidly becoming an invaluable epigenetic marker in breast cancer detection, treatment and prognosis. DNA methylation of CpG islands within the promoter of tumour suppressor genes, particularly *BRCA1*, is a phenomenon that is observed in sporadic breast tumours (Bianco *et al.,* 2000).

Numerous studies have shown compelling evidence between decreased *BRCA1* protein expression and aberrant methylation of CpG islands within the *BRCA1* promoter (Hsu *et al.,* 2013; Birgisdottir *et al.,* 2006). Such findings illustrate the significant role played by DNA methylation in silencing transcription of the *BRCA1* gene, and its contribution to breast carcinogenesis.

Methylation status of promoter regions in tumour suppressor genes has been elucidated in a number of studies comparing breast cancer patients and healthy women (Dobrovic and Simpfendorfer 1997; Wong *et al.*, 2011; Vos *et al.*, 2017). *BRCA1* promoter methylation was detected in peripheral blood DNA of patients diagnosed with sporadic breast cancer, as opposed to healthy controls who did not have detectable *BRCA1* methylation. DNA methylation that is detectable in healthy tissue is known as constitutional methylation, and will be further discussed in the sections to follow.

1.4. Constitutional Methylation

1.4.1. Constitutional *BRCA1* methylation in breast cancer

Constitutional methylation is an aberration in gene expression that is detectable in normal tissues, either in a complete or mosaic fashion (Wong *et al.,* 2011). This epigenetic event is rapidly being considered as an alternative to gene mutation for cancer predisposition. An association between constitutional methylation of *BRCA1* and greater risk of breast cancer development has been postulated (Dobrovic and Simpfendorfer 1997; Wong *et al.,* 2011; Gupta *et al.,* 2014). Detection of this epigenetic aberration is feasible in peripheral blood, and may prove to be a powerful, yet non-invasive approach to breast cancer diagnosis, prognosis and predisposition.

Breast cancer is commonly recognised as a genetic disease, however, overwhelming research has shown that aberrant DNA methylation is present in some breast cancer cases. Over the past decade, detectable methylation of tumour suppressor genes in normal human tissue has been identified (Gazzoli *et al.,* 2002; Wong *et al.,* 2011).

A study of 255 women under the age of 40 diagnosed with breast cancer in the absence of a *BRCA1* germline mutation, examined levels of *BRCA1* promoter methylation detectable in peripheral blood (Wong *et al.,* 2011). Two independent, yet complementary

methodologies were implemented to ascertain methylation levels among these women; i) MethyLight, a highly quantitative and sensitive method for methylation detection based on TaqMan probes, and ii) Methylation-Sensitive High Resolution Melting (MS-HRM). The latter is a semi-quantitative assay used in the present study to validate results obtained by MethyLight.

Wong and colleagues (2011) reported that women presenting with five or more *BRCA1* mutation-associated morphological features had high *BRCA1* promoter methylation (30.8%) when compared to unaffected controls (4% *BRCA1* methylation frequency). Women with less than five of the distinct morphological features had detectable promoter methylation in peripheral blood, between 5-10%. The variation in levels of detectable methylation in peripheral blood of affected women ($p = 0.000002$) versus control women (0.004) was highly significant, suggesting a strong association between detectable *BRCA1* methylation in normal tissue (i.e. peripheral blood) and risk of breast cancer.

Earlier research had also demonstrated that *BRCA1* promoter methylation was present in a mosaic fashion in somatic tissue of some breast cancer patients (Snell *et al.,* 2008). Both peripheral blood and buccal mucosa DNA were assessed for *BRCA1* methylation using three independent methods; MethyLight, MS-HRM, and digital MS-HRM (an adaptation of MS-HRM that quantitates the number of methylated and unmethylated alleles). Two patients were found to have extremely low *BRCA1* methylation in their peripheral blood (1%), though tumour DNA from these women were close to 100% methylated. Interestingly, one control individual displayed exceptionally low levels of peripheral blood methylation (0.1%). These findings demonstrate that even remarkably low levels of *BRCA1* methylation detected in normal tissue may be telling of potential future breast cancer diagnosis. Additional research also analysed constitutional *BRCA1* promoter methylation in peripheral blood in a case-control study, and found that methylation was frequently

detected in patients in the absence of a *BRCA1* mutation, whereas *BRCA1* mutation carriers did not show any *BRCA1* promoter methylation (Gupta *et al.,* 2014).

Further work has also shown that constitutive (soma-wide) *BRCA1* promoter methylation is evident in early-onset sporadic breast cancers, in mutation negative patients (Hansmann *et al.,* 2012). Like many studies, peripheral blood was the tissue of choice when assessing levels of methylation, as collection of blood is generally considered non-invasive and is readily available. Bisulfite pyrosequencing was used to screen over 600 mutation-negative patients for hypermethylation of several tumour suppressor genes, including *BRCA1*. Bisulfite pyrosequencing enables almost exact quantification of methylation at individual CpG sites with high accuracy, making it an exceedingly valuable tool for epimutation screening. Patients with putative epimutations (i.e. over 6% promoter methylation) determined by bisulfite pyrosequencing were subjected to further analysis by bisulfite plasmid sequencing for identification of hypermethylated alleles. Findings illustrate that *BRCA1* promoter methylation in blood cells occurred in a mosaic manner, consistent with preceding work (Kristensen *et al.*, 2012). This methylation event seems to be a frequent "first hit" in sporadic breast cancers, similar to that of an inherited germline mutation (Baylin 2005).

More recently, constitutional *BRCA1* methylation was assessed in white blood cells of breast cancer patients and cancer-free women (Al Moghrabi *et al.,* 2014). *BRCA1* promoter methylation was present in white blood cells of over 14% of women with breast cancer, and most of these women also displayed *BRCA1* methylation in paired tumour DNA. These results suggest that methylation of *BRCA1* in white blood cells may prompt breast carcinogenesis. Interestingly, more than 9% of healthy women had detectable *BRCA1* promoter methylation in their white blood cells, which may be suggestive of an elevated risk of breast cancer development.

Most studies choose peripheral blood as the ideal normal somatic tissue for DNA methylation analysis of the promoter region of tumour suppressor genes (Dobrovic & Simfendorfer 1997; Snell *et al.,* 2008; Wong *et al.,* 2011). While blood is a convenient tissue for DNA methylation analyses especially in the context of cancer, it is important to note that analysis of such epigenetic events in alternative healthy tissues including normal breast tissue, as well as peripheral blood and the tumour itself, would likely provide valuable insight and shed light on the subject of constitutional methylation of tumour suppressor genes as an early predictor of breast cancer.

Though evidence is mounting in support of constitutional *BRCA1* methylation (as well as other tumour suppressor genes) as a cancer predisposition factor, conflicting research exists. Women with a strong family history of breast cancer but are negative for *BRCA1* and *BRCA2* germline mutations were assessed for *BRCA1* promoter methylation in their peripheral blood leukocytes (Chen *et al.,* 2006). Interestingly, no significant variance was found in promoter methylation between healthy controls and women with hereditary cancer, hence prompting the conclusion that epimutations are an unlikely explanation for hereditary breast cancer in women without *BRCA1/2* germline mutations.

Chen and colleagues (2006) applied bisulfite sequencing to determine the methylation status of 30 CpG sites in the *BRCA1* promoter, and only 9 of the 11 clones were sequenced for each sample. Although a CpG was only considered methylated when at least 20% of clones were methylated, using such a minimal number of clones is inadequate when inferring that *BRCA1* promoter methylation detected in healthy tissue is dissociated with increased breast cancer risk in women who lack germline mutation. A secondary independent technique should have been implemented in this case, to solidify and validate results attained.

The frequency of constitutional *BRCA1* methylation detected in peripheral blood is low in breast cancer patients, and methylation at low levels (below 5%) can be difficult to accurately quantify. For this reason, highly sensitive methodologies are pivotal when detecting low level methylation. Droplet digital PCR (ddPCR) is a highly sensitive droplet generation method based on fractionation of a sample into ~20,000 droplets, and provides absolute quantification of methylated and unmethylated target DNA. ddPCR can detect methylation at a single-molecule level, and therefore requires very little DNA input. Further approaches to assess tumour suppressor gene methylation in apparently normal tissue are based on Next-generation sequencing (NGS) techniques. NGS allows for precise quantification of DNA methylation, and can be applied for population based studies. Various methodologies used for DNA methylation analyses are outlined in Table 1-3.

Table 1-3. Comparison of methods for DNA methylation analysis. Characteristics of common methodologies used to analyse DNA methylation are outlined below. +++ defines the best sensitivity or specificity of a method (+++ > ++ > +).

Method	Coverage	Sensitivity	Specificity	Starting material	Tissue type	References
MS-HRM (Methylation Sensitive High Resolution Melting)	Gene-specific	+++	++	25-1000 ng	Colorectal carcinomas	Wojdacz and Dobrovic 2007
LUMA (Luminometric based Assay)	Genome-wide	++	++	250-500 ng	Colon cancer cell lines	Karimi *et al.,* 2006
Whole Genome Bisulfite Sequencing (WGBS)	Genome-wide	+++	+++	50-100 ng	CD4/CD8 T cells, CD184+ cells, primary adult liver tissue, brain cortex tissue, human ESCs	Ziller *et al.,* 2015; Gatzmann and Lyko 2019
LINE-1 + Pyrosequencing	17% of genome	+++	++	50 ng	61 cancer cell lines, 60 colorectal carcinomas and adjacent normal colorectal tissue	Estécio *et al.,* 2007
HumanMethylation450 BeadChip array	482,000 CpG sites (99% of known genes)	+++	+++	0.5-1 µg	Infinium HumanMethylation 450K BeadChip (485,577 sites)	Marabita *et al.,* 2013
Droplet digital PCR (ddPCR)	Gene-specific	+++	+++	1-120,000 copies/20 µl reaction	N/A	Droplet Digital™ PCR Applications Guide

1.4.2. Constitutional methylation in monozygotic twin models

Epigenetic analysis on MZ twins provides an understanding into epigenetic effects of DNA methylation in complex human disease, including cancer. Research of this nature is valuable in identical twins, given that a genetically identical state is obtained at every allele. This allows researchers to determine whether DNA methylation at certain genes is intrinsically variable at certain loci within twin pairs. Furthermore, MZ twins have the unambiguous division of maternal inheritance, and serve as matched controls for many environmental factors.

Gene expression and repression via epigenetic modifications may explain, in part, why monozygotic (MZ) twins can be discordant for levels of DNA methylation. Despite having a shared intrauterine environment during development, there is evidence demonstrating that MZ twins discordant for certain diseases including cancer, have variable methylation levels at key genomic regions associated with their disease (Kaminsky *et al*., 2009).

Hannon *et al.* (2018) quantified genome-wide DNA methylation in whole blood of MZ twins aged 18 years (n = 426 pairs) and dizygotic (DZ) twins (n = 306 pairs), in attempt to characterise the genetic and environmental determinants of variations in DNA methylation. They report that site-specific DNA methylation levels was more strongly correlated between MZ twins compared to DZ twins.

In the context of breast cancer, Heyn and colleagues (2012) analysed DNA methylation in the blood of 15 MZ twin pairs discordant for breast cancer, using high-resolution 450k analysis. They identified that the *DOK7* gene promoter is hypermethylated in the blood years before cancer diagnosis, and that *DOK7* hypermethylation may serve as a blood-based, breast cancer-specific epigenetic biomarker.

Research of this nature can help determine the molecular mechanisms of disease, and define the extent of environmental influence. This may, in turn lead to the treatment and possible prevention of complex diseases.

1.5. Methylation markers in melanoma

1.5.1. *RASSF1A* promoter methylation in melanoma

RASSF1A is a tumour suppressor gene responsible for vital cellular processes including inducing apoptosis, regulating cell cycle and mitosis, and maintaining microtubule stability (Chow *et al.,* 2012). *RASSF1A* gene expression is low in various cancer cells, including lung, liver and breast cancers (Dammann *et al.,* 2000; Yu *et al.,* 2002; Agathanggelou *et al.,* 2001). Earlier studies attributed the low *RASSF1A* expression in cancer cells to DNA methylation of the promoter. Consequently, a link between methylation of the *RASSF1A* promoter and tumour formation has since been demonstrated in numerous studies (Jiang *et al.,* 2012; Liu *et al.,* 2013; 2014; Ge *et al.,* 2014).

Hypermethylation of *RASSF1A* has been observed at significantly lower levels in primary melanomas compared to metastatic melanomas (Hoon *et al.,* 2004). Hoon and colleagues (2004) reported a 42% increased rate of *RASSF1A* methylation in metastatic melanomas versus primary tumours. Hoon *et al.* (2004) report that only 15% of primary tumours had *RASSF1A* methylation; however, the number of primary tumours analysed were limited. These findings suggest that methylation of the *RASSF1A* gene may be acquired during tumour progression.

In 2005, Mori *et al.* analysed methylation of multiple cancer-related genes by methylation-specific polymerase chain reaction (MS-PCR) in serum DNA of patients receiving treatment for metastatic melanoma (n = 47). Patients were classified as either responders (n = 23) if they showed complete or partial response to treatment, or non-responders (n =

24) if they experienced disease progression. Mori and colleagues (2005) found that patients who responded to treatment had a significantly lower frequency of *RASSF1A* methylation detected in the blood (3/23 patients; 13%) compared to non-responders (10/24 patients; 42%). Melanoma patients with *RASSF1A* methylation (or an additional methylated gene) had significantly worse overall survival (P = 0.013, and 0.01 respectively) than patients who lack methylation in tumour-related genes. Additionally, *RASSF1A* methylation was the only methylated gene found to have a significant association with overall survival and response to treatment (risk ratio, 2.38; 95% CI, 1.16 to 4.86; P = 0.018; odds ratio = 0.21; 95% CI, 0.05 to 0.90; P = 0.036).

Reactivation of *RASSF1A* expression in cancer cells by inhibiting DNA methylation has also been explored (Dammann *et al.,* 2017). Natural compounds classed as polyphenols have been shown to inhibit DNMT (Campbell and Collett, 2005; Shu *et al.,* 2011), thus enhancing *RASSF1A* expression by decreasing *RASSF1A* promoter methylation (Du *et al.,* 2012). These findings demonstrate the clinical utility of *RASSF1A* methylation in identifying patients who are likely to respond to certain anti-cancer treatment.

1.5.2. *RARβ* promoter methylation in melanoma

The retinoic acid receptor beta (*RARβ*) gene is implicated in cellular signalling processes during embryonic morphogenesis, cell differentiation and cell growth (Rasmussen *et al.,* 2018). Loss of *RARβ* expression through aberrant DNA methylation has been observed in many cancer types including melanoma, prostate cancer and breast cancer, and its suppression is associated with cancer initiation and progression (Nesvet *et al.,* 2019).

Melanoma methylation frequencies of *RARβ* have been observed at rates of up to 70% in both primary and metastatic melanoma (Hoon *et al.,* 2004). Hoon and colleagues (2004) also reported a significant association between hypermethylated *RARβ* and increased

primary tumour thickness; an important feature recognised as a crucial prognostic factor in early stage melanoma (Bostick *et al.,* 1999).

More recently, de Unamuno Bustos *et al.* (2018) analysed 170 formalin-fixed paraffin-embedded melanoma tumour samples. They assessed methylation in a panel of tumour suppressor genes, and found that *RARβ* was the most prevalently methylated, in 31% (53/170) of tumours. Additionally, the rate of *RARβ* methylation showed a significant age-association, with methylation increasing with age ($P < 0.001$, OR = 40 (95% CI 21–76) (de Unamuno Bustos *et al.,* 2018). Once clinicopathological features were considered, aberrant methylation of the promoter of tumour suppressor genes, including *RARβ,* was associated with aggressive tumour pathology and poorer overall survival (de Unamuno Bustos *et al.,* 2018). Collectively, these findings provide additional insight into DNA methylation and its influence on pathogenesis in melanoma. They also highlight the potential of *RARβ* methylation as a prognostic marker in the clinical setting.

1.6. Study Rationale, Hypotheses and Aims

Based on a review of the literature regarding constitutional DNA methylation of cancer-associated genes in cancer, it is likely that constitutional methylation detected in peripheral blood of healthy individuals will play a significant role as a non-invasive method of identifying individuals who may be predisposed to developing a certain cancer type. In light of the finding that constitutional methylation is detected in peripheral blood of cancer patients and their matched tumour DNA (outlined in section 1.3), expanding on this research will further our understanding of this epigenetic event to determine the role of somatic tumour suppressor gene methylation in the development of particular cancers. As such, the primary objective of this PhD research project is based on understanding the role of constitutional methylation in cancer predisposition.

DNA methylation of tumour suppressor genes has been identified as an initiating event in various cancer types. Despite common mutations associated with breast cancer and melanoma, cancer-associated methylation markers including *BRCA1* in breast cancer, has been identified in peripheral blood and matching primary tumours of women. As outlined in **section 1.5**, *RASSF1A* and *RARβ* methylation have been detected in primary and metastatic melanoma lesions, with *RASSF1A* methylation associated with later stage disease.

This research project analysed constitutional DNA methylation in the context of breast cancer and melanoma. The aims and hypotheses for this thesis are as follows:

Breast Cancer

HYPOTHESIS 1. Individuals with *BRCA1* methylated tumours will have detectable *BRCA1* methylation in peripheral blood.

Aim 1A. To establish the frequency of *BRCA1* methylation in peripheral blood of women with breast cancer and healthy women, to further understand the tole of constitutional methylation of tumour suppressor genes in cancer predisposition.

Aim 1B. To quantify *BRCA1* methylation in breast tumours of women with breast cancer who have detectable peripheral blood methylation.

Aim 1C. To examine the link between the degree of peripheral blood *BRCA1* methylation and breast cancer predisposition. This study is presented in Chapter 4 of this thesis.

HYPOTHESIS 2. Monozygotic twin pairs will be concordant for *BRCA1* promoter methylation if there is a strong genetic basis.

Aim 2. To understand the role of genetics versus environment on DNA methylation and disease predisposition using a monozygotic twin model to test for constitutional *BRCA1* methylation. This study is presented in Chapter 5 of this thesis.

Melanoma

HYPOTHESIS 1. Melanoma patients with *RASSF1A* and/or *RARβ* methylation in primary melanoma tumours will have detectable constitutional methylation in peripheral blood DNA.

Aim 1A. To determine the frequency of *RASSF1A* and *RARβ* methylation in primary melanoma tumours. This study is presented in Chapter 6 of this thesis.

Aim 1B. To examine the link between tumour methylation and peripheral blood methylation in matched patient tissue samples.

CHAPTER 2.

MATERIALS AND METHODS

2.1. Accrual of monozygotic twin samples

2.1.1. Buccal mucosa and white blood cell DNA for *BRCA1* methylation studies

DNA from buccal mucosa of 73 female monozygotic (MZ) twin pairs was used for constitutional *BRCA1* methylation analysis. Buccal swabs from these 73 twins were collected at birth. Additional buccal DNA samples were provided for selected twin pairs (n = 9 pairs) at 6 years old. Methylation analysis was performed on an alternative tissue (white blood cell DNA from the umbilical cord) and was provided for twins who tested positive for methylation in buccal mucosa DNA at birth (n = 18 pairs)**.** Buccal mucosa DNA of male MZ twin pairs (n = 15 pairs) was also provided and assessed for *BRCA1* methylation (see Chapter 4**)**.

Genomic DNA for all twin samples was extracted and kindly provided by Associate Professor Jeffrey Craig and Dr Jane Loke (Murdoch Children's Research Institute, Melbourne, Australia) for *BRCA1* methylation analysis (Saffery *et al.,* 2012).

2.2. Accrual of melanoma samples

2.2.1 Formalin-Fixed Paraffin Embedded tissue (FFPE) and blood cell pellets for *RASSF1A* and *RARb* methylation studies

Primary melanoma FFPE tumour tissue blocks from 25 patients were obtained from Melanoma Research Victoria (MRV). All patients provided informed consent for their tissue to be used for the purpose of research, and are enrolled in the MRV study. Matched

blood cell pellets for each patient were also provided to the Translational Genomics and Epigenomics Laboratory.

Methylation analysis was performed on all FFPE tumour tissue and blood cell pellets. All samples were kindly provided by Sonia Mailer (MRV, Peter MacCallum Cancer Centre, Melbourne, Australia).

All melanoma FFPE tumour tissue samples were microscopically reviewed and confirmed by an Anatomical Pathologist, Dr Louise Jackett, at Austin Health (Austin Hospital, Melbourne, Australia). Melanoma sections with highest tumour purity were specified by Dr Louise Jackett prior to microdissection and methylation analysis.

2.3. DNA from the LifePool Project

2.3.1. Whole blood DNA from healthy women and women with breast cancer for *BRCA1* methylation analysis

LifePool is a resource that collects DNA from a subset of women who were mammographically screened through BreastScreen Victoria. All women involved in the LifePool project have provided informed consent. Women eligible for free mammography in Australia must be 40+ years of age, and can access the LifePool project consent forms from http://www.lifepool.org/forms.htm to donate tissue contributing to breast cancer research. Women can also join the LifePool project through the National Breast Cancer Foundation's research database, Register4 (www.register4.org.au).

Peripheral blood samples are collected from women at the time of mammography. Additional tumour tissue remaining from biopsy or surgery are provided to LifePool from pathology laboratories once diagnosis is confirmed and treatment is planned. All personal patient information is deidentified to researchers.

DNA from whole blood of 327 healthy women (controls) and 300 women with breast cancer (cases) were obtained from LifePool and used for *BRCA1* methylation analysis. Cases and controls were aged between 29 and 86 years at the time of blood donation, and both cohorts were age-matched.

Genomic DNA for all LifePool samples were extracted by LifePool, and kindly provided by Lisa Devereux (LifePool) at a concentration of 50 ng/µL. Prior to methylation analysis, 500 ng of genomic DNA was subjected to bisulfite modification (see section 2.4.3).

2.4. DNA bisulfite conversion

2.4.1 EZ DNA Methylation-Lightning kit (Zymo Research)

Most of the DNA bisulfite conversions described in this thesis were performed using the EZ DNA Methylation Lightning kit (200 preps, Zymo Research) (Cat no. D5031). However, occasionally when specified, the EZ DNA Methylation Lightning kit (2x96 preps, Zymo Research) (Cat no. D5032) was used.

2.4.2. EZ DNA Methylation Lightning kit (200 preps) (Zymo Research)

In clear 0.2 mL 8-tube PCR strips (Bio-Rad) (Product no. TLS0801) PCR-grade water was added to the required volume of DNA, creating a final reaction volume of 20 µL. 130µL of Lightning Conversion Reagent was then added to each reaction, followed by mixing by pipetting up and down five times. The PCR tubes were centrifuged and placed in the C1000 Touch^{TM} thermal cycler (Bio-Rad). Cycling conditions were as follows: (1) denaturation at 98^{o}C for 8 minutes, (2) incubation at 54^{o}C for 60 minutes, (3) hold at 4^{o}C for up to 20 hours.

Following cycling, bisulfite modified DNA was cleaned up by adding 600 µL of M-Binding Buffer to a Zymo-Spin^{TM} IC Column placed into a provided Collection Tube. The bisulfite converted DNA was added into the Zymo-Spin^{TM} IC Column containing M-Binding

Buffer, and inverted five times to mix. All columns were centrifuged for 1 minute at 2424,000 x gx g. Flow through was discarded and spin columns were placed back into collection tubes. 100 µL of M-Wash Buffer was added to each spin column, and centrifuged for 1 minute at 1424,000 x g . 200 µL of L-Desulphonation Buffer was added to each spin column and incubated at room temperature for 20 minutes. Spin columns were centrifuged for 1 minute at 2424,000 x gx g. 200 µL of M-Wash Buffer was added to the spin columns and centrifuged for 1 minute at 24,000 x g. This step was repeated once. Following centrifugation, spin columns were transferred into new collection tubes and a dry spin was performed for 1 minute at 2424,000 x gx g. Collection tubes were discarded, and spin columns were transferred into new Eppendorf tubes. 10 µL of M-Elution Buffer was added directly onto the membrane of each spin column and incubated at room temperature for 5 minutes. Spin columns were centrifuged for 1 minute at 24,000 x g. The elution step was repeated once to obtain a final elution volume of 20 µL of bisulfite converted DNA. DNA was stored at 4^{o}C for short-term storage, or -80^{o}C for long-term storage (more than two months).

2.4.3. EZ-96 DNA Methylation Lightning kit (Shallow-Well, 2x96 preps) (Zymo Research)

The following protocol outlines the process of bisulfite conversion using the EZ DNA Methylation Lightning kit (2x96 preps, Zymo Research) (Cat no. D5032).

In the Conversion Plate provided, 20 µL of genomic DNA was added to 130 µL of Lightning Conversion Reagent. Where the volume of DNA was less than 20 µL, PCR-grade water was added to create a final reaction volume of 20 µL. Samples were mixed by pipetting up and down 5 times, and the conversion plate was sealed with the provided film. The conversion plate was centrifuged and placed in the C1000 Touch^{TM} thermal cycler

(Bio-Rad). Cycling conditions were as follows: (1) denaturation at 98ºC for 8 minutes, (2) incubation at 54ºC for 60 minutes, (3) hold at 4ºC for up to 20 hours.

Following cycling, bisulfite modified DNA was cleaned up by adding 400 µL of M-Binding Buffer to the wells of a Silicon-A^{TM} Binding Plate mounted on a Collection Plate. The bisulfite converted DNA was then added into the wells of the Silicon-A^{TM} Binding Plate containing M-Binding Buffer. Samples were mixed by pipetting up and down 5 times, followed by centrifugation for 3 minutes at 3000 x *g*. Flow through was discarded.

After centrifugation, 400 µL of M-Wash Buffer was added into each well of the plate followed by centrifugation for 5 minutes at 3000 x *g*. 200 µL of L-Desulphonation Buffer was then added to each well and incubated at room temperature for 20 minutes. Following incubation, plates were centrifuged for 5 minutes at 3000 x *g* and flow through was discarded.

400 µL of M-Wash Buffer was added into each well of the plate followed by centrifugation for 5 minutes at 3000 x *g*. Flow through was discarded. An additional 400 µL of M-Wash Buffer was added into the wells and plates were centrifuged for 10 minutes at 3000 x *g*.

Following centrifugation, the Silicon-A^{TM} Binding Plate was placed onto an Elution Plate. 30 µL of M-Elution Buffer was added directly to each well. The plate was incubated at ambient temperature for 10 minutes, then centrifuged for 3 minutes at 3000 x *g*. Bisulfite converted DNA was stored at 4ºC for short-term storage, or -80ºC for long-term storage (more than two months).

2.5. DNA extraction

2.5.1. DNA extraction from whole blood

DNA was extracted from whole blood using the DNeasy® Blood & Tissue Kit (Qiagen, Cat no. 69506). 36 µL of Proteinase K (provided) was added into labelled 1.5 mL screw cap tubes for each sample and NEC. 100 µL of whole blood was transferred into the corresponding 1.5 mL screw cap tube (Neptune, Cat no. 3744.S.X) containing Proteinase K, and into each NEC. 100 µL of PBS (1X) (Life Technologies) was added into each screw cap tube containing whole blood, into each NEC. Each screw cap tube containing solution was mixed by vortexing for 10 seconds then centrifuged for 2 seconds.

200 µL of Buffer AL was then added into each 1.5 mL screw up tube. Each solution was mixed by vortexing for at least 15 seconds (until solution appears homogenous) then centrifuged for 2 seconds. Each 1.5 mL screw cap tube containing cell line samples and NEC was placed in an incubation oven at 56ºC for at least 30 minutes, but no longer than 24 hours.

Following the incubation, all extracted DNA was cleaned up. Buffer AE was added to a microcentrifuge tube and incubated at 72ºC on a block heater. The amount of Buffer AE was calculated by adding 100 µL of Buffer AE for each sample and NEC, and an additional 20 µL of Buffer AE.

The 1.5 mL screw cap tubes were removed from the incubation oven and cooled at ambient temperature for 5 minutes. After cooling, all screw cap tubes were pulse vortexed for 5 seconds and centrifuged for 2 seconds. 200 µL of molecular-grade absolute Ethanol was added to each sample and NEC, followed by vortexing for 15 seconds and centrifugation for 1 second.

The contents of the 1.5 mL screw cap tube of each sample and NEC were transferred into the corresponding DNeasy® Spin Columns, placed in provided Collection Tubes. Spin

columns were centrifuged at 7,800 x g for 1 minute. Collection tubes were discarded and spin columns were placed into new collection tubes.

500 µL of Buffer AW1 was added to each spin column, and centrifuged at 7,800 x g for 1 minute. Collection tubes were discarded and spin columns were placed into new collection tubes.

500 µL of Buffer AW2 was added to each spin column, and centrifuged at 7,800 x g for 1 minute. Collection tubes were discarded and spin columns were placed into new collection tubes, followed by centrifugation at 2424,000 x gx g for 3 minutes.

Collection tubes were discarded, and spin columns were placed into clean 1.5 mL microcentrifuge tubes. 50 µL of pre-warmed Buffer AE was placed directly onto the centre of each spin column. Spin column caps were closed and incubated at ambient temperature for 5 minutes. Following incubation, spin columns were centrifuged at 24,000 x g for 3 minutes. Spin columns were discarded.

Extracted DNA was stored at 4ºC for short term storage (no more than 3 months) and at -80ºC for long term storage.

2.5.2. DNA extraction from buffy coat

DNA extraction from buffy coat was performed using the DNeasy® Blood & Tissue Kit (Qiagen, Cat no. 69506). The protocol for extracting DNA from buffy coat is defined in section 2.5.1, however 100 µL of buffy coat was used in place of whole blood.

2.5.3. DNA extraction from plasma

DNA extraction from plasma was performed using the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Cat no. 55114). A single 50 mL Falcon tube (In Vitro Technologies, Cat no.

FAL352070) was prepared for each plasma sample and NEC. The volume of reagents outlined in the following protocol is for extraction of DNA from 4 mL of plasma.

Prior to commencing the plasma DNA extraction, Buffer ACL and Carrier RNA mix was made in a 50 mL Falcon tube, according to Table 2-1 below:

Table 2-1. Buffer ACL and Carrier RNA concentrations for DNA extraction from plasma.

Number of Plasma Samples	Buffer ACL (mL) for 4 mL of Plasma	Carrier RNA (µL)
1	3.5	5.6
2	7.0	11.3
3	10.5	16.9
4	14.0	22.5
5	17.5	28.1
6	21.0	33.8
7	24.5	39.4
8	28.0	45.0
9	31.5	50.6
10	35.0	56.3

400 µL of Proteinase K was pipetted into each 50 mL Falcon tube, followed by 4 mL of plasma into the corresponding Falcon tubes. 3.2 µL of Buffer ACL/carrier RNA mix was added to each Falcon tube and NEC. The cap was closed and tubes were vortexed for 30 seconds using the Ratek Personal Vortex Mixer (Ratek, Model no: VM1), followed by incubation at 56ºC for 40 minutes.

Falcon tubes were removed from the incubator and 7.2 mL of Buffer ACB was added into each sample and NEC. Caps were closed and Falcon tubes were mixed by vortexing for 30 seconds. All tubes were incubated on ice for 5 minutes.

The QIAvac 24 vacuum manifold (Qiagen, Cat no. 19413) was set up according to the manufacturer's instructions. The lysate-Buffer ACB mixture was applied in the tube extender of the QIAamp Mini Column. Vacuum force was applied to draw lysate through

the column. Once all lysates have been drawn through the column completely, the vacuum valve was closed and pressure was released from the vacuum system using the Vacuum Regulator.

Once all lysates were drawn, 600 µL of Buffer ACW1 was added to the QIAamp Mini Column. The caps of the columns were left open, and vacuum force was applied. After all of Buffer ACW1 was drawn through the column, the vacuum valve was closed and pressure was released using the Vacuum Regulator.

750 µL of Buffer ACW2 was then added to the QIAamp Mini Column. With the cap of the column open, vacuum force was applied. Once all of the buffer was drawn through the column, the vaccum valve was closed and pressure was released using the Vacuum Regulator.

750 µL of absolute ethanol was added into the QIAamp Mini Column. The column caps were left open and vacuum force was applied. Once all ethanol had been drawn through the column, the vacuum valve was closed to release pressure using the Vacuum Regulator. Once pressure was released, the spin column caps were closed.

Each QIAamp Mini Column was placed into a new 2 mL collection tube, and centrifuged at 24,000 x g for 3 minutes. Following centrifugation, spin columns were transferred to clean collection tubes and incubated at 56^{o}C for 10 minutes on a heat block. Caps were left open, to ensure the membrane was completely dry.

Following the 10 minute incubation, each QIAamp Mini Column was placed in a clean pre-labelled 1.5 mL elution tube. 50 µL of Buffer AVE was added to the centre of the QIAamp Mini membrane. Caps were closed and tubes were incubated at ambient temperature for 3 minutes. After the incubation, elution tubes were centrifuged for 3 minutes at 24,000 x g.

Following the centrifugation, the 50 µL of Buffer AVE was re-eluted to the centre of the QIAamp Mini membrane, and incubated for 3 minutes at ambient temperature with caps closed. The QIAamp Mini column was then centrifuged at 24,000 x g for 3 minutes, and then the column was discarded. Eluted plasma DNA was stored at 4ºC for short term storage (no longer than 1 month), and at -15ºC to -25ºC for long term storage.

2.5.4. DNA extraction from Formalin-Fixed Paraffin Embedded (FFPE) tissue

DNA extraction from Formalin-Fixed Paraffin-Embedded (FFPE) tissue was performed using the DNeasy® Blood & Tissue Kit (Qiagen, Cat no. 69506).

Into pre-labelled 1.5 mL screw cap tubes, 100 µL of Buffer ATL was added into each sample and NEC. Methyl green sections of a sample were oriented to the corresponding H&E section and marked tumour (or normal) area. Each methyl green section had a border scratched around the marked tumour or normal tissue using a needle and the corresponding H&E section. This approach was used to minimise sample 'contamination' with normal cells.

Depending on the size of the tissue area, 1 – 5 µL of Buffer ATL was pipetted onto the tissue area to be scraped, in order to promote clumping of tissue particles. The tumour-enriched or normal cell material from the marked area was scratched and transferred into the corresponding 1.5 mL screw cap tube containing Buffer ATL. This step was repeated until methyl green sections of a sample were macrodissected.

Once all tissue had been macrodissected, solutions were vortexed for 5 seconds and centrifuged for 1 second. Each 1.5 mL screw cap tube was then placed in a heat block at 93ºC for 15 minutes.

Following the incubation, screw cap tubes were cooled at ambient temperature for 5 minutes and centrifuged for 1 second. 36 µL of Proteinase K was added to each sample and NEC, and all tubes were vortexed for 15 seconds then centrifuged for 2 seconds.

Each 1.5 mL screw cap tube containing and NEC was placed in an incubation oven at 56ºC for at least 12 hours.

Following the incubation, all samples and NEC were removed from the oven, and vortexed for 5 seconds followed by centrifugation for 2 seconds. 110 µL of Buffer AL was added to each sample and NEC, followed by a 15 second vortex and 2 second centrifugation. Each tube was then placed in a heat block at 72ºC for 10 minutes.

Following incubation, tubes were cooled at ambient temperature for 5 minutes and centrifuged for 1 second. 110 µL of molecular grade absolute ethanol was then added to each sample and NEC. Each solution was pulse vortexed for 15 seconds and centrifuged for 1 second.

The contents of the 1.5 mL screw cap tubes were transferred into pre-labelled corresponding spin columns, and spun for 1 minute at 7,800 x g. Collection tubes were discarded and spin column were placed into new collection tubes.

280 µL of Buffer AW1 was added to each spin column, and columns were centrifuged for 1 minute at 7,800 x g. 280 µL of Buffer AW2 was then added into each spin column, and columns were centrifuged for 1 minute at 7,800 x g.

Collection tubes were discarded with flow-through and spin columns were placed into new collection tubes, followed by centrifugation for 3 minutes at 24,000 x g.

Collection tubes were discarded and spin columns were placed into clean 1.5 mL microcentrifuge tubes. 50 µL of Buffer AE pre-warmed at 72ºC was added onto the centre

of each spin column. Caps were closed and spin columns were incubated at ambient temperature for 5 minutes. Following incubation, spin columns were centrifuged for 3 minutes at 7,800 x g.

Buffer AE was re-eluted onto the centre of the corresponding spin column, and incubated for 5 minutes at ambient temperature. Columns were centrifuged for 3 minutes at 24,000 x g, and spin columns were discarded.

Eluted FFPE DNA was stored at 4^{o}C for short term storage (no longer than 3 months), and at -80^{o}C for long term storage.

2.5.5. DNA extraction from cell pellet

DNA extraction from cell pellets was performed using the DNeasy® Blood & Tissue Kit (Qiagen, Cat no. 69506). For each 1.5 mL screw cap tube containing the cell pellet, 20 µL of Proteinase K was added. This step was replicated for the NEC. Solutions were vortexed for 10 seconds and centrifuged for 2 seconds.

200 µL of PBS (1X) (Life Technologies) was added to each sample and NEC, followed by vortexing for 10 seconds and centrifugation for 2 seconds. After centrifugation, 200 µL of Buffer AL was added to each sample and NEC. All tubes were vortexed for at least 15 seconds, until samples appear to be homogenised. Each sample was then centrifuged for 2 seconds. Screw cap tubes containing samples and NEC were placed in an incubation oven at 56^{o}C for at least 12 hours but no longer than 24 hours.

Following incubation, all extracted DNA was cleaned up. Tubes were initially cooled at ambient temperature for 5 minutes. Once cooled, all samples were vortexed for 5 seconds and centrifuged for 2 seconds.

200 µL of molecular grade absolute ethanol was then added to each sample and NEC. Each solution was pulse vortexed for 15 seconds and centrifuged for 1 second. The contents of the 1.5 mL screw cap tubes were transferred into the corresponding DNeasy® Spin Columns which were placed in the provided Collection Tubes. Each spin column was centrifuged for 1 minute at 7,800 x g. Collection tubes were discarded and spin column were placed into new collection tubes.

500 µL of Buffer AW1 was added to each spin column, and columns were centrifuged for 1 minute at 7,800 x g. 500 µL of Buffer AW2 was then added into each spin column, and columns were centrifuged for 1 minute at 7,800 x g.

Collection tubes were discarded with flow-through and spin columns were placed into new collection tubes, followed by centrifugation for 3 minutes at 24,000 x g.

Collection tubes were discarded and spin columns were placed into clean 1.5 mL microcentrifuge tubes. DNA was eluted in 50 µL of Buffer AE pre-warmed at 72ºC, which was added onto the centre of each spin column. Caps were closed and spin columns were incubated at ambient temperature for 5 minutes. Following the incubation, spin columns were centrifuged for 3 minutes at 24,000 x g, and spin columns were discarded. Extracted genomic DNA was stored at 4ºC for short term storage (less than 1 month) and at -80ºC for long term storage.

2.6. Droplet Digital PCR (ddPCR)

BRCA1 methylation was quantified using the probe-based ddPCR methodology. The assay outlined in section 2.6.1.1 was initially being used until issues with background and PCR efficiency were identified. Subsequently, this assay was discontinued and the assay outlined in section 2.6.1 has since been used. The particular assay used to generate *BRCA1* methylation data throughout this thesis will be specified where applicable.

2.6.1. Quantification of *BRCA1* methylation using the ddPCR QX200™

Droplet Generator System: Current assay

The required number of clear 0.2 mL 8-tube PCR strips (Bio-Rad) (Product no. TLS0801) were placed into a 96-well rack, and labelled accordingly.

The QX200™ ddPCR™ Supermix for Probes (2X; no dUTP) (BioRad, Cat no. 186-3024) and the *BRCA1* primers and probes were mixed by pulse vortexing for 5 seconds and centrifugation for 1 second.

Primers were designed to amplify bisulfite modified DNA (see section 2.4.2). MGB TaqMan probes were designed and labelled with either of two fluorescent dyes (FAM and VIC) hybridizing to methylated or unmethylated DNA respectively.

The ddPCR Master Mix was prepared in a 1.5 mL microcentrifuge tube, and contained 900 nM of the forward primer (5'-GCGGGAATTATAGATAAATTAAAATTG-3'); 900 nM of the reverse primer (5'-CTATCCCCCNTCCAAAAAATC-3'); 250 nM of the methylated *BRCA1* probe (5'-FAM-ACTCACGCCGCGCAA-3'); 250 nM of the unmethylated *BRCA1* probe (5'-VIC-ACTCACACCACACAATC-3'), 1X ddPCR Supermix for Probes (2X; no dUTP; Bio-Rad) and ultra-pure H_2O to create a final reaction volume of 21 µL. The Master Mix was vortexed for 5 seconds and centrifuged for 1 second. 20 µL of Master Mix was then aliquoted into the designated wells of the 8-tube PCR strips, followed by 2 µL of test sample DNA to be analysed for methylation. Reactions were mixed by pipetting up and down 10 times using a Multi-channel (5 – 50 µL) (Finnpipette Cat no. FA8-50R). The 0.2 mL 8-tube PCR strips (Bio-Rad) were sealed with the Optical Flat 8-Cap Strips (Bio-Rad, Cat no. TCS-0803) and centrifuged for 2 seconds.

Droplets were generated by first inserting the DG8™ Cartridge (Bio-Rad, Cat no. 1864008) into the DG8™ Cartridge Holder (Bio-Rad, Cat no. 1863051). Using the Multi-channel (5

– 50 µL) (Finnpipette), 20 µL of each sample was transferred to the sample wells of the DG8™ Cartridge (Bio-Rad). Where less than 8 samples were being tested, 40 µL of H_2O was dispensed into the empty wells. Once all samples have been transferred, 70 µL of Droplet Generation Oil for Probes (Bio-Rad, Cat no. 186-3005) was dispensed into the designated wells of the DG8™ Cartridge (Bio-Rad), and sealed with the DG8™ Gasket (Bio-Rad, Cat no. 186-3009).

The DG8™ Cartridge Holder (Bio-Rad) was placed into the QX200™ Droplet Generator (Bio-Rad, Cat no. 186-4002), and the door was closed. When droplet generation was complete, the DG8™ Cartridge Holder (Bio-Rad) was removed from the unit, and the DG8™ Gasket (Bio-Rad) was discarded.

50 µL of generated droplets were transferred into a twin.tec unskirted PCR plate (Eppendorf, Cat no. 0030133374) using the Multi-channel Pipet-Lite XLS (5 – 50 µL LTS) (Rainin, Cat no. L8-50XLS+). Once droplet transfer was complete, the twin.tec PCR plate (Eppendorf) was covered with Piercable Foil Heat Seal™ (Bio-Rad, Cat no. 181-4040), and sealed with the PX1™ PCR Plate Sealer (Bio-Rad, Cat no. 181-4000) for 3 seconds at 180ºC.

Once heat sealing was complete, the twin.tec PCR plate (Eppendorf) was placed into a C1000 Touch™ Thermal Cycler (Bio-Rad, Cat no. 185-1196) and a PCR under the following conditions was performed: 95°C for 10 minutes followed by 40 cycles of 94°C for 30 seconds and 57°C for 1 minute, 98°C for 10 minutes, and 4°C storage. Data analysis was performed with the QX200™ Droplet Reader (Bio-Rad, Cat no. 1864003) and the QuantaSoft™ software (Bio-Rad, version 1.7.4) (see section 2.6.2).

2.6.2. Quantification of *BRCA1* methylation using the ddPCR QX200™

Droplet Generator System: Discontinued assay

The required number of clear 0.2 mL 8-tube PCR strips (Bio-Rad) (Product no. TLS0801) were placed into a 96-well rack, and labelled accordingly.

The QX200™ ddPCR™ Supermix for Probes (2X; no dUTP) (BioRad, Cat no. 186-3024) and the *BRCA1* primers and probes were mixed by pulse vortexing for 5 seconds and centrifugation for 1 second.

Primers were designed to amplify bisulfite modified DNA (see section 2.2.3). MGB TaqMan probes were designed and labelled with either of two fluorescent dyes (FAM and VIC) hybridizing to methylated or unmethylated DNA respectively.

The ddPCR Master Mix was prepared in a 1.5 mL microcentrifuge tube, and contained 1800 nM of the forward primer (5'-GCGGGAATTATAGATAAATTAAAATTG-3'); 1800 nM of the reverse primer (5'-TATCCCCCGTCCAAAAAATCTCA-3'); 500 nM of the methylated *BRCA1* probe (5'-FAM-ACTCACGCCGCGCAA-3'); 750 nM of the unmethylated *BRCA1* probe (5'-VIC-ACTCACACCACACAATC-3'); 1 U HotStarTaq DNA polymerase; 1X PCR Buffer (10X; 15 mM $MgCl_2$); 1X ddPCR Supermix for Probes (2X; no dUTP; Bio-Rad) and ultra-pure H_2O to create a final reaction volume of 21 µL. The Master Mix was vortexed for 5 seconds and centrifuged for 1 second. 20 µL of Master Mix was then aliquoted into the designated wells of the 8-tube PCR strips, followed by 2 µL of test sample DNA to be analysed for methylation. Reactions were mixed by pipetting up and down 10 times using a Multi-channel (5 – 50 µL) (Finnpipette Cat no. FA8-50R). The 0.2 mL 8-tube PCR strips (Bio-Rad) were sealed with the Optical Flat 8-Cap Strips (Bio-Rad, Cat no. TCS-0803) and centrifuged for 2 seconds.

Droplets were generated by first inserting the DG8™ Cartridge (Bio-Rad, Cat no. 1864008) into the DG8™ Cartridge Holder (Bio-Rad, Cat no. 1863051). Using the Multi-channel (5 – 50 µL) (Finnpipette), 20 µL of each sample was transferred to the sample wells of the DG8™ Cartridge (Bio-Rad). Where less than 8 samples were being tested, 40 µL of H_2O was dispensed into the empty wells. Once all samples have been transferred, 70 µL of Droplet Generation Oil for Probes (Bio-Rad, Cat no. 186-3005) was dispensed into the designated wells of the DG8™ Cartridge (Bio-Rad), and sealed with the DG8™ Gasket (Bio-Rad, Cat no. 186-3009).

The DG8™ Cartridge Holder (Bio-Rad) was placed into the QX200™ Droplet Generator (Bio-Rad, Cat no. 186-4002), and the door was closed. When droplet generation was complete, the DG8™ Cartridge Holder (Bio-Rad) was removed from the unit, and the DG8™ Gasket (Bio-Rad) was discarded.

50 µL of generated droplets were transferred into a twin.tec unskirted PCR plate (Eppendorf, Cat no. 0030133374) using the Multi-channel Pipet-Lite XLS (5 – 50 µL LTS) (Rainin, Cat no. L8-50XLS+). Once droplet transfer was complete, the twin.tec PCR plate (Eppendorf) was covered with Piercable Foil Heat Seal™ (Bio-Rad, Cat no. 181-4040), and sealed with the PX1™ PCR Plate Sealer (Bio-Rad, Cat no. 181-4000) for 3 seconds at 180°C.

Once heat sealing was complete, the twin.tec PCR plate (Eppendorf) was placed into a C1000 Touch™ Thermal Cycler (Bio-Rad, Cat no. 185-1196) and a PCR under the following conditions was performed: 95°C for 10 minutes followed by 40 cycles of 94°C for 30 seconds and 57°C for 1 minute, 98°C for 10 minutes, and 4°C storage. Data analysis was performed with the QX200™ Droplet Reader (Bio-Rad, Cat no. 1864003) and the QuantaSoft™ software (Bio-Rad, version 1.7.4) (see section 2.5.2).

2.6.3. ddPCR using the QX200™ Automated Droplet Generator (AutoDG™) System

The required PCR Master Mix was prepared in a 1.5 mL microcentrifuge tube (Eppendorf, Cat no. EPP0030125150) according to the relevant assay. The Master Mix was pipetted up-and-down 10 times to mix. The appropriate volume of Master Mix was aliquoted into the ddPCR™ 96-well Semi-skirted PCR plate (clear well/clear shell, Bio-Rad, Cat no. 12001925).

The appropriate volume of sample DNA and control DNA was added into the designated wells of the 96-well Semi-skirted PCR plate (Bio-Rad), creating a final reaction volume of 23 µL. Reactions were mixed by pipetting up and down 5 times using a multi-channel pipette set at 15 µL.

The 96-well plates (Bio-Rad) were then sealed with Optical Flat 8-Cap Strips (Bio-Rad, Cat no. TCS-0803) and centrifuged for 5 seconds using a Mini Plate Spinner (Pacific Laboratory Products, Cat no. C1000). Samples were checked to ensure no bubbles were seen after spinning. Following centrifugation, the Optical Flat 8-Cap Strips (Bio-Rad) were removed prior to loading the plate on the AutoDG™.

To prepare for droplet generation, the DG32™ AutoDG Cartridges (Bio-Rad, Cat no. 188-4106) and AutoDG™ Pipet Tips (Bio-Rad, Cat no. 186-4120) were placed in their designated compartments within the instrument according to the manufacturer's instructions. The 96-well PCR plate containing the prepared ddPCR reaction was also placed in the instruments allocated position.

The Droplet Plate assembly was then loaded by removing the cooling block from the -20°C freezer and placing it in the designated compartment in the AutoDG™ instrument. A clean 96-well Semi-skirted PCR collection plate (Bio-Rad, Cat no. 12001925) was placed into

the cooling block. Once all consumables were correctly loaded into the instrument, the run was started and droplets were generated (Figure 1A).

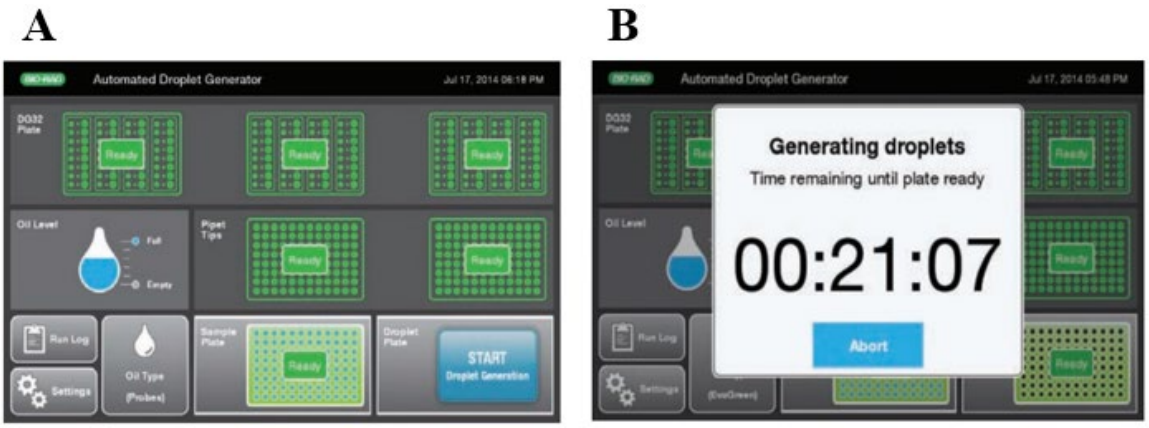


Figure 2-1. Parameters for AutoDG setup. (A) Home screen of AutoDG. All consumables are correctly loaded into the instrument, and the system is ready to commence droplet generation. **(B)** Countdown timer displaying the time remaining until droplet generation is complete and the Droplet Plate can be removed.

Once droplet generation was complete, the 96-well PCR plate was removed from the AutoDG™ and covered with Piercable Foil Heat Seal™ (Bio-Rad, Cat no. 181-4040), and sealed with the PX1™ PCR Plate Sealer (Bio-Rad, Cat no. 181-4000) for 3 seconds at 180ºC.

Once heat sealing was complete, the 96-well PCR plate (Bio-Rad) was placed into a C1000 Touch™ Thermal Cycler (Bio-Rad, Cat no. 185-1196) and a PCR under the following conditions was performed: 95°C for 10 minutes followed by 40 cycles of 94°C for 30 seconds and 57°C for 1 minute, 98°C for 10 minutes, and 4°C storage. Data analysis was performed with the QX200™ Droplet Reader (Bio-Rad, Cat no. 1864003) and the QuantaSoft™ software (Bio-Rad, version 1.7.4) (see section 2.6.2).

2.7. Methylation Sensitive High Resolution Melting (MS-HRM)

2.7.1. *COL2A1* control assay

The required PCR Master Mix was prepared in a 1.5 mL microcentrifuge tube (Eppendorf, Cat no. EPP0030123328). The Master Mix was mixed by pulse vortexing for 3 seconds to mix, followed by centrifugation for 1 second. 19 µL of Master Mix was aliquoted into the 96-well PCR plate (Bio-Rad, Cat no. HSP-9955). 1 µL of bisulfite modified DNA, NEC or NBC, and NTC was added into the appropriate well. The 96-well plate was then sealed with the Microseal® plate sealing film (Bio-Rad, Cat no. MSB1001), followed by centrifugation for 5 seconds in the Mini Plate Spinner (Labnet, EQP2012).

The MS-HRM was performed on the CFX Connect ™ Real-Time System (Bio-Rad, EQP1867). The 96-well plate containing Master Mix and DNA was loaded into the CFX Connect Real-Time System instrument. The Bio-Rad CFX Manager software was used to select the *COL2A1* MS-HRM protocol and label each well with the designated sample name. The PCR cycling conditions were activation at 95°C for 15 minutes, followed by 45 cycles of 95°C for 10 seconds, 64°C for 20 seconds and 72°C for 20 seconds. This was immediately followed by 97°C for 1 minute and a HRM step from 72°C to 95°C rising at 0.2°C per second. Data analysis was performed using the Precision Melt Analysis™ Software (Bio-Rad).

2.7.2. Measuring *RASSF1A* methylation using MS-HRM (Rotor-Gene® 6000 System)

The required PCR Master Mix was prepared in a 1.5 mL microcentrifuge tube (Eppendorf, Cat no. EPP0030123328). The Master Mix was mixed by pulse vortexing for 3 seconds to mix, followed by centrifugation for 1 second.

Primers were designed to amplify bisulfite modified DNA (see section 2.2.3). The Master Mix contained 200 nM of forward primer (5'-GTTTTAGATGAAGTCGTTATAGAGGT-

3'); 200 nM of reverse primer (5'- CCCACACGACAACTAATCCCTAA-3'); 0.5 U of HotStarTaq DNA polymerase; 1X PCR Buffer (10X; 15 mM $MgCl_2$); 2.5 mM of $MgCl_2$ (25mM); 200 µM of dNTP-Mix (2.5 mM each); 5 µM of SYTO 9 (100µM) and ultra-pure H_2O to create a final reaction volume of 18 µL. The Master Mix was vortexed for 5 seconds and centrifuged for 1 second. 18 µL of Master Mix was then aliquoted into the designated wells of the 0.2 mL PCR tubes (QIAGEN, Cat No. 981005) followed by 2 µL of test sample DNA to be analysed for methylation. The PCR tubes were then sealed with corresponding caps.

The MS-HRM was performed on the Rotor-Gene® 6000 (CORBETT/QIAGEN, Cat no. 1070452EN. The sealed PCR tubes containing Master Mix and DNA were then placed in the Rotor-Disc 72 Rotor (CORBETT/QIAGEN, Cat no. 9018899), followed by attachment of the Locking Ring (CORBETT/QIAGEN, Cat no. 9018900). The PCR cycling conditions were activation at 95°C for 15 minutes, followed by 55 cycles of 95°C for 10 seconds, 58°C for 20 seconds and 72°C for 30 seconds. This was immediately followed by 97°C for 1 minute and a HRM step from 68°C to 95°C rising at 0.2°C per second. Data analysis was performed using Corbett Rotor-Gene 6000 Application Software, version 1.7.

2.7.3. Measuring *RARβ* methylation using MS-HRM (Rotor-Gene® 6000 System)

The required PCR Master Mix was prepared in a 1.5 mL microcentrifuge tube (Eppendorf, Cat no. EPP0030123328). The Master Mix was mixed by pulse vortexing for 3 seconds to mix, followed by centrifugation for 1 second.

Primers were designed to amplify bisulfite modified DNA (see section 2.4.2). The Master Mix contained 200 nM of forward primer (5'- CGAGTTGTTTGAGGATTGGGATGT - 3'); 300 nM of reverse primer (5'- ACGATACCCAAACAAACCCTACTC - 3'); 0.5 U of HotStarTaq DNA polymerase; 1X PCR Buffer (10X; 15 mM $MgCl_2$); 2.5 mM of $MgCl_2$

(25 mM); 200 µM of dNTP-Mix (2.5 mM each); 5 µM of SYTO 9 (100 µM) and ultra-pure H_2O to create a final reaction volume of 18 µL. The Master Mix was vortexed for 5 seconds and centrifuged for 1 second. 18 µL of Master Mix was then aliquoted into the designated wells of the 0.2 mL PCR tubes (QIAGEN, Cat No. 981005) followed by 2 µL of test sample DNA to be analysed for methylation. The PCR tubes were then sealed with corresponding caps.

The MS-HRM was performed on the Rotor-Gene® 6000 (CORBETT/QIAGEN, Cat no. 1070452EN. The sealed PCR tubes containing Master Mix and DNA were then placed in the Rotor-Disc 72 Rotor (CORBETT/QIAGEN, Cat no. 9018899), followed by attachment of the Locking Ring (CORBETT/QIAGEN, Cat no. 9018900). The PCR cycling conditions were activation at 95°C for 15 minutes, followed by 55 cycles of 95°C for 10 seconds, 69°C for 20 seconds and 72°C for 30 seconds. This was immediately followed by 97°C for 1 minute and a HRM step from 65°C to 90°C rising at 0.2°C per second. Data analysis was performed using Corbett Rotor-Gene 6000 Application Software, version 1.7.

CHAPTER 3.

AN OPTIMISED DROPLET DIGITAL PCR METHOD FOR DETECTING LOW LEVEL *BRCA1* METHYLATION

3.1. Introduction

DNA methylation of specific genes is important in disease processes like cancer, and may serve both as a predictive marker and as a marker for early detection, prognosis and post-treatment surveillance of patients. Therefore, accurate detection with high analytical sensitivity and precise quantification of methylation levels in target DNA regions is essential.

Up to now, quantifying DNA methylation using standard PCR-based techniques and next generation sequencing has been challenging (Candiloro *et al.,* 2017). The recent development of droplet digital PCR (ddPCR) has enabled the absolute quantification of both methylated and unmethylated templates while providing high specificity and analytical sensitivity. ddPCR partitions a PCR reaction mix into thousands of individual droplets. The partitioning allows quantification of target molecules without reference to standards or controls. PCR amplification only occurs in the droplets containing the specific DNA template. The partitioning also eliminates common issues normally encountered in PCR-based methodologies, including PCR-bias (reviewed in Candiloro *et al.,* 2017).

ddPCR is unique in quantifying DNA methylation for a number of reasons, as it bypasses the need for standard curves in order to achieve absolute quantification of the target of interest (Hudecova 2015). However, the most distinct feature of ddPCR is the partitioning of a PCR reaction into approximately 20,000 droplets, with each droplet acting as a micro-reaction prior to amplification. This allows precise measurements of both methylated and unmethylated DNA templates. Additionally, ddPCR markedly reduces the effect of PCR inhibitors, and this is attributable to the end-point fluorescent signals that are generated, as

well as the counting of binomial events (i.e. methylated or unmethylated droplets). Taken together, these attributes combined with a Poisson algorithm (based on 95% confidence intervals) form a high-confidence measurement of the initial sample concentration (Hindson *et al.,* 2011; Pinheiro *et al.,* 2012).

Quantifying DNA methylation and gene expression by alternative PCR-based techniques such as quantitative PCR (qPCR) was previously considered gold standard and the method of choice (Redshaw *et al.,* 2014). However, more recently, considerable research has demonstrated the superiority of ddPCR in comparison to alternative methodologies, for the precise quantification of both DNA methylation and gene expression analysis (Pharo *et al.,* 2018), even in highly fragmented DNA such as FFPE tissue, or samples that have a low concentration. Despite the low amount of input DNA, ddPCR is advantageous in that replicates are highly reproducible with a little variation between replicates.

Here, the measurement of *BRCA1* methylation using ddPCR is addressed. The sensitivity of ddPCR in quantification of *BRCA1* methylation is important both in the context of assessing *BRCA1* methylation dosage in tumours (Kondrashova *et al.,* 2018), and in exploring constitutional methylation in normal tissues as a prospective breast and ovarian cancer predisposition factor (Wong *et al.,* 2011; Snell *et al.,* 2008; Lønning *et al.,* 2018).

A highly sensitive and quantitative *BRCA1* methylation ddPCR assay based on the quantification of both methylated and unmethylated target DNA post-bisulfite modification was developed (refer to Chapter 2, section 2.2.3). The assay utilises a dual-probe reaction system in which each probe recognises either methylated or unmethylated *BRCA1* alleles. Primers were designed to amplify bisulfite modified DNA, with careful consideration in avoiding amplification of the *BRCA1* pseudogene. MGB TaqMan^{TM} probes labelled with either of two fluorescent dyes (FAM and VIC) hybridizing to methylated or unmethylated DNA respectively were designed (Figure 3-1). Detection of target DNA is largely

dependent on fluorescent TaqMan™ probes (Holland *et al*., 1991; Livak 1991). In brief, probes anneal to their target DNA within the primer binding sites. The probes have a fluorescent modification at the 5' end, and a quencher at the 3' end. At the annealing step of the PCR, the probe binds to their target DNA. During primer extension, *Taq* polymerase cleaves the probe at the 3' end, which releases the quencher and consequently the fluorophore. As a result, a fluorescent signal is generated during the PCR cycling step, and is read when each droplet is passed through the droplet reader. Assay details and conditions are outlined in Chapter 2, section 2.5.1.

The *BRCA1* methylation assay outlined in Chapter 2, section 2.5.1 is the successor of an earlier *BRCA1* methylation assay that was previously being used. This assay was discontinued and re-optimised due to failed ddPCR runs on the automated droplet generator (AutoDG™), despite successful ddPCR runs achieved using the manual droplet generation system. Troubleshooting revealed that failed runs using the AutoDG™ were a consequence of the added constituents to the ddPCR mastermix, including HotStarTaq and 10x Buffer, which hindered efficient generation of droplets. The addition of reagents into the mastermix deviated from the BioRad recommendations. Details of the discontinued *BRCA1* methylation assay are outlined in Chapter 2, section 2.5.1.1.

Given the large number of samples to be tested for *BRCA1* methylation in Chapter 5, the AutoDG™ system was utilised as it has the capacity to run 96 samples at any one time. Additionally, these samples were bisulfite modified with the EZ DNA Methylation Lightning kit (2x96 preps, Zymo Research) (Chapter 2, section 2.2.3). Differences in amplitude of *BRCA1* methylation were observed in samples modified using the 96-well Zymo Research kit compared to the EZ DNA Methylation Lightning kit (200 preps, Zymo Research) (Chapter 2, section 2.2.2). These findings are presented in section 3.2.6, and will be addressed later in this chapter.

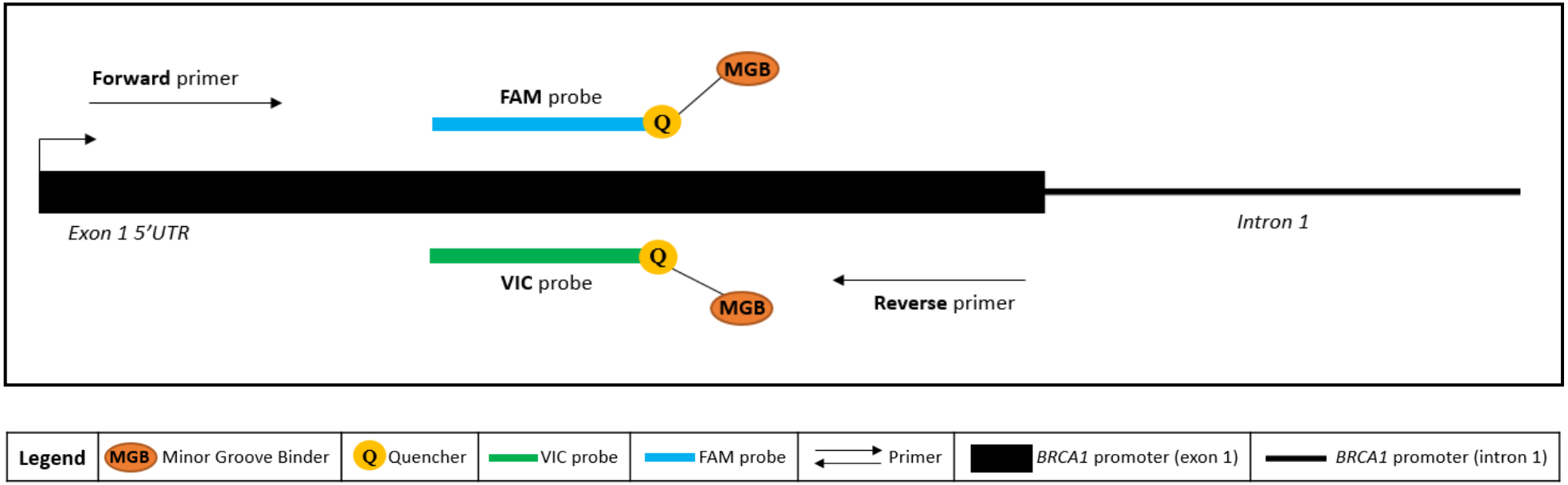


Figure 3-1. Map of the *BRCA1* promoter showing location of primers and probes. The sequence shown begins at the transcription start site and continues through the proximal 5'UTR, ending before the first intron. The position of the forward and reverse primers are indicated by arrows flanking the MGB TaqMan^{TM} probes. FAM and VIC probes are denoted by the blue and green blocks, and bind specifically to methylated or unmethylated DNA respectively. The quencher attached to the 3' end of each probe is indicated by the yellow circle.

3.2. Results

For this study, a highly sensitive probe-based methylation assay was designed to target the *BRCA1* promoter region. The *BRCA1* methylation assay was optimised by ddPCR using a dilution series, prior to being applied to study samples. Each dilution was tested in replicates for reproducibility and consistency. The fully methylated control (100%) used was the WEHICS62 cell line provided by Professor Clare Scott at the Walter and Eliza Hall Institute of Medical Research. WEHICS62 is a cell line generated from a patient derived xenograft (PDX) model, and retains homozygous *BRCA1* methylation (Kondrashova *et al.,* 2018). The unmethylated cell line used was HCT-116; a human colorectal carcinoma cell line. WEHICS62 and HCT-116 cell lines were normalised using the *COL2A1* assay (Chapter 2, section 2.6.1) to bring bisulfite-converted DNA from both cell lines to equivalent concentrations. Once this was achieved, the methylated and unmethylated control DNAs were mixed to create a methylation dilution series of 50%, 10%, 3% and 1%.

3.2.1. Assessment of amplifiable *BRCA1* methylated templates at varying concentrations in control sample DNAs.

An assessment of *BRCA1* methylation was performed on cell line DNA to determine the reproducibility and specificity of the assay. Figure 3-2 demonstrates the amplification of methylated and unmethylated *BRCA1* alleles across a dilution series. Methylated *BRCA1* templates appear in the FAM-channel, whereas unmethylated (i.e. wild-type) *BRCA1* templates appear in the VIC-channel. In a successful ddPCR run, two bands appear; one band appears at very low amplitude and represents droplets that contain no amplifiable target template. The second band however fluoresces at a higher amplitude, and represents *BRCA1* methylated (or unmethylated) droplets. The *BRCA1* methylation assay successfully amplified the target *BRCA1* promoter region, though intermediate droplets sitting between the two bands eluded to earlier are present in both the FAM and VIC channels. The

intermediate droplets are often referred to as ‘rain’, and as the amount of methylated *BRCA1* alleles in the FAM-channel decrease, the rain is also minimised, as demonstrated in Figure 3-2, E and G.

This assay also demonstrated high repeatability, as all three replicates within a single run for each dilution produce matching *BRCA1* methylation results. Once this assay was optimised and applied in subsequent ddPCR runs, the dilutions outlined in Figure 3-2 produced consistent *BRCA1* methylation levels. Furthermore, ddPCR MasterMix reagents originating from various orders with unique batch identifiers were used overtime; again, *BRCA1* methylation results were consistent. Similarly, we observe reproducible results across replicate samples when the assay is run by other scientists (data not shown).

Figure 3-2.

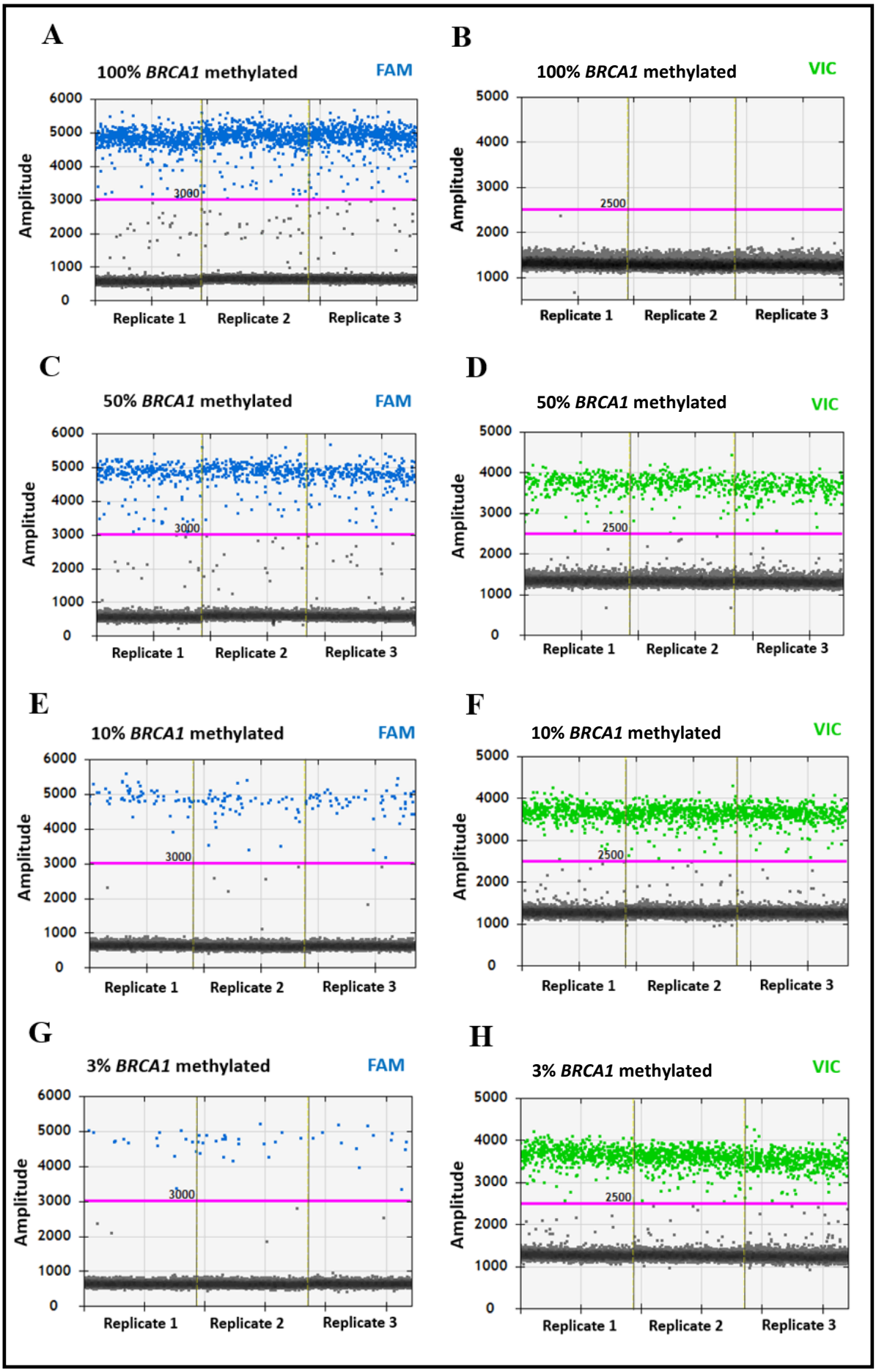

A
100% BRCA1 methylated
FAM
Amplitude
6000
5000
4000
3000
2000
1000
0
3000
Replicate 1
Replicate 2
Replicate 3
B
100% BRCA1 methylated
VIC
Amplitude
5000
4000
3000
2000
1000
2500
Replicate 1
Replicate 2
Replicate 3
C
50% BRCA1 methylated
FAM
Amplitude
6000
5000
4000
3000
2000
1000
0
3000
Replicate 1
Replicate 2
Replicate 3
D
50% BRCA1 methylated
VIC
Amplitude
5000
4000
3000
2000
1000
0
2500
Replicate 1
Replicate 2
Replicate 3
E
10% BRCA1 methylated
FAM
Amplitude
6000
5000
4000
3000
2000
1000
0
3000
Replicate 1
Replicate 2
Replicate 3
F
10% BRCA1 methylated
VIC
Amplitude
5000
4000
3000
2000
1000
0
2500
Replicate 1
Replicate 2
Replicate 3
G
3% BRCA1 methylated
FAM
Amplitude
6000
5000
4000
3000
2000
1000
0
3000
Replicate 1
Replicate 2
Replicate 3
H
3% BRCA1 methylated
VIC
Amplitude
5000
4000
3000
2000
1000
0
2500
Replicate 1
Replicate 2
Replicate 3

Figure 3-2. *Continued*

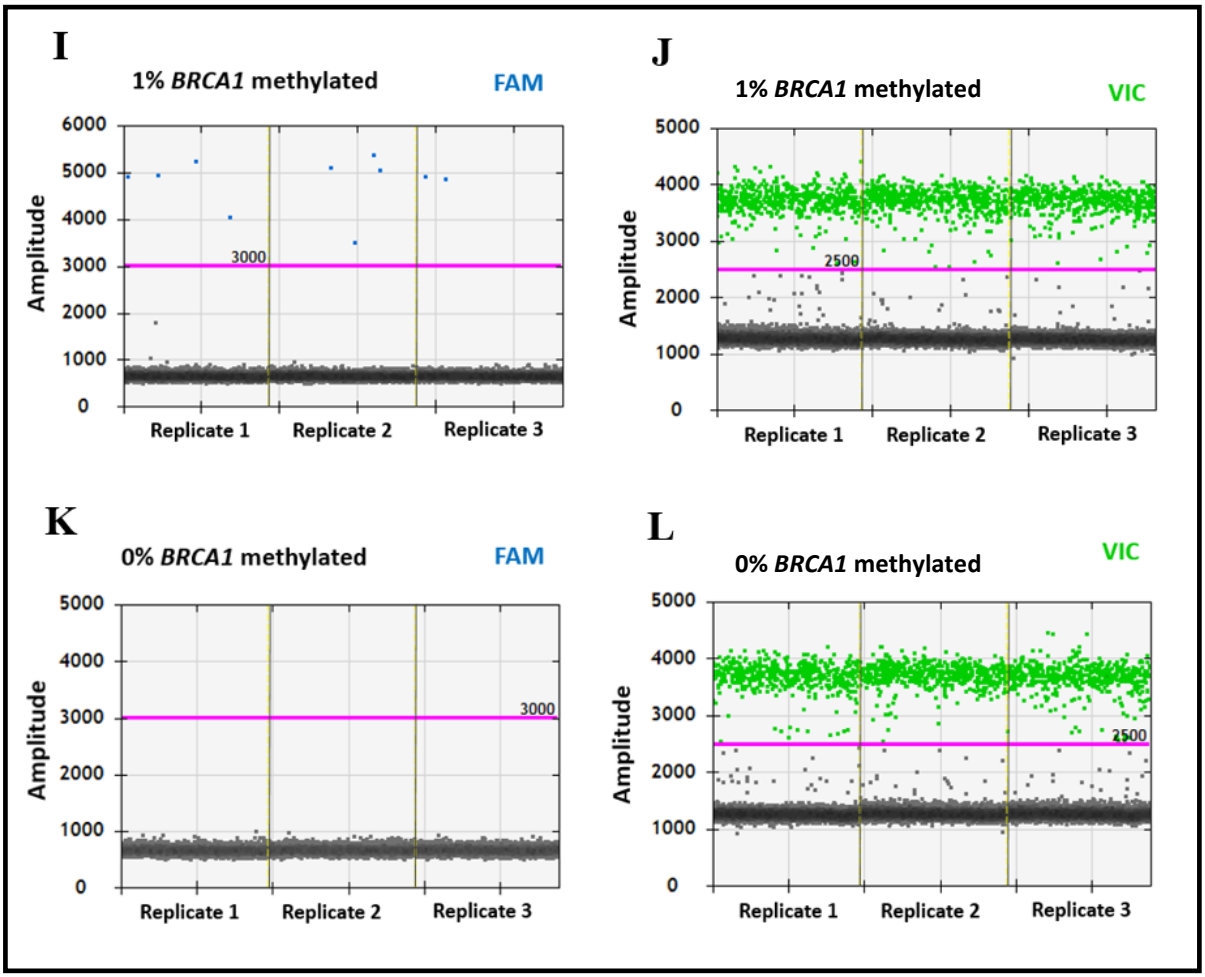


Figure 3-2. 1-D plot demonstrating detection of *BRCA1* methylation in a dilution series by ddPCR. Droplets from a dilution series are displayed in a 1-D plot generated by ddPCR. The methylated (FAM) signal for each dilution is presented in panels on the left, and the corresponding unmethylated (VIC) signal for each dilution is shown in panels on the right. All droplets containing methylated *BRCA1* templates (FAM - represented by blue droplets) or unmethylated *BRCA1* templates (VIC - represented by green droplets) are indicated above the pink threshold line. All droplets above the threshold are assigned a value of 1. All droplets below the threshold are scored as negative and assigned a value of 0. *BRCA1* methylation was assessed in a fully methylated (100%; WEHICS62) cell line (**A and B**), 50% methylated dilution **(C and D)**, 10% methylated dilution **(E and F)**, 3% methylated dilution **(G and H)**, 1% methylated dilution **(I and J)** and an unmethylated

(0%; HCT-116) cell line **(K and L)**. Each dilution was run in triplicate, with each replicate outlined by the grey vertical lines.

3.2.2. Duplex amplification using a probe-based assay allows highly sensitive quantification of *BRCA1* methylation in the presence of wild-type *BRCA1* alleles.

ddPCR allows the visualisation and quantification of multiple genomic targets within a single sample. To assess the amplitude of methylated and unmethylated *BRCA1* templates, and to evaluate the specificity of the FAM and VIC probes in targeting *BRCA1* alleles with a specific methylation status, viewing the 2-D ddPCR plot on the Quantasoft™ Software is informative.

2-D amplitude plots were generated for the 100% methylated WEHICS62 cell line, the 50% methylated dilution and the 0% methylated HCT-116 cell line (Figure 3-3).

Methylated and unmethylated *BRCA1* droplets appeared in two distinct and separated clusters on the Y and X axes respectively (Figure 3-3). This enabled accurate detection and quantification of methylated and unmethylated *BRCA1* alleles within a sample, and demonstrates assay specificity. Panel A highlighting the fully methylated cell line displays only one cluster containing *BRCA1* methylated template, and no unmethylated *BRCA1* alleles, as expected. The inverse was observed in Panel C in the HCT-116 cell line. The 50% methylated dilution had three droplets present as orange double-positive droplets (Panel B). This was not surprising, as DNA is distributed into droplets at random during droplet generation. Importantly, double-positive droplets have no impact on quantifying methylation percentage, as these droplets are each given a value of 1 in both the FAM and VIC signals by the Quantasoft™ Software.

Figure 3-3.

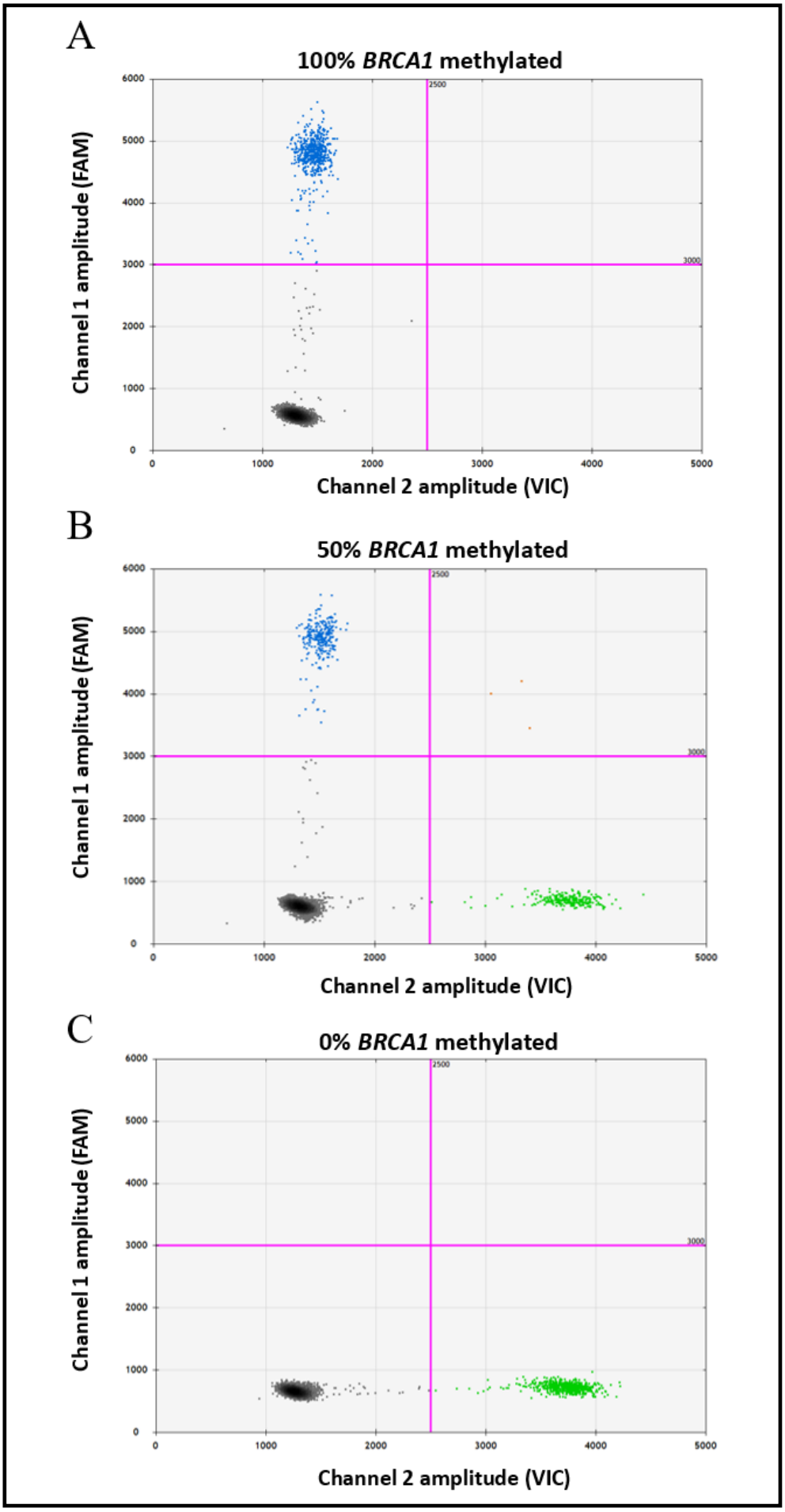

A
100% BRCA1 methylated
Channel 1 amplitude (FAM)
Channel 2 amplitude (VIC)
B
50% BRCA1 methylated
Channel 1 amplitude (FAM)
Channel 2 amplitude (VIC)
C
0% BRCA1 methylated
Channel 1 amplitude (FAM)
Channel 2 amplitude (VIC)

Figure 3-3. 2-D fluorescence plot of methylated and unmethylated *BRCA1* templates targeted by FAM and VIC probes respectively. 2-D fluorescence amplitude plot generated by QuantaSoft™ software shows distinct clustering of methylated and unmethylated *BRCA1* molecules in cell line DNA. Each dilution was run in replicates of three, and each 2-D plot demonstrates pooled data from three replicates of either 100%, 50% or 0% methylated cell line DNA. **(A)** 100% methylated WEHICS62 cell line displaying fully methylated droplets clustered in the FAM channel (blue). **(B)** 50% methylated dilution made from a mixture of WEHICS62 (100%) and HCT-116 cell lines (0%). The blue cluster (FAM-channel 1) represents droplets that are only *BRCA1* methylated. The green cluster (VIC-channel 2) represents unmethylated *BRCA1* droplets only. The orange cluster comprised of three droplets indicates double-positive droplets that are contain both *BRCA1*-methylated and *BRCA1*-unmethylated DNA. **(C)** 0% methylated HCT-116 cell line showing fully unmethylated droplets clustered in the VIC channel (green). The grey cluster in Panels A, B and C are droplets without amplifiable *BRCA1* template. The pink line denotes the manually set threshold.

3.2.3. Concentration estimates of methylated and unmethylated *BRCA1* templates in a dilution series determined by Quantasoft™ Software.

ddPCR has recently emerged as a robust technique for providing highly accurate DNA concentrations within a sample (Hindson *et al.,* 2011), predominantly due to the partitioning of the input DNA into discrete droplets. In this case, the concentration of DNA molecules that were either methylated or unmethylated at the *BRCA1* promoter were determined by utilising target-specific fluorescent TaqMan™ probes.

The concentrations of the fully methylated DNA (100%) and fully unmethylated DNA (0%) used to create the dilution series were initially normalised on MS-HRM using the *COL2A1* assay. The *COL2A1* MS-HRM assay is detailed in Chapter 2, section 2.6.1. Once

normalisation of the 100% and 0% methylated cell line DNA was achieved, the dilutions outlined in Figure 3-4 were created. To determine the true concentration of methylated and unmethylated *BRCA1* alleles, each dilution was run in triplicate on ddPCR. Results show that the Expected methylation percentages versus Observed concentrations on ddPCR varied slightly (Figure 3-4). This was particularly the case for dilutions with lower quantities of methylated templates, including the 3% and 1% methylated dilution. Again, these findings were anticipated given the high level of sensitivity achieved by ddCPR.

Figure 3-4.

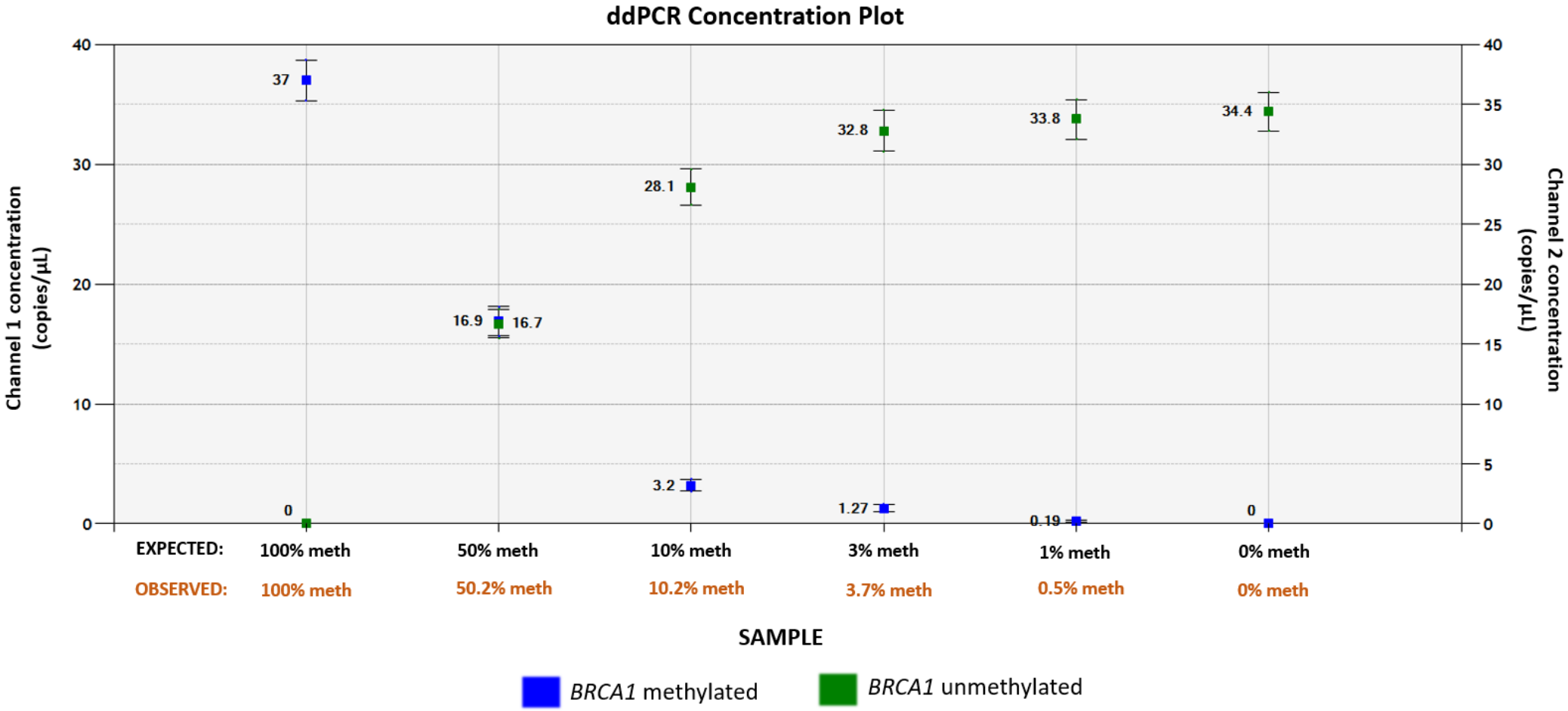

ddPCR Concentration Plot
Channel 1 concentration (copies/µL)
Channel 2 concentration (copies/µL)
40
30
20
10
0
35
25
15
5
37
0
16.9
16.7
28.1
3.2
32.8
1.27
33.8
0.19
34.4
0
EXPECTED:
100% meth
50% meth
10% meth
3% meth
1% meth
0% meth
OBSERVED:
100% meth
50.2% meth
10.2% meth
3.7% meth
0.5% meth
0% meth
SAMPLE
BRCA1 methylated
BRCA1 unmethylated

Figure 3-4. Concentrations plotted as copies/µL from each dilution showing varying degrees of *BRCA1* methylation. Concentration of methylated and unmethylated *BRCA1* templates in each dilution series were plotted as copies/µL. All dilutions were performed in triplicate, and data was merged to inform the concentration of each dilution. Blue blocks indicate the concentration of *BRCA1*-methylated DNA as copies/µL. Green blocks indicate unmethylated *BRCA1* templates as copies/µL. Expected methylation values for each dilution are outlined in black, and observed values obtained by ddPCR are listed below in brown. Observed methylation percentages were calculated using the following equation; M÷(M+UM) × 100, where M indicates methylated, and UM indicates unmethylated. All error bars generated by the Quantasoft™ Software represent the 95% confidence interval.

3.2.4. Total droplet events in a *BRCA1* methylation dilution series produce analogous methylation values to that of concentration values.

ddPCR enables counting of fluorescent positive and negative *BRCA1* methylated droplets. To assess whether *BRCA1* methylation is best quantified using the raw (or total) droplet counts versus the concentration (or Poisson-corrected values), this section calculated the number of *BRCA1*-methylated alleles using the raw droplet numbers (presented in Figure 3-5).

Samples with less than 10,000 total droplets were excluded from all ddPCR analyses as low droplet counts are indicative of technical issues, although such low total droplet counts were not observed in this study. Methylation percentages were calculated by applying the following equation to the total droplet counts; M÷(M+UM) × 100, where 'M' indicates methylated, and 'UM' indicates unmethylated. The methylation percentages calculated using raw droplet counts (Figure 3-4) provide comparable methylation values when compared to methylation percentages obtained by using concentration values (outlined in

section 3.2.3). Therefore, either raw droplet numbers or concentration values can be used interchangeably to quantify *BRCA1* methylation.

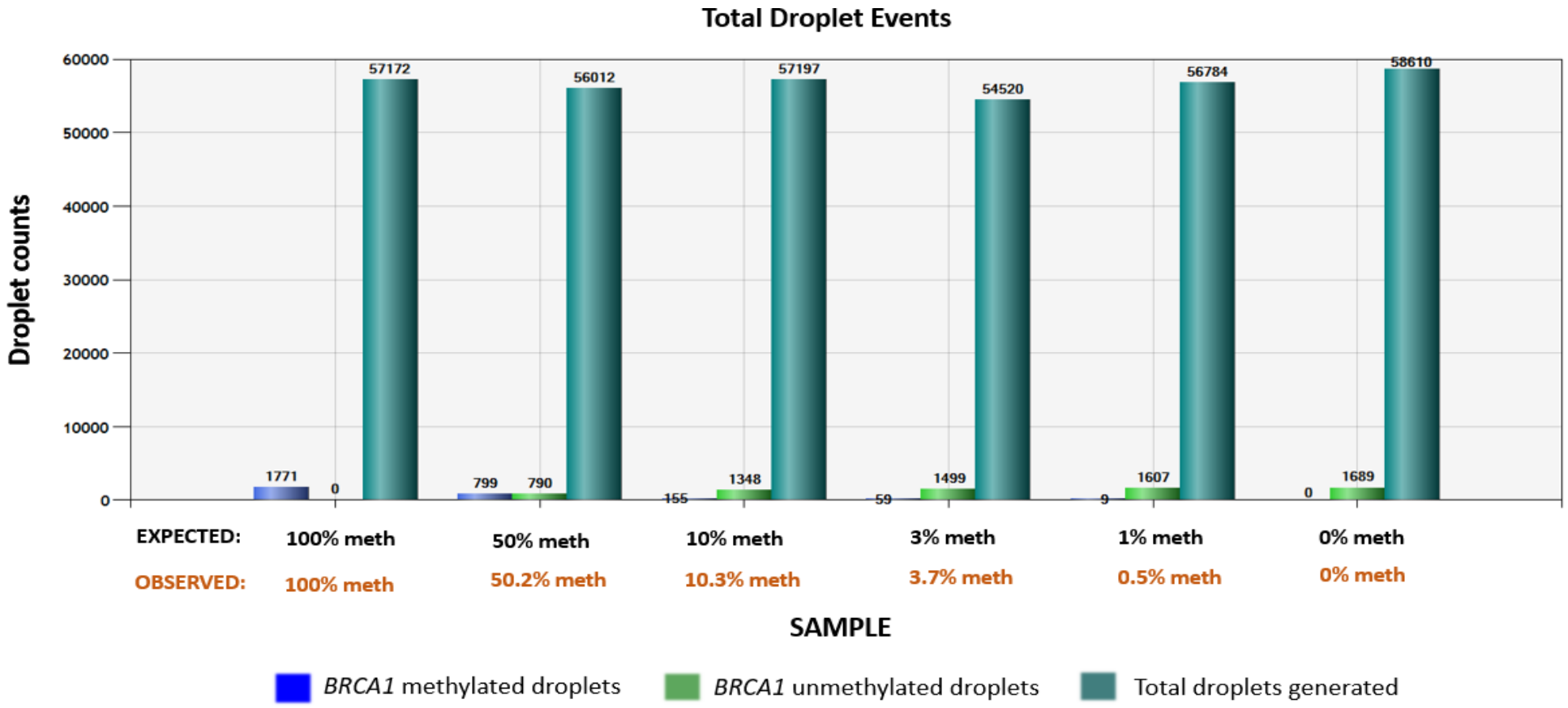


Figure 3-5. Total number of droplets with copies of target DNA in a *BRCA1* methylation dilution series. The number of methylated and unmethylated *BRCA1* droplets detected in each dilution are presented as a histogram. Each dilution was run in triplicate. Droplet counts are listed above each bar, and are representative of three merged wells per sample. Methylated *BRCA1* droplets are indicated by the dark blue bars. Green bars represent unmethylated *BRCA1* droplets. Teal bars represent total droplets generated.

3.2.5. Observing the effects of high DNA input on ddPCR.

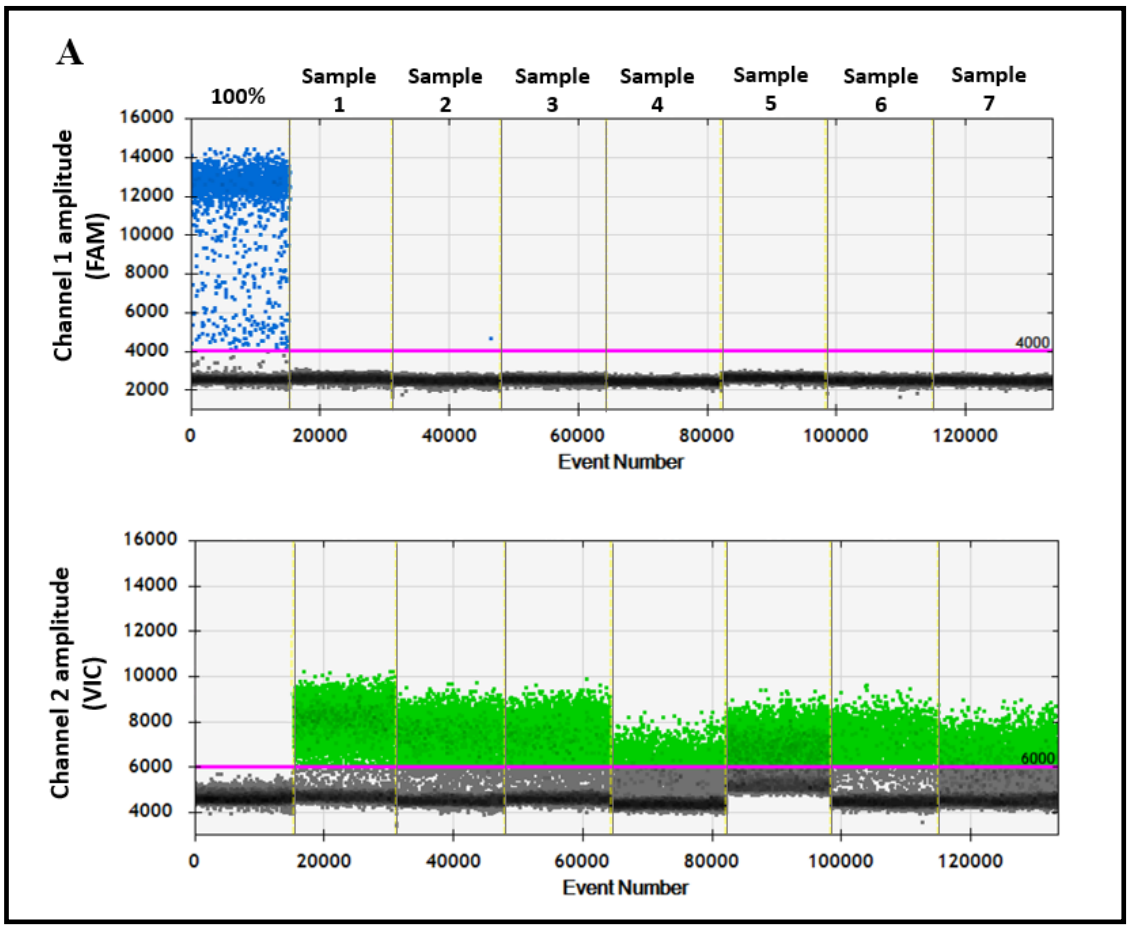
BRCA1 methylation was assessed in high quality peripheral blood DNA at high concentrations and at standard concentrations (Figure 3-6). The DNA samples analysed in this section each had 50ng of genomic DNA subjected to bisulfite modification. Samples 1-7 were modified using the EZ DNA Methylation Lightning kit (2x96). The 100% methylated control (CpGenome) was converted using the EZ DNA Methylation Lightning kit (200 prep [single-column]). Converted DNA was run on ddPCR to quantify *BRCA1* methylation, using high DNA input to ensure that even extremely low level methylation was captured (Figure 3-6, Panel A).

According to Zymo Research, bisulfite modification of genomic DNA using their EZ DNA Methylation Lightning kits results in an 80% recovery of single-stranded DNA. Given that DNA becomes single-stranded post-bisulfite modification, it cannot be reliably quantified using a spectrophotometer; therefore, it is difficult to provide an exact measurement of DNA input per reaction. For this reason, the amount of input DNA will be referred to in microlitres (µL) instead of nanograms (ng).

As observed in Figure 3-6, all 7 peripheral blood samples analysed with high and standard DNA input provided the same *BRCA1* methylation status, with all samples showing no methylation. Interestingly, when the samples were highly concentrated (Panel A), extremely poor separation of unmethylated droplets from the baseline was observed, compared to Panel B where the same samples were run using reduced DNA input.

The assay used to quantify *BRCA1* methylation in Figure 3-6 has since been discontinued (Chapter 2, section 2.5.1.1). However, Sample 1 to 7 (Figure 3-6) were subsequently tested for *BRCA1* methylation using the current re-optimised assay (Chapter 2, section 2.5.1), and methylation results between the two assays were concordant (Chapter 5).

Figure 3-6.


A
100%
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Channel 1 amplitude (FAM)
4000
Event Number
Channel 2 amplitude (VIC)
6000
Event Number

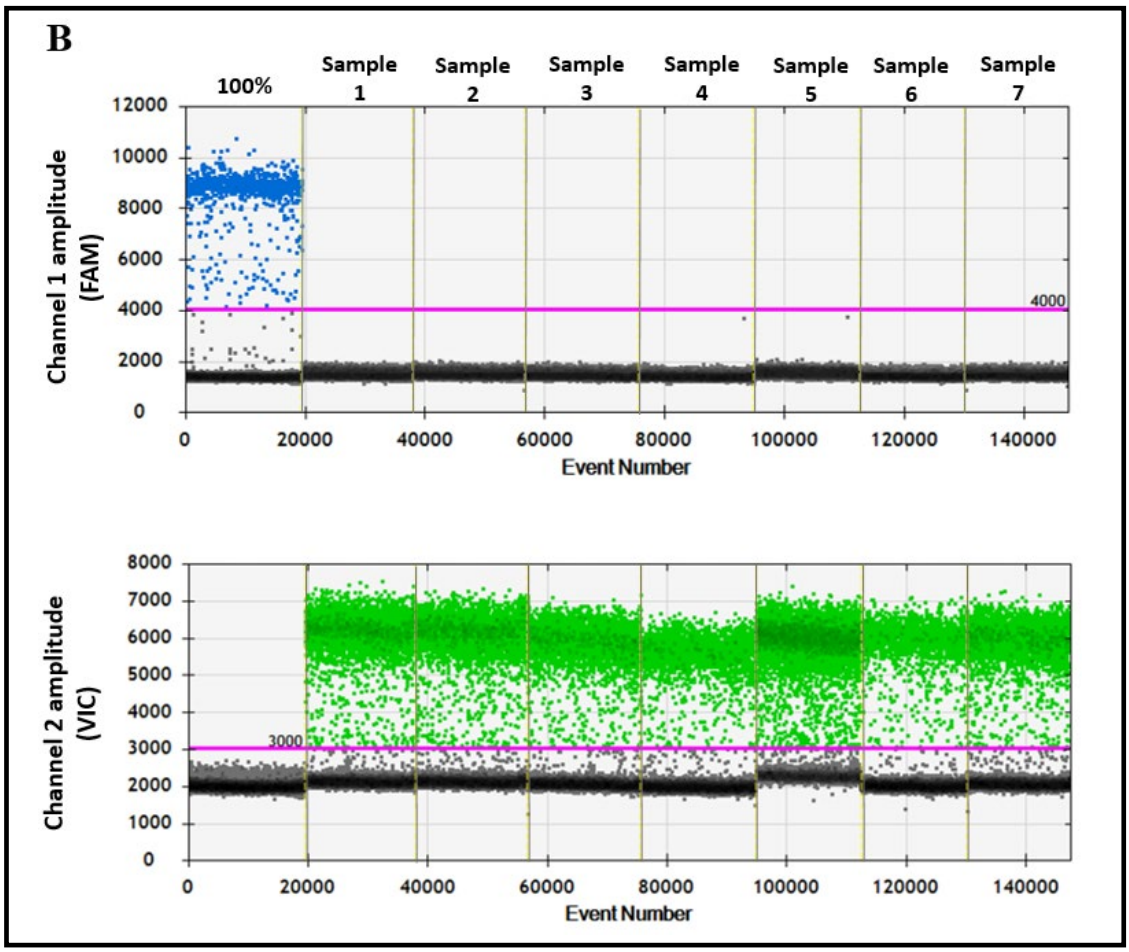

B
100%
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Channel 1 amplitude (FAM)
4000
Event Number
Channel 2 amplitude (VIC)
3000
Event Number

Figure 3-6. DNA input concentrations influence ddPCR amplitude. Seven peripheral blood DNA samples were assessed for *BRCA1* methylation using the (now) discontinued *BRCA1* methylation assay, at both high and standard concentrations. **(A)** 4 µL of bisulfite converted DNA input for *BRCA1* methylation analysis. The 100% methylated control (CpGenome) is represented by blue droplets in the FAM signal. Peripheral blood samples show no positive *BRCA1* methylated droplets. **(B)** 2 µL of bisulfite modified DNA input for each peripheral blood sample. Blue droplets indicate methylated droplets in the 100% methylated control. Green droplets are unmethylated *BRCA1* templates.

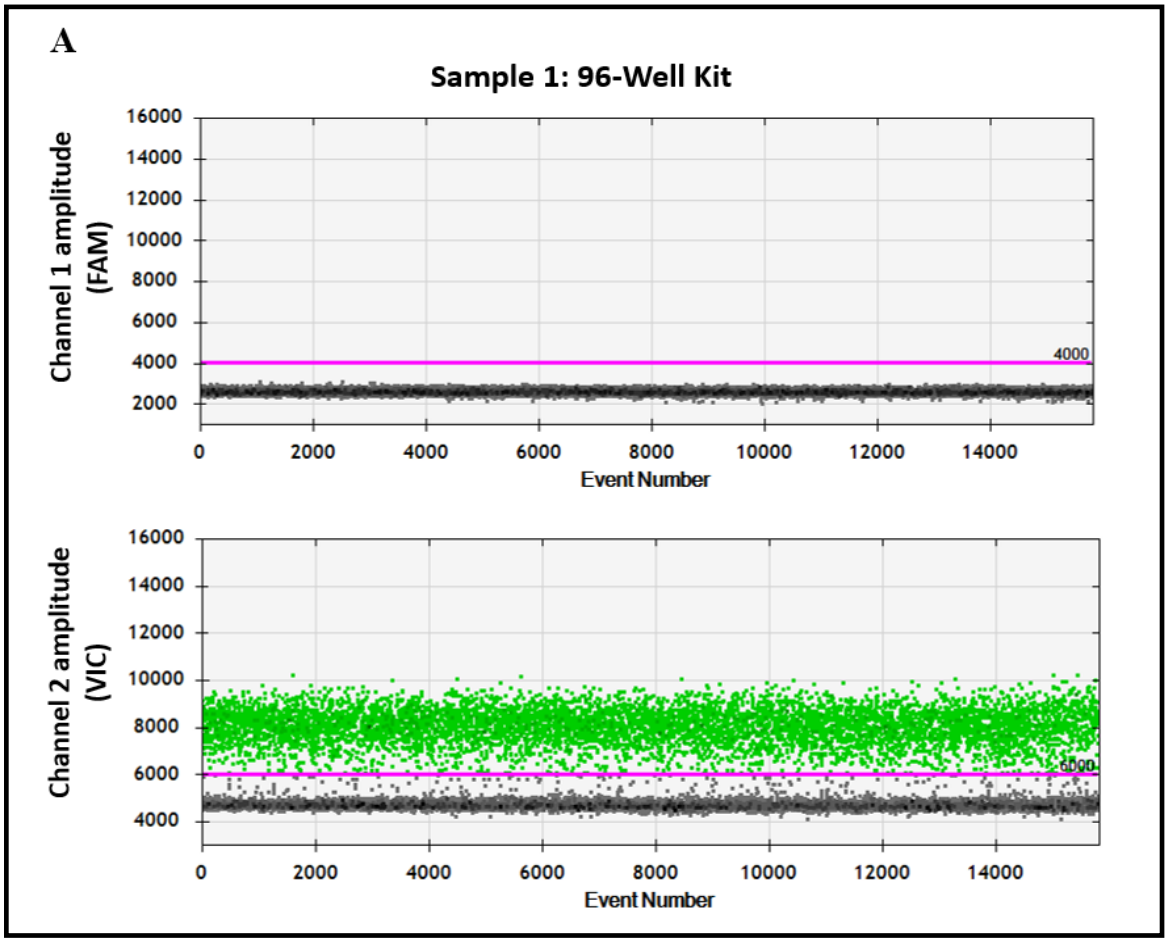
3.2.6. Assessment of *BRCA1* ddPCR efficiency using two distinct Zymo DNA Methylation Lightning kits.

Prior to any ddPCR methylation analyses, genomic DNA must be treated with sodium bisulfite to convert unmethylated cytosine bases to thymine. Most samples throughout this thesis were bisulfite modified using the EZ DNA Methylation Lightning kit (200 preps [single-column], Zymo Research). However, in cases where a large number of samples needs to be bisulfite modified, the EZ DNA Methylation Lightning kit (2x96 preps, Zymo Research) is the ideal kit to use. The key advantage of the 2x96 prep kit is that up 96 samples can be subjected to bisulfite conversion simultaneously. However, PCR efficiency and amplitude are reduced in samples that underwent bisulfite modification using the 96 well kit.

In this section, we incidentally observed poor PCR efficiency and separation of droplets from the baseline in a series of peripheral blood DNA samples. As demonstrated in Figure 3-7 Panel B, the single-column bisulfite modification kit results in substantially improved ddPCR efficiency and enhanced baseline separation compared to the 96-well kit (Panel A).

It is important to note that the assay used to assess *BRCA1* methylation in Figure 3-7 has since been discontinued (details of this assay are outlined in Chapter 2, section 2.5.1.1). However, Sample 1 (Figure 3-7) and 6 others (Figure 3-6) were subsequently tested for *BRCA1* methylation using the current assay (Chapter 2, section 2.5.1), and methylation results between the two assays were concordant.

Figure 3-7.


A
Sample 1: 96-Well Kit
Channel 1 amplitude (FAM)
16000
14000
12000
10000
8000
6000
4000
2000
4000
0
2000
4000
6000
8000
10000
12000
14000
Event Number
Channel 2 amplitude (VIC)
16000
14000
12000
10000
8000
6000
4000
6000
0
2000
4000
6000
8000
10000
12000
14000
Event Number

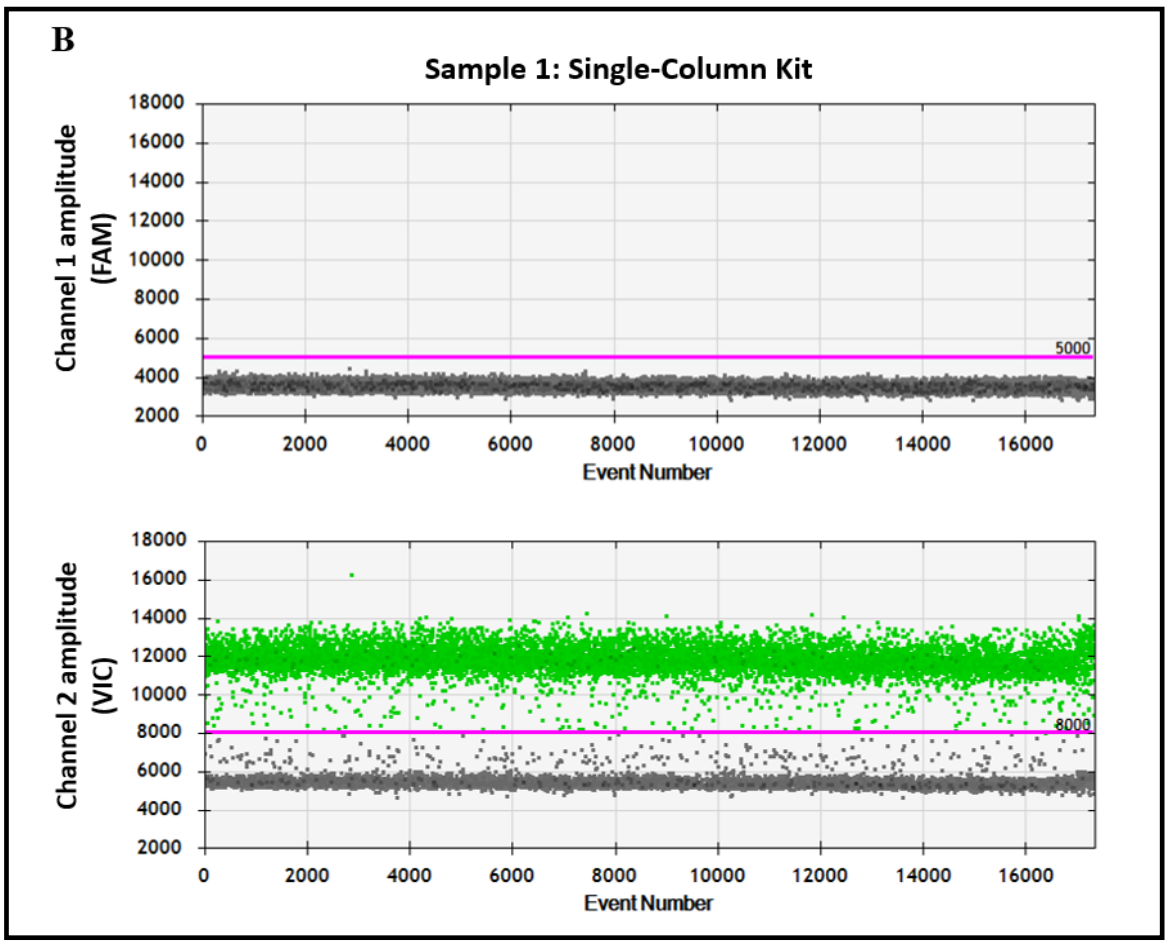

B
Sample 1: Single-Column Kit
Channel 1 amplitude (FAM)
18000
16000
14000
12000
10000
8000
6000
4000
2000
5000
0
2000
4000
6000
8000
10000
12000
14000
16000
Event Number
Channel 2 amplitude (VIC)
18000
16000
14000
12000
10000
8000
6000
4000
2000
8000
0
2000
4000
6000
8000
10000
12000
14000
16000
Event Number

Figure 3-7. Discrepancies in ddPCR efficiency depending on bisulfite modification modality. DNA from peripheral blood was bisulfite modified with two independent EZ DNA Methylation Lightning kits, and assessed for *BRCA1* methylation. **(A)** Bisulfite modification of Sample 1 with the EZ DNA Methylation Lightning kit (2x96 preps, Zymo Research). Channel 1 (FAM) contains no positive *BRCA1* methylated droplets. Channel 2 (VIC) demonstrates poor separation of unmethylated *BRCA1* droplets (green) from baseline (horizontal grey line). **(B)** Bisulfite modification of Sample 1 with the EZ DNA Methylation Lightning kit (200 preps [single-column], Zymo Research) shows improved amplitude and droplet separation from baseline.

3.3. Discussion

The performance of ddPCR for methylation analysis of bisulfite converted DNA was evaluated. The current probe-based *BRCA1* methylation assay has the capacity to accurately detect and quantify *BRCA1* methylated templates in a background of unmethylated unmethylated (i.e. wild-type) *BRCA1* molecules within a sample.

As mentioned earlier in this chapter, the original *BRCA1* assay was re-optimised. Initially, droplets were generated solely using the manual droplet generator (Chapter 2, section 2.5.1). However, the manual droplet generation system can only generate droplets in 8 samples at any one time. Manual droplet generation would not suffice given the time consuming nature of the method, and the scale of samples that needed to be quantified for *BRCA1* methylation by ddPCR (details on this project are outlined in Chapter 5). For this reason, the automated droplet generation system (AutoDG^{TM}) was ideal. However, major issues regarding poor PCR amplification and efficiency were identified during the AutoDG runs, as well as low total droplet counts (below 10,000 per sample). Interestingly, these findings were not observed using the manual droplet generation method.

To rule out issues with the AutoDG instrument, ddPCR was run on the AutoDG according to the BioRad recommended conditions. More specifically, the mastermix constituents were restored to the BioRad recommendations, with additional temperature re-optimisation. The current *BRCA1* methylation assay is detailed in Chapter 2, section 2.5.1. By adhering to the BioRad recommended conditions, the AutoDG run was successful, with satisfactory amplification and no background. All results from the AutoDG runs assessing *BRCA1* methylation using the current assay are outlined in Chapter 5.

The results presented in sections 3.2.1 to section 3.2.4 were all performed using the current re-optimised assay, whereas sections 3.2.5 and 3.2.6 were run on the discontinued *BRCA1* methylation assay. However, the seven peripheral blood DNA samples outlined in the latter sections are part of the sample cohort in Chapter 5 of this thesis, and were therefore repeated with the current ddPCR *BRCA1* methylation assay. Both assays produced comparable methylation results.

All samples underwent bisulfite modification with either the 96-well Zymo Lightning kit or the single-column kit, and clear differences were observed (Section 3.2.6). ddPCR amplification efficiency was evidently enhanced in samples converted with the single-column Zymo kit compared to the 96-well conversion kit (Figure 3-7, Panel B and A respectively). Notably, unmethylated *BRCA1* droplets showed improved separation from the baseline using the single-column conversion kit (Figure 3-7, B). Appropriate droplet separation from the baseline is crucial, particularly when establishing a threshold. Discrepancies between the two kits in producing efficient PCR amplification suggest that the 96-well Zymo Lightning kit may contain elements that result in partial inhibition of the PCR, despite producing the same methylation status. The PCR inhibition can partly be explained by the slight variation in procedure between the two kits, where discrepancies in

wash buffer volume, elution volume and centrifugation speeds exist. Details on each bisulfite modification kit are outlined in Chapter 2, sections 2.2.2 and 2.2.3.

Fluorescent measurements in alternative methodologies such as standard RT-PCR are unable to differentiate between *BRCA1* methylated and unmethylated templates, as fluorescence is measured as total amplification within the mastermix. In contrast, ddPCR partitions a reaction into thousands of droplets, hence enabling the distinction between methylated and unmethylated *BRCA1* molecules. Droplets containing either methylated or unmethylated molecules are displayed as two distinct populations of droplets, as depicted in Figure 3-3.

Quantification of DNA methylation by ddPCR can be erroneous if droplets are incorrectly classified (Querci *et al*., 2016). Therefore, precise separation of methylated and unmethylated droplets is critical. When droplets are generated, the distribution of DNA into each droplet occurs at random, and droplets cluster into four groups; Group 1: FAM-/VIC- (double-negative droplets); Group 2: FAM+/VIC-; Group 3: FAM-/VIC+ and Group 4: FAM+/VIC+ (double-positive droplets). The *BRCA1* methylation assay described herein highlights the distinct clustering of droplets with varying methylation status (Figure 3-3).

When quantifying DNA methylation, the concentration values or alternatively, the raw droplet counts of methylated and unmethylated *BRCA1* alleles can be used (Figure 3-4 and 3-5 respectively). Here, we observe comparable results using both measurements. However, it is important to note that using the raw droplet count values will result in slight variations when quantifying methylation, depending on where the manually set threshold is fixed. Therefore, it is important to establish a consistent algorithm for setting the threshold value for the *BRCA1* methylation assay that can be applied across all *BRCA1* ddPCR runs to acquire consistency in quantification across various runs.

The *BRCA1* methylation ddPCR assay showed no false positive droplets with the negative control cell line HCT-116, indicating its high target specificity. In addition, peripheral blood samples negative for *BRCA1* methylation were tested using both 2 μL and 4 μL of input bisulfite-modified DNA (Figure 3-6). Again, no false positive droplets were present. For this study, the *BRCA1* methylation assay was optimised using a dilution series, therefore ascertaining the precise false-positive rate was limited. However, since optimisation, this assay has been used to test over 600 samples for *BRCA1* methylation, and most of these samples showed no methylation, hence highlighting the low false positive rate. This study is outlined in depth in Chapter 5 of this thesis. More recently, Van Wesenbeeck *et al.* (2018) demonstrated that precision of ddPCR in methylation analyses is largely dependent on the quantity of amplifiable template rather than the amount of input DNA.

Earlier studies have used alternative methodologies for quantification of *BRCA1* methylation, including MethyLight and MS-HRM (Wong *et al*., 2011). We now consider that ddPCR is a superior method for quantification of *BRCA1* methylation, especially as it allows the determination of methylated allele frequencies, without the need of endogenous internal controls and standard curves.

BRCA1 methylation in particular has newly identified utility in the clinical setting for predicting patient response to PARP inhibitors (Kondrashova *et al*., 2018). Such findings highlight the significance of precision quantification of *BRCA1* methylation. As ddPCR allows the accurate determination of methylation levels within a sample, we suggest that this is the method of choice for quantification of *BRCA1* methylation.

3.4. Limitations of the study and suggested improvements

Although this assay has been used to quantify *BRCA1* methylation in DNA extracted from FFPE, this study could be improved by implementing fragmented or chemically modified DNA in the optimisation process. FFPE DNA is prone to generating false positive results. Hence, analysing FFPE tissue with known *BRCA1* methylation status would provide valuable information into the false positive rate of this assay.

Additionally, more rigorous validation studies would provide assurance of reliability of the assay results, and will allow estimation of the analytical performance of the assay. For example, one such validation study may focus on limit of detection, to provide comprehensive insight into the lowest degree of *BRCA1* methylation that can be detected and quantified with precision.

3.5. Summary

The primers and probes used in this assay were designed to target both methylated and unmethylated *BRCA1* alleles with high precision and absolute quantification. A distinct feature of the ddPCR technology is the ability to compartmentalise DNA molecules into discrete droplets, where some droplets are comprised of one or more target DNA molecules, whereas others contain no template. This study was able to detect and precisely quantify methylation across a dilution series of control DNA, as well as peripheral blood DNA.

Collectively, the data presented in this chapter demonstrates that ddPCR is a desirable tool for accurate quantification of *BRCA1* methylation, and can be used for methylation detection in clinical samples.

CHAPTER 4.

IDENTIFYING CONSTITUTIONAL *BRCA1* METHYLATION IN A MAMMOGRAPHICALLY SCREENED COHORT: A CASE-CONTROL STUDY

4.1. Introduction

Inactivating *BRCA1* and *BRCA2* germline mutations are key drivers of breast cancer. However, this is only the case for approximately 5% of breast cancer cases (Langston *et al.,* (1996); Whittemore *et al.,* (1997); Southey *et al.,* (1999); Tung *et al.,* (2016)). However, a proportion of mutation-negative women have tumours that share morphological features with *BRCA1*-mutant tumours (Loughrey *et al.,* 2008; Snell *et al.,* 2008). Interestingly, approximately 20% of these cancers that share morphological characteristics have detectable *BRCA1* methylation, implicating epigenetic mechanisms as a potential means of *BRCA1* inactivation and possibly breast cancer predisposition.

Extensive evidence has emerged proposing a direct role for DNA methylation in tumorigenesis (Baylin *et al.,* 2000; Esteller *et al.,* 2001; reviewed in Dobrovic and Kristensen 2009; Subramaniam *et al.,* 2014). The first case of an epimutation in a tumour suppressor gene in relation to cancer development was identified for the retinoblastoma (*RB1*) gene (Greger *et al.,* 1989). Subsequent tumour suppressor genes that have the capacity to become methylated including *MLH1* and *BRCA1* have since been associated with various forms of sporadic cancers (Dobrovic and Simpfendorfer 1997; Herman *et al.,* 1998; Gazzoli *et al.,* 2001; Snell *et al.,* 2008).

Earlier work has identified detectable methylation of the *BRCA1* gene in normal human tissue (i.e. constitutional methylation) has been identified (Snell *et al.,* 2008; Wong *et al.,* 2011). Notably, constitutional methylation has been considered as an alternative to genetic mutation for cancer predisposition. Constitutional *BRCA1* methylation in particular has

been considered a possible risk factor for breast cancer development in some women (Snell *et al.,* 2008; Wong *et al.,* 2011). Detection of this epigenetic aberration is feasible in peripheral blood, and may prove to be a powerful, yet non-invasive approach to identifying breast cancer predisposition.

In this chapter, a case-control study was carried out to assess constitutional *BRCA1* methylation in peripheral blood of healthy women (controls) and women with breast cancer (cases). All samples were obtained from age-matched case and control women who were enrolled in the Lifepool Project (Chapter 2, section 2.3). This study aimed to establish the frequency of peripheral blood methylation of the *BRCA1* gene in women with and without breast cancer, and who are representative of the population. Additionally, following the development of a quantitative method to determine *BRCA1* methylation, the association between the degree of peripheral blood methylation and breast cancer predisposition was also examined.

4.2. Results

Women who are part of the Lifepool Project were identified during mammographic screening with BreastScreen Victoria, and provided consent to donate peripheral blood and tumour tissue (where applicable). Women were able to provide consent via the Lifepool website (www.lifepool.org) or through the National Breast Cancer Foundation's (NBCF) research database Register 4. Peripheral blood DNA was provided by Lifepool to the Translational Genomics and Epigenomics Laboratory for 300 cases and 327 age-matched control women.

BRCA1 methylation was quantified using the assay outlined in Chapter 2, section 2.5.1. All results in this chapter were generated using the Automated Droplet Generator (AutoDG^{TM})

system (Chapter 2, section 2.5.2). All samples with ≤ 2 positive droplets were considered unmethylated.

4.2.1. Age-matching of Lifepool women with and without breast cancer.

The age range of the Lifepool participants in this study ranged from 29.6 years to 86.7 years for healthy control women at the time of blood donation (n = 327), and 29.6 years to 86.3 years for women with breast cancer (cases) (n = 300). All participants were age-matched as closely as possible to reduce age as a confounding variable in our dataset. As demonstrated by the histogram in Figure 4-1, both cases and controls appear effectively matched for age.

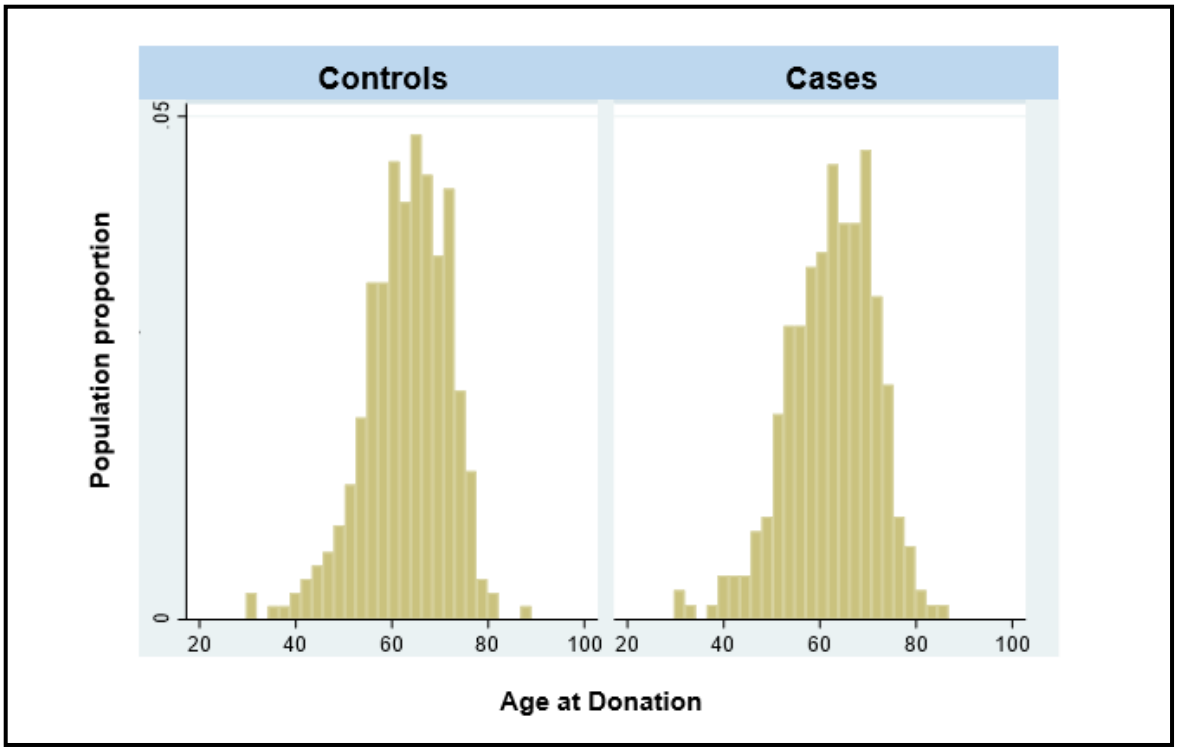


Figure 4-1. Age distribution of cases and controls. The age distributions of women without breast cancer (controls) and women with breast cancer (cases) are represented as a histogram. The X axis represents age at blood donation, and the Y axis represents the proportion of women.

4.2.2. Constitutional *BRCA1* methylation detected in peripheral blood of women with and without breast cancer.

BRCA1 methylation was assessed by ddPCR in a total of 627 women with and without breast cancer (300 cases and 327 controls) to determine the frequency and level of methylation in each cohort. Figure 4-2 shows varying levels of methylated *BRCA1* alleles across peripheral blood samples from 6 selected Lifepool cases and controls with detectable *BRCA1* methylation. The WEHICS62 cell line was used as a fully methylated (100%) control, and the RKO cell line was used as the unmethylated (0%) control.

Most women had no detectable *BRCA1* methylation in their peripheral blood DNA. The *BRCA1* methylation frequency detected in cases and controls was almost identical in the two populations of women (6.7% and 6.4% respectively). Women with detectable *BRCA1* methylation were categorised into one of three groups depending on the degree of methylation present in their peripheral blood DNA (Table 4-1). Interestingly, higher levels of methylation (above 4%) were only detected in women that developed breast cancer, and not in healthy controls. The average level of constitutional *BRCA1* methylation was significantly higher in cases compared to controls (IRR 4.49, 95% CI:1.92-10.49, p = 0.001).

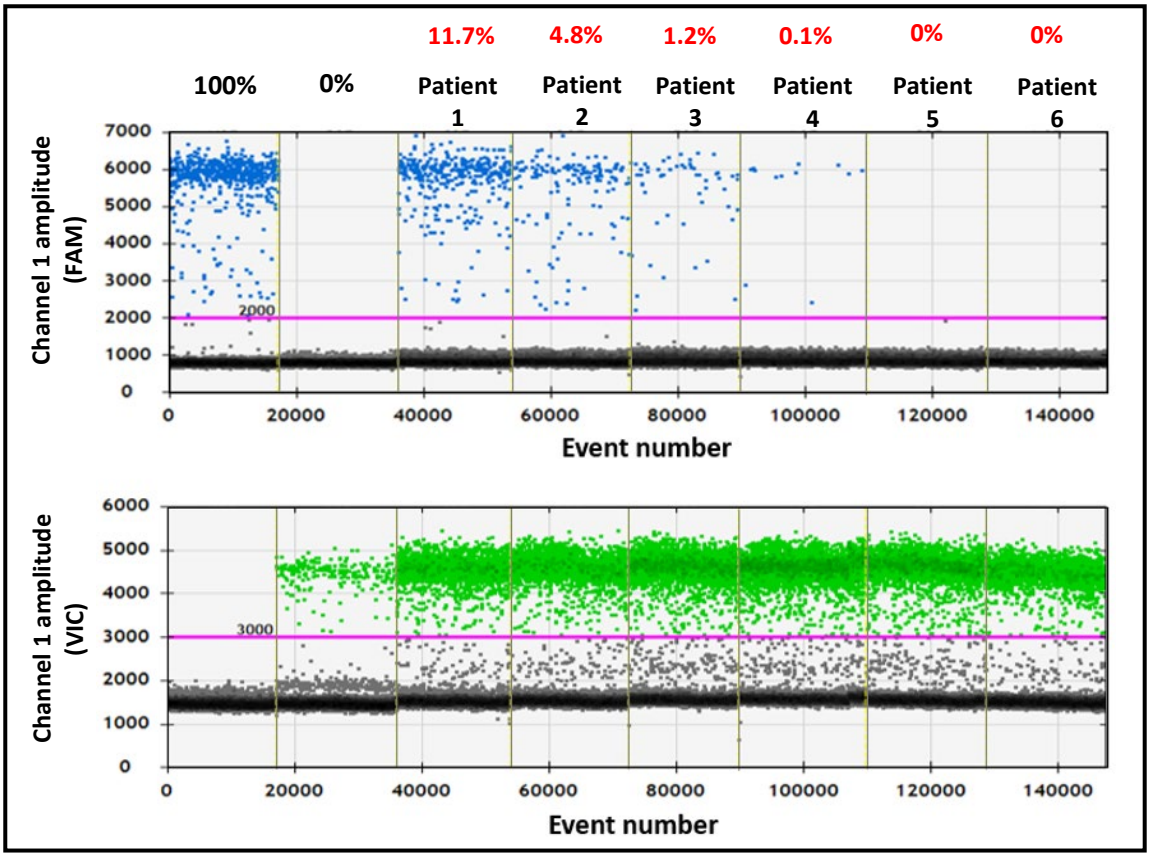


Figure 4-2. 1-D plots of variability in *BRCA1* methylation levels detected by ddPCR in peripheral blood of 6 women. *BRCA1* methylation detected at varying degrees across a random selection of 6 selected case and control samples is displayed. Blue droplets (FAM-channel) correspond to methylated *BRCA1* alleles. Green droplets correspond to unmethylated (i.e. wild-type) *BRCA1* alleles (VIC-channel). The 100% methylated control cell line and 0% methylated control cell line are presented in columns 1 and 2 respectively, followed by 6 peripheral blood samples belonging to individual mammographically screened women.

Table 4-1. Frequency of varying degrees of *BRCA1* methylation observed in cases and controls. Cases and controls with detectable *BRCA1* methylation were grouped by the degree of methylation observed. The total *BRCA1* methylation frequency in case and control cohorts is also presented.

Table 4-1. *BRCA1* methylation frequencies across Lifepool cases and controls.

	BRCA1 meth. **(Total)**	*BRCA1* meth. **(<1%)**	*BRCA1* meth. **(1-4%)**	*BRCA1* meth. **(>4%)**
CASE (breast cancer)	20/300 **(6.7%)**	14/20 (70%)	2/20 (10%)	4/20 (20%)
CONTROL (cancer-free)	21/327 **(6.4%)**	17/21 (80%)	4/21 (20%)	0/21

4.2.3. Identifying age-associated trends in *BRCA1* methylation frequency in age-sorted cases and controls.

All case and control samples were sorted by the age of blood donation, following collation of *BRCA1* methylation data by ddPCR. Age-sorting was carried out to determine whether there was an association between constitutional *BRCA1* methylation frequency and age in both healthy women and women with breast cancer. Interestingly, higher *BRCA1* methylation frequencies were observed in women under 40 years of age compared to women over 40 in both cases and controls (20% versus 6.3% respectively) (Figure 4-3). Cases and controls in the 60-69 year age group shared similar methylation frequencies, as did women in the 70+ year age group. Age at donation was not significantly associated with *BRCA1* methylation frequency in either case or control women when the two groups were considered independently ($p = 0.127$ and $p = 0.250$ respectively). However, when cases and controls were considered collectively ($n = 627$ women), age at blood donation was significantly associated with *BRCA1* methylation frequency (Fisher's exact test, $p = 0.045$) (Table 4-2).

Table 4-2. Presence of *BRCA1* methylation in cases and controls sorted by age and categorised by decade. All cases and controls were grouped into 5 age-group categories. Methylation (0) indicates absence of *BRCA1* methylation, and Methylation (1) indicates presence of *BRCA1* methylation. Fisher's exact test shows a significant association between *BRCA1* methylation and age (p = 0.045).

Age group	Methylation (0)	Methylation (1)	Total
Group 1: <40	n = 8	n = 2	n = 10
Group 2: 40-49 years	n = 39	n = 1	n = 40
Group 3: 50-59 years	n = 164	n = 12	n = 176
Group 4: 60-69 years	n = 240	n = 22	n = 262
Group 5: 70+	n = 135	n = 4	n = 139
TOTAL:	**n = 586**	**n = 41**	**n = 627**
Pearson Chi²: **Pr:** **Fisher's exact:**	8.5499 0.073 p = 0.045		

Figure 4-3.

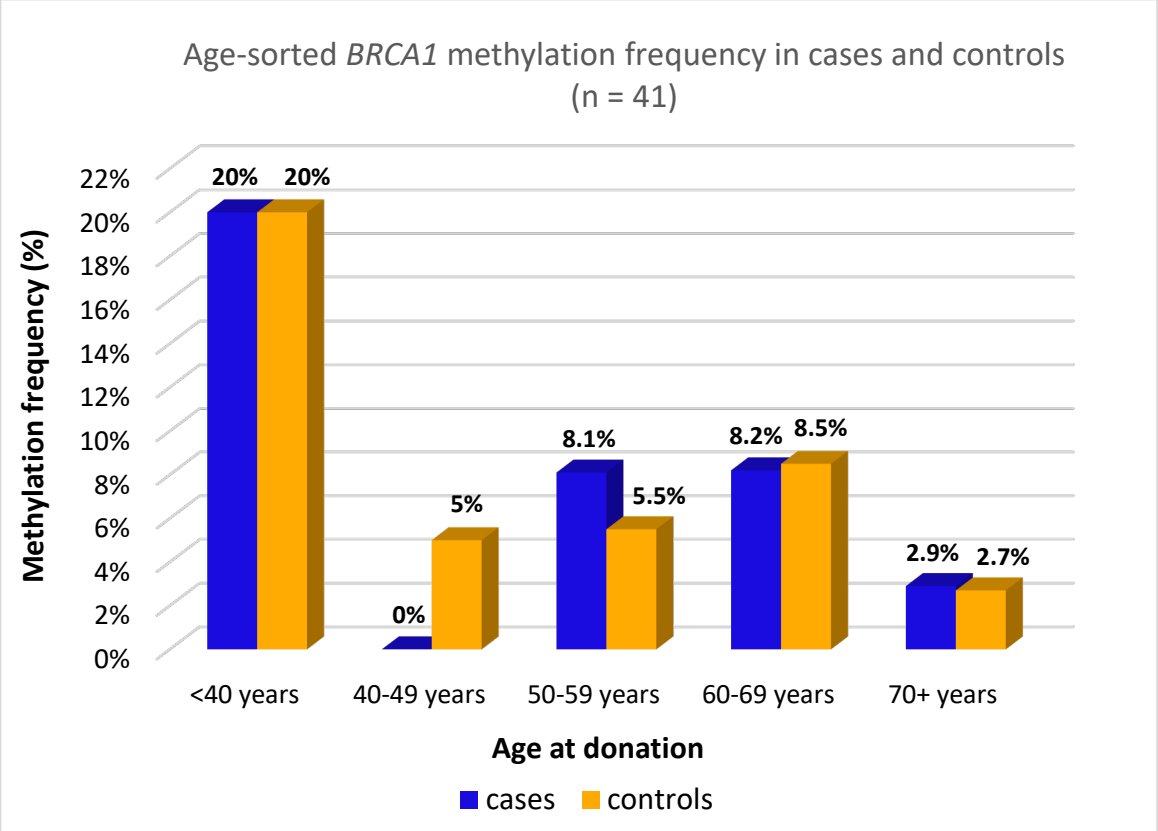


Figure 4-3. *BRCA1* methylation frequencies detected in peripheral blood DNA of cases and controls. *BRCA1* methylation frequencies across various age groups in women with and without breast cancer are presented. A total of 41 cases and controls showed detectable peripheral blood methylation. Women under 40 years of age showed a higher *BRCA1* methylation frequency compared to women over 40. Similar methylation frequencies were observed across the two groups in all age groups, however no case-women had detectable *BRCA1* methylation in the 40-49 year age group.

4.2.4. Assessing the relationship between *BRCA1* methylation in peripheral blood and tumour hormone receptor status.

Hormone receptor (HR) status was obtained for most women with breast cancer (n = 284 of 300). 16 women had completely missing hormone receptor information due to unavailable data from the time of diagnosis. 15 women had partially missing HR information, where data was missing for one or two hormones per patient. Constitutional *BRCA1* methylation has been reported at higher frequencies in women with triple negative breast cancers (TNBC) (Gupta *et al.,* 2014). In this dataset, 19 of the 284 cases with available HR status had TNBC, and only three of these women had detectable peripheral blood *BRCA1* methylation. Interestingly, most of the women who tested positive for peripheral blood methylation had tumours that were HR positive (14/20 [70%]). Details of HR status for all cases with *BRCA1* methylation detected in peripheral blood are outlined in Table 4-3.

4.2.5. Measuring *BRCA1* methylation in tumours of women with detectable peripheral blood methylation.

For some women with breast cancer who had detectable peripheral blood methylation, breast tumours were obtained and assessed for *BRCA1* methylation. As outlined in Table

4-3, only 8 of the 20 case women with detectable peripheral blood methylation were able to have tumours retrieved and analysed for *BRCA1* methylation. High levels of tumour methylation were observed in patients 9 and 11 (55.3% and 31.6% respectively) (Table 4-3). Interestingly, patients 9 and 11 had *BRCA1* methylation detected in peripheral blood at 0.57% and 9% respectively, indicating that despite low levels of *BRCA1* methylation detected in peripheral blood, corresponding tumours can show high levels of methylation. Most tumours corresponding to women with detectable peripheral blood methylation were unavailable, and therefore could not be assessed for *BRCA1* methylation. For this reason, it was difficult to draw a definitive conclusion regarding the association between peripheral blood methylation and tumour methylation.

Table 4-3. Hormone receptor status and tumour methylation in cases with detectable peripheral blood methylation of *BRCA1*. Tumour methylation results and HR status are presented for available case samples. 20 of 300 women diagnosed with breast cancer had detectable *BRCA1* methylation in peripheral blood DNA. Three of the 20 patients had triple negative breast cancers (yellow), while 14 women had breast tumours that were positive for one or more hormone receptors. Three patients had no HR data available, and were scored as N/A.

Patient no.	Age at Diagnosis	Age at Donation	Peripheral blood methylation (%)	Tumour methylation (%)	Estrogen Receptor (ER)	Progesterone Receptor (PR)	HER2
1	28.7	30.6	0.44	DNA unavailable	+	+	-
2	49.1	50.5	0.05	0	+	+	-
3	39.5	51	3.9	DNA unavailable	-	-	-
4	49.3	52.3	0.36	0	-	-	+
5	50.3	52.4	0.33	DNA unavailable	+	-	-
6	53	52.7	0.57	DNA unavailable	-	-	+
7	53.9	54.1	0.77	DNA unavailable	+	+	-
8	52.4	54.3	0.44	0	+	+	-
9	60.9	61.6	0.57	55.3	-	-	-
10	64.3	62.5	0.42	DNA unavailable	+	+	-
11	62.8	63.7	9.0	31.6	-	-	-
12	60.8	64.8	0.62	0.6	+	+	-
13	63.2	65.3	9.4	DNA unavailable	+	-	+
14	43.5	68.3	1.1	DNA unavailable	N/A	N/A	N/A
15	31.5	68.5	4.8	DNA unavailable	N/A	N/A	N/A
16	69.3	69.4	11.7	DNA unavailable	+	+	-
17	42.9	69.5	0.2	DNA unavailable	N/A	N/A	N/A
18	68.8	69.9	0.18	0	+	+	-
19	68.3	71.5	0.26	0.11	-	-	+
20	72	72.8	0.4	DNA unavailable	+	-	-

4.3. Discussion

The present study explored constitutional *BRCA1* methylation patterns *across women with breast cancer and women without breast cancer.* The methylation frequency in both groups of women was established using a case-control study design. This study revealed unexpected similarities in peripheral blood *BRCA1* methylation frequencies between Lifepool cases and controls.

Constitutional *BRCA1* methylation data was analysed in peripheral blood of all women (n = 627), and almost identical methylation frequencies in cases and controls were observed (6.7% and 6.4% respectively). Despite similar methylation frequencies in cases and controls, levels of *BRCA1* methylation above 4% were detected in cases alone (Table 4-1), and that the overall levels of detectable *BRCA1* methylation in cases were significantly higher when compared to controls ($p = 0.001$). These findings suggest that high peripheral blood methylation may increase risk of breast cancer by hindering the activity of the *BRCA1* gene.

Our finding of similar methylation frequencies between the two populations of women somewhat contrasts to previously published literature, demonstrating that constitutional *BRCA1* methylation is detected at a frequency of approximately 4% in healthy adult women (Wong *et al.,* 2011). More recently, Sharp *et al.* (2019) used Illumina 450k array data to analyse the methylation profiles of more than 23,000 individuals, and corroborated low rates of methylation amongst healthy adults. They reported *BRCA1* hypermethylation at a population frequency of approximately 1 in 3000; much lower than our findings.

The significant differences observed in *BRCA1* methylation frequencies compared to the published literature can partly be explained by the methodologies used to quantify methylation. An abundance of literature exists outlining the high analytical sensitivity and

superiority of ddPCR in comparison to alternative methodologies (Hindson *et al.,* 2013; Wiencke *et al.,* 2014; Demuth *et al.,* 2018), particularly in samples with minimal amounts of starting material and poor quality DNA (Taylor *et al.,* 2017).

Once age was considered, cases and controls shared similar *BRCA1* methylation frequencies across all age groups (Figure 4-3). The association between age and *BRCA1* methylation frequency was prominent, with results revealing significantly higher methylation rates in women under 40 years old compared to women over 40 (20% versus 6.3% respectively) (section 4.2.3). Two of 10 women under 40 (one case and one control) showed *BRCA1* methylation. The high incidence of *BRCA1* methylation detected in peripheral blood of younger women may be indicative of increased risk of developing a *BRCA1*-methylated tumour.

Following *BRCA1* methylation assessment in peripheral blood, tumours were retrieved for some of the cases and assessed for *BRCA1* methylation to determine the correlation between constitutional methylation and tumour methylation (section 4.2.5). *BRCA1* methylation in tumours was detected at significantly higher levels compared to levels detected in peripheral blood, suggesting that low levels of *BRCA1* methylation (below 1%) can indicate a *BRCA1*-methylated breast tumour.

Research has shown that *BRCA1*-methylated breast carcinomas are mainly reported in triple-negative breast cancers (TNBC) (Stirzaker *et al.,* 2015). TNBC is an aggressive subtype of breast cancer, accounting for approximately 20% of all breast cancers (Temian *et al.,* 2018). TNBC is often diagnosed in younger women and lacks targeted and effective therapeutic regimens (Gretchen *et al.,* 2010). For this reason, hormone receptor status of all women with breast cancer was considered when analysing *BRCA1* methylation in peripheral blood and tumour tissue. In this study, only three of the 20 case women with

detectable peripheral blood methylated had TNBC breast tumours. Importantly, two of these women had *BRCA1* tumour methylation (Table 4-3). Due to insufficient tumour DNA, the *BRCA1* methylation status of Patient 3 with TNBC was unable to be obtained.

Contrary to previously published data, most women with peripheral blood *BRCA1* methylation had breast tumours that were hormone receptor positive. This was observed in case women irrespective of the level of detectable peripheral blood methylation, which ranged from as little as 0.05% to more than 11%. This data suggests that hormone receptor status has little impact on the presence of constitutional *BRCA1* methylation.

Collectively, our findings demonstrate that there is no difference in methylation frequency between cases and controls. However, high levels of peripheral blood methylation (above 4%) were observed in cases alone, indicating that higher levels of constitutional *BRCA1* methylation may be indicative of increased breast cancer predisposition. Due to incomplete access to tumour DNA of case women, limited conclusions were drawn regarding the association between peripheral blood methylation and corresponding tumour methylation in women with breast cancer. Limitations of this study and suggestions for future research are addressed in section 4.4.

4.4. Limitations of the study and suggested improvements

The DNA from peripheral blood and tumours of all women in this study were of extremely high quality. However, a few limitations relating to tissue availability and sample size were encountered, therefore limiting observations and conclusions. This section will review the limitations throughout this study as well as suggested future research.

Assessing whether breast carcinomas are *BRCA1*-methylated in women who also present with constitutional *BRCA1* methylation is crucial in understanding the relationship between

tumour methylation and detectable healthy tissue methylation. A major limitation of this study was the inability to measure *BRCA1* methylation in tumours of all case women due to limited availability of tumour DNA provided by Lifepool. Consequently, definitive conclusions could not be drawn regarding the association between tumour methylation and the presence of constitutional methylation in this cohort of patients. A study of this nature necessitates access to all matched tumours in order to precisely define the association of tumour methylation and peripheral blood methylation with breast cancer risk.

Section 4.2.3 of this study assessed whether there was an association between the age at the time of blood donation and the frequency of constitutional *BRCA1* methylation. When case and control groups were considered independently, statistical significance could not be achieved, as the sample size in each group was not large enough (n = 300 cases; n = 327 controls). However, when *BRCA1* methylation data was pooled from both case and control women, statistical significance was reached due to satisfactory sample size. Hence, future research should involve larger cohorts of women; approximately 600 for each case and control group, in order to improve statistical analyses and confidence in estimated values.

Most Australian women who are diagnosed with breast cancer are above 50 years of age (79%) (AIHW 2019). In this study, 92% of women were above 50 years of age at the time of donation (n = 577) (Table 4-2), thus reflecting the true Australian population of women diagnosed with breast cancer. Given that only a small proportion of women in this study were under 50, the findings in this chapter cannot accurately inform *BRCA1* methylation frequencies in tumour or peripheral blood in women with early-onset breast cancer.

For this reason, assessing *BRCA1* methylation in tissue from a larger and younger population of case and control women (<40 years) would provide value in determining with higher precision the effects of age on constitutional *BRCA1* methylation in particular, and

its effects on breast cancer predisposition in women who are diagnosed with early-onset breast cancer.

4.5. Summary

This is the first study to quantify *BRCA1* methylation in peripheral blood of healthy women and women with breast cancer using ddPCR methodology. The use of this sensitive method shows that the population frequency of constitutional *BRCA1* methylation is more abundant than initially thought, and is indeed comparable between cases and controls. There is also an indication that the level of peripheral blood methylation is important. Peripheral blood methylation levels above 4% were solely observed in women with breast cancer. Collectively, our data suggests that constitutional *BRCA1* methylation alone may not strongly predispose women, particularly post-menopausal women, to developing breast cancer; however, levels above 4% may identify women with an increased risk of breast cancer during their lifetime. Furthermore, most women had hormone-receptor positive tumours, whereas few had TNBC. Nevertheless, further research is required to determine the extent of the correlation between peripheral blood methylation and corresponding tumour methylation.

CHAPTER 5.

ASCERTAINING CONCORDANCE RATES OF CONSTITUTIONAL *BRCA1* METHYLATION IN BUCCAL MUCOSA AND WHITE BLOOD CELL DNA OF MONOZYGOTIC TWIN PAIRS

5.1. Introduction

Epigenetics has become a key component of disease epidemiology as it has the capacity to unravel biological mechanisms underlying various disease (Feinberg 2018). DNA methylation in particular has the potential to serve as a marker of disease prognosis and progression (Pandith *et al.,* 2018; Kristensen *et al.,* 2016). Epigenetic modifications have been described in many disease types, including cancer (Feinberg and Tycko 2004), Alzheimer's disease (Sanchez-Mut *et al*., 2013), autoimmune diseases (Ballestar 2010), and many other complex human diseases. Most studies assessing epigenetic effects on disease phenotype often use cohorts of unrelated individuals. However, instances of identical twins discordant for various diseases continue to be reported, and a number of studies have explored the epigenetic profiles of identical twins who show disease discordance (Lonning *et al.,* 2018; Heyn *et al.,* 2012; Galetzka *et al.,* 2012).

Gene expression and repression via epigenetic modifications may explain, in part, why monozygotic (MZ) twins can be discordant for levels of DNA methylation. There is evidence demonstrating that MZ twins discordant for certain diseases have variable methylation levels at key genomic regions associated with their disease (Gordon *et al.,* 2012; Wong *et al.,* 2010). One such example was demonstrated in 2002 by Weksberg *et al.* who analysed methylation patterns from skin fibroblasts of monozygotic twin pairs discordant for Beckwith-Wiedemann syndrome (BWS) (n = 5). They found that only the affected twin showed altered methylation of the CpG island upstream of *KCNQ1OT1*, as well as biallelic expression of *KCNQ1OT1.*

Most evidence supporting phenotypic variation by epigenetic modifications has arisen from studies of defects in genomic imprinting: an epigenetic phenomenon that allows monoallelic expression of a subset of genes depending on parental origin (Hanna and Kelsey 2017). Epigenetic modifications (or epimutations) can cause gene inactivation (Jiang *et al.,* 2004). Many genes can be targeted by epimutations, giving rise to various diseases. DNA methylation of imprinted genes can alter the expression of normal genes, resulting in disease phenotypes. Therefore, it is probable that DNA methylation may have the capacity to alter expression of any gene.

Epigenetic analysis on MZ twins provides an understanding into epigenetic effects of DNA methylation in complex human disease, including cancer. Research of this nature is valuable in MZ twins as a genetically identical state is obtained at every allele, allowing us to identify whether DNA methylation at certain genes is intrinsically variable at certain loci within twin pairs. Furthermore, MZ twins have the unambiguous division of maternal inheritance, and serve as matched controls for many shared genetic and environmental factors.

Given that MZ twins originate from a single fertilised egg and have the same genome, differences observed between a twin pair can be attributed to environmental influences rather than genetics. Though MZ twins share identical genomes, recent data has shown that they often have non-shared twin-specific factors, including the amnion, chorion and placenta, and that these non-shared features may explain MZ twin discordance (Figure 5-1) (Craig *et al.,* 2020*, in press*, "The environmental differences between twins in utero and their importance for downstream development – a need for standardized monitoring in obstetric research). In contrast, dizygotic twins (DZ) arise from two independent zygotes, and share 50% of their genomic sequence, as siblings do. Research has shown that many differences observed between identical twins are environmentally induced, and these differences are reflected in the epigenome (Czyz *et al.,* 2012; Kunio *et al.,* 2013).

DNA methylation of the cytosine base on CpG dinucleotides is known to vary throughout the lifespan, even between identical twin pairs (Ollikainen *et al.,* 2010; Gordon *et al.,* 2012; Martino *et al.,* 2013). A number of studies have analysed DNA methylation patterns in MZ twins, and have identified differentially methylated CpG sites between identical twin pairs. In 2013, Li *et al.* (2013) used BeadChip methodology to analyse DNA methylation in more than 27,000 CpG sites in 22 MZ twin pairs. They found that 92 CpG sites were significantly differentially methylated within twin pairs. Similarly, Du *et al.* (2015) identified 38 differentially methylated regions in four MZ twin pairs, and showed within-pair methylation differences.

Analysing the degree to which phenotypic traits are genetically inherited is particularly powerful in cohorts of MZ twins. Such studies play a critical role in determining the degree of environmental influence on complex diseases, and identifying molecular mechanisms of these diseases, which may, in turn, aid in improved therapeutic outcomes and more importantly, disease prevention.

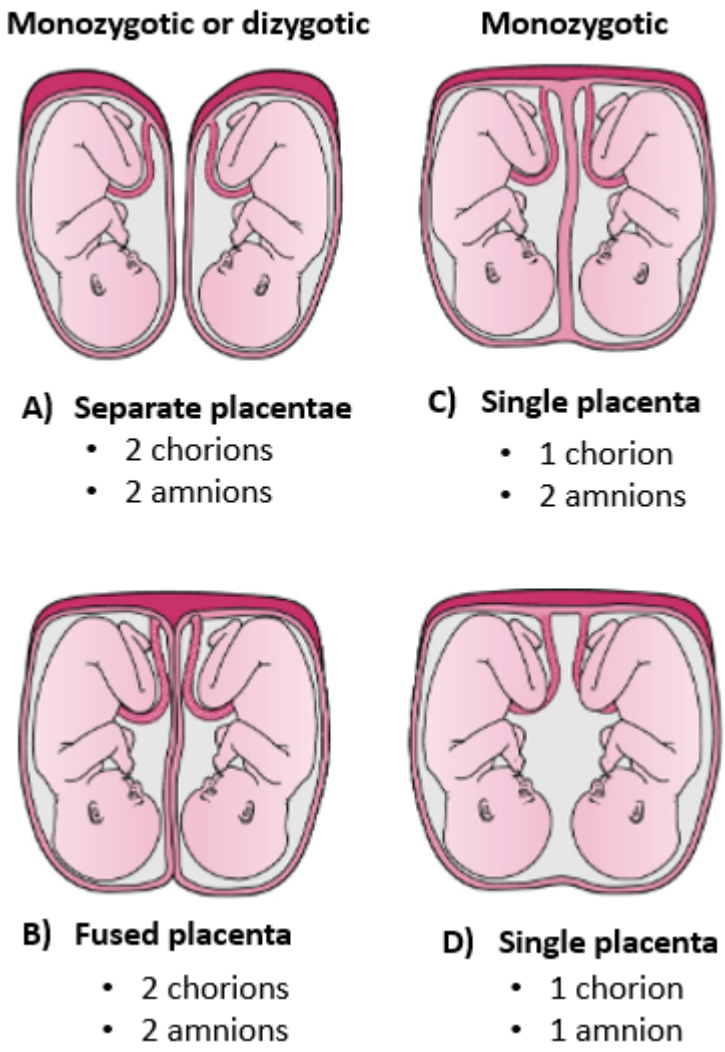


Figure 5-1. Types of twin pregnancy and the uterine environment. (A) Dichorionic-diamniotic twins. These twins have their own placenta, amniotic sac and umbilical cord, and can be monozygotic or dizygotic. In some cases, each placenta may grow in close proximity and fuse (B). (C) Monochorionic-diamniotic twins. Twins share a placenta but have separate amniotic sacs and umbilical cords. (D) Monochorionic-monoamniotic twins. Both twins share one placenta and one amniotic sac and are always monozygotic.

In this chapter, we evaluate *BRCA1* methylation in male and female monozygotic twin pairs to determine the extent of heredity and environmental influence on DNA methylation of the *BRCA1* gene. To address this, we assess constitutional *BRCA1* promoter methylation in buccal mucosa DNA and cord blood DNA collected from healthy female monozygotic twin

pairs at birth and at 6 years old. We also measure constitutional *BRCA1* methylation in buccal mucosa DNA of identical twin males at 6 years old. The highly sensitive droplet digital PCR (ddPCR) methodology was applied to quantify methylated and unmethylated target DNA in all samples.

5.2. Results

The *BRCA1* ddPCR assay outlined in Chapter 2, section 2.6.1.1 was used to quantify DNA methylation in MZ twins in section 5.2.1, 5.2.2 and 5.2.3. Although this assay has been discontinued since this project was carried out, it produced robust *BRCA1* methylation results using the manual droplet generation system, and was able to quantify *BRCA1* methylation below 1% in this twin cohort. ddPCR results presented in section 5.2.4 of this chapter were generated using the current optimised *BRCA1* methylation detection assay. For more detail on this assay, see Chapter 2, section 2.6.1.

5.2.1. Assessing constitutional *BRCA1* methylation in buccal mucosa DNA of female monozygotic twin pairs at birth.

Constitutional *BRCA1* methylation was assessed in buccal mucosa DNA obtained at birth from female identical twins (n = 73 pairs). However, due to the poor quality of buccal mucosa DNA in some cases, 41 twin pairs were excluded from *BRCA1* methylation analysis. Therefore, this section reviews data from the remaining 32 MZ twin pairs. Samples were excluded from this dataset if any of the following criteria were met: i) if one twin had less than 350 wild-type (i.e. unmethylated) *BRCA1* droplets; ii) if both twins had less than 350 wild-type (i.e. unmethylated) *BRCA1* droplets; and iii) if one or both twins had less than 3 *BRCA1*-methylated droplets. The exclusion criteria for this segment of the twin study is outlined in detail in Table 5-1.

MZ twins were classified into three categories: i) concordant negative (-/-), where both twins in a pair had no detectable *BRCA1* methylation; ii) discordant (+/-), where one twin in a pair is methylated and the other is not; and iii) concordant positive (+/+), where both twins in a pair are methylated. As observed in Figure 5-2, 3 of the 32 MZ twin pairs showed discordant *BRCA1* methylation (i.e. where one twin is methylated and the other is not). The observed *BRCA1* methylation frequency in this population was 4.7%, which is similar to the 2-4% frequency previously observed in healthy adult women (Lonning *et al.,* 2018). However, most twin pairs had no detectable *BRCA1* methylation. *BRCA1* methylation frequencies as well as the level of methylation observed in discordantly methylated twins is summarised in Table 5-2.

Table 5-1. Exclusion criteria of MZ twin pairs following *BRCA1* methylation analysis by ddPCR.

Exclusion Criteria	No. of Excluded Twin Pairs
1 twin has <350 WT droplets	N = 30 pairs
Both twins have <350 WT droplets	N = 7 pairs
1 or both twins have <3 methylated droplets	N = 4 pairs

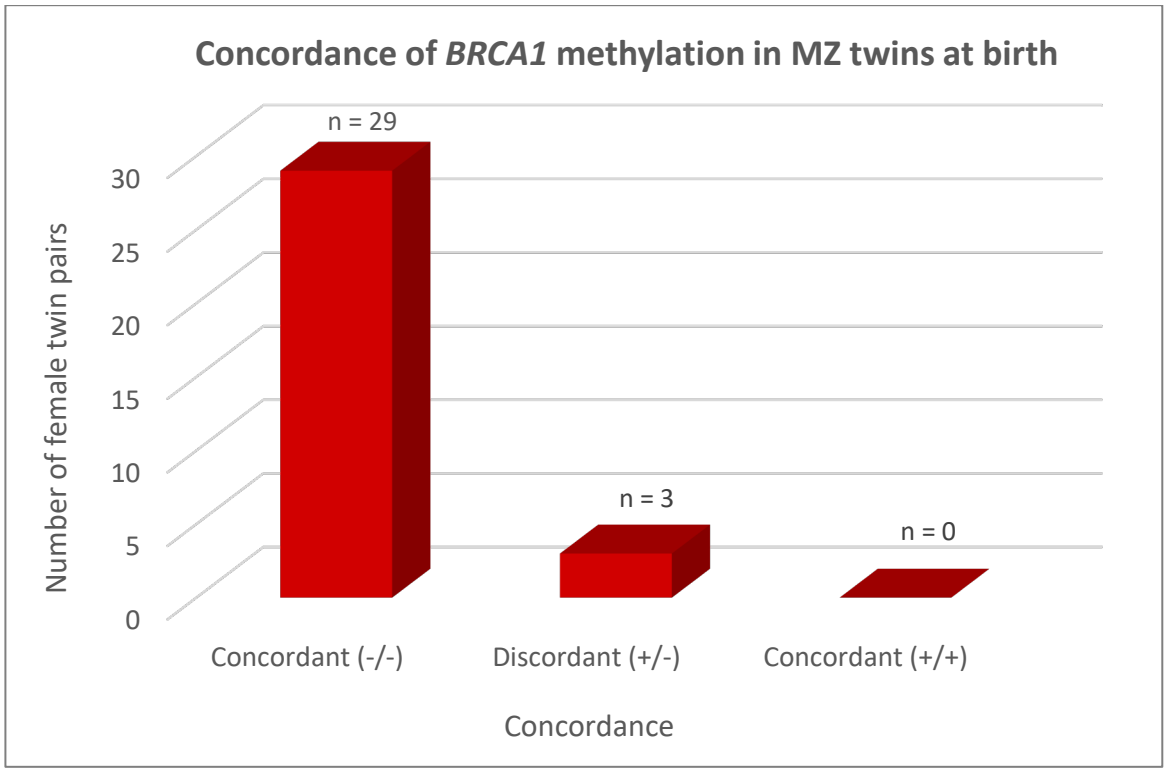


Figure 5-2. Histogram representation of *BRCA1* methylation rates detected in buccal mucosa of female MZ twins at birth. Constitutional *BRCA1* methylation was assessed in buccal mucosa DNA of 32 identical female twin pairs at birth. Most twin pairs had no detectable *BRCA1* methylation in buccal DNA (n = 29), and were classified as concordant negative. Three MZ twin pairs were discordant for *BRCA1* methylation, where one twin in a pair was methylated and the other was not. No twin pairs showed concordant positive *BRCA1* methylation.

Table 5-2. *BRCA1* methylation frequencies in buccal mucosa DNA of MZ female twin pairs at birth (n = 32 pairs). Values represent the number of MZ twins that are methylated. Percentage values represent the *BRCA1* methylation frequencies. Individual twins who presented with methylation were categorised into methylation <1% or between 1-3%.

	***BRCA1* methylated (total pairs)**	***BRCA1* methylated (<1%)**	***BRCA1* methylated (1-3%)**
Concordant (+/+)	0/32	0	0
Concordant (-/-)	29/32 (90.6%)	N/A	N/A
Discordant (+/-)	3/32 (9.3%)	2/3	1/3

5.2.2. *BRCA1* methylation frequency detected in buccal mucosa DNA of newborn females.

Given that some MZ twins had to be excluded from methylation analysis due to their co-twin meeting any of the exclusion criteria (Table 5-1), the frequency of *BRCA1* methylation was assessed in newborn girls from the same twin cohort outlined in section 5.2.1, by considering the twins as independent rather than as pairs (n = 94 newborns). Of the 94 girls assessed for *BRCA1* methylation in buccal DNA at birth, seven had detectable *BRCA1* methylation (methylation frequency = 7.4%). These findings are summarised in Figure 5-3.

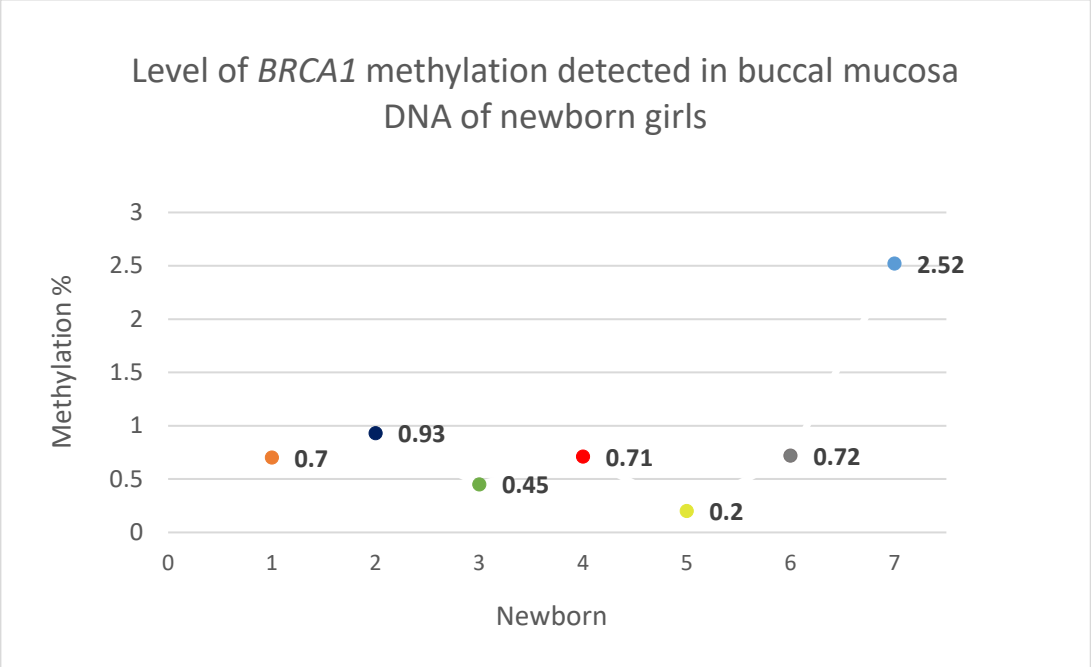


Figure 5-3. Degree of *BRCA1* methylation in buccal mucosa of seven newborn females. Seven of 94 newborn girls had detectable *BRCA1* methylation in buccal mucosa DNA. Each newborn is numbered from 1 to 7 on the X-axis. Each coloured spot corresponds to the single newborn numbered directly below. Methylation percentage observed for each newborn is outlined beside the coloured spot.

5.2.3. Assessing constitutional *BRCA1* methylation in white blood cell DNA from cord blood of monozygotic twins at birth.

Eighteen MZ twin pairs (n = 36 individuals) who were assessed for *BRCA1* methylation in buccal mucosa DNA at birth (section 5.2.1) were assessed for *BRCA1* methylation in an alternative tissue, to determine whether methylation observed in buccal DNA is maintained across other tissue types. Due to limited availability, DNA from cord blood was not attainable for all MZ twin pairs analysed for methylation at birth (see section 5.2.1).

Twin pairs were tested for *BRCA1* methylation in white blood cell (WBC) DNA isolated from their umbilical cord blood. DNA methylation analysis was performed using the *BRCA1* ddPCR assay outlined in Chapter 2, section 2.5.1.1.

Table 5-3 shows that *BRCA1* methylation was detected in cord blood DNA from four individuals in three MZ twin pairs (methylation frequency: 11%). Only one twin pair (Pair 1) was concordant positive (+/+), where both twins had detectable *BRCA1* methylation. Interestingly, twin Pair 1 showed no methylation in buccal mucosa DNA collected at birth. The remaining two MZ twin pairs (Pair 2 and Pair 4) showed discordant (+/-) *BRCA1* methylation in WBC DNA (i.e. only Twin B in each pair had detectable methylation, while Twin A did not). These twins also had detectable *BRCA1* methylation in buccal mucosa DNA at birth. Twin Pair 3 also showed discordant methylation, however, this pair was excluded from methylation analysis given that only two droplets were *BRCA1*-methylated in the positive twin.

Table 5-3. *BRCA1* methylation detected in white blood cell DNA of four MZ twin pairs. *BRCA1* methylation was assessed in WBC DNA of female MZ twin pairs. Twins were classified as concordant positive (+/+) or discordant (+/-). Whether *BRCA1*

methylation was detected in buccal mucosa at birth is also indicated. Twin pair 3 (shaded in grey) was excluded from final analysis due to having <3 *BRCA1*-methylated droplets.

Twin Pair		Methylated? (WBC DNA)	Methylation (%)	Concordance	Methylated? (buccal – birth)
Pair 1	**Twin A**	✓	0.7%	Concordant (+/+)	×
	Twin B	✓	0.3%		×
Pair 2	**Twin A**	×	0	Discordant (-/+)	×
	Twin B	✓	0.3%		✓ 0.4%
Pair 3	**Twin A**	×	0	Discordant (-/+)	×
	Twin B	✓	0.07%		×
Pair 4	**Twin A**	×	0	Discordant (-/+)	×
	Twin B	✓	0.7%		✓ 2.3%

5.2.4. Evaluating maintenance of *BRCA1* methylation in buccal mucosa DNA of female MZ twin pairs at 6 years old.

BRCA1 methylation was assessed in buccal mucosa DNA of 9 healthy MZ twin pairs (n = 18 individuals) at 6 years old to determine whether *BRCA1* methylation detected at birth is maintained overtime in the same individuals, and whether any twins who were unmethylated at birth gained methylation at 6 years. Findings show that at 6 years of age, only 1 twin pair (Pair 4, Table 5-4) was concordant for *BRCA1* methylation. However, when considered as individuals and not as pairs, methylation was present in five of the 18 individuals in this twin cohort, bringing the overall *BRCA1* methylation frequency to a significantly high 27% in 6 year olds. Interestingly, 3 of the 5 methylated 6-year-old twins also showed *BRCA1* methylation at birth, while 2 of the 5 methylated twins at 6 years had no detectable methylation at birth (Pair 1, Twin B and Pair 4, Twin B) (Table 5-4). Despite the high frequency of *BRCA1* methylation observed in this population, most 6-year-old females had no detectable *BRCA1* methylation in buccal mucosa DNA. The proportion of twins in this cohort who have gained, maintained or showed no *BRCA1* methylation since birth is demonstrated in Figure 5-4.

Table 5-4. *BRCA1* methylation observed in buccal mucosa DNA of four female MZ twins at 6 years of age. *BRCA1* methylation was assessed in buccal mucosa DNA of female MZ twin pairs at 6 years old. Twins were classified as concordant (+/+) where both twins in a pair are methylated, or discordant (+/-) if only one twin in a pair was methylated. Whether *BRCA1* methylation was detected in buccal mucosa at birth is also indicated.

Twin Pair		Methylated? (6 years)	Methylation (%)	Concordance	Methylated (buccal – birth)
Pair 1	**Twin A**	×	0	Discordant (-/+)	×
	Twin B	✓	1%		×
Pair 2	**Twin A**	×	0	Discordant (-/+)	×
	Twin B	✓	0.62%		✓ 0.3%
Pair 3	**Twin A**	✓	1.7%	Discordant (-/+)	✓ 0.85%
	Twin B	×	0		×
Pair 4	**Twin A**	✓	0.5%	Concordant (+/+)	✓ 0.58%
	Twin B	✓	0.4%		×

Figure 5-4.

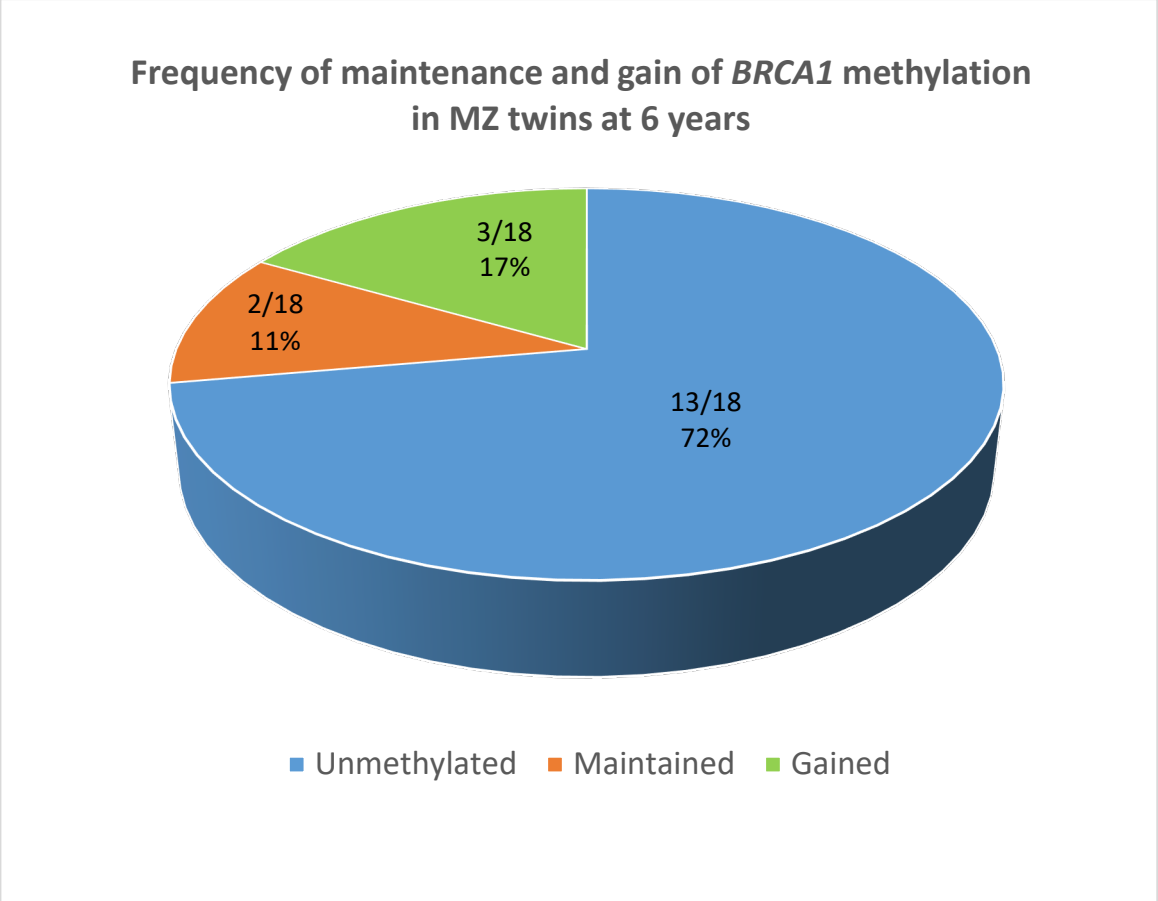


Figure 5-4. Pie Chart representation showing the proportion of MZ twins who gained or maintained *BRCA1* methylation since birth. Constitutional *BRCA1* methylation was assessed in buccal mucosa DNA of 18 individual MZ twins (n = 9 pairs) at 6 years of age. Most individuals had no detectable *BRCA1* methylation (blue). Frequency of females who maintained methylation at the same levels is demonstrated in orange, and the frequency of females who gained methylation since birth is indicated in green.

5.2.5. Determining *BRCA1* methylation frequency in buccal mucosa DNA of male MZ twins at 6 years of age.

Given that MZ twin girls showed high rates of discordant *BRCA1* methylation both at birth and 6 years, we anticipated that similar trends would be observed in male MZ twins. In this section, *BRCA1* methylation was analysed in buccal mucosa DNA of 6-year-old monozygotic twin boys (n = 15 pairs), to determine the *BRCA1* methylation frequency and rate of concordance. As demonstrated in Figure 5-5, most twin pairs had undetectable *BRCA1* methylation, while one twin pair showed discordant *BRCA1* methylation. Interestingly, only one twin pair was concordant for *BRCA1* methylation (i.e. where both twins in a pair were methylated). In total, 3 of the 30 individuals showed methylation, bringing the *BRCA1* methylation frequency in this cohort to 10%.

Figure 5-5.

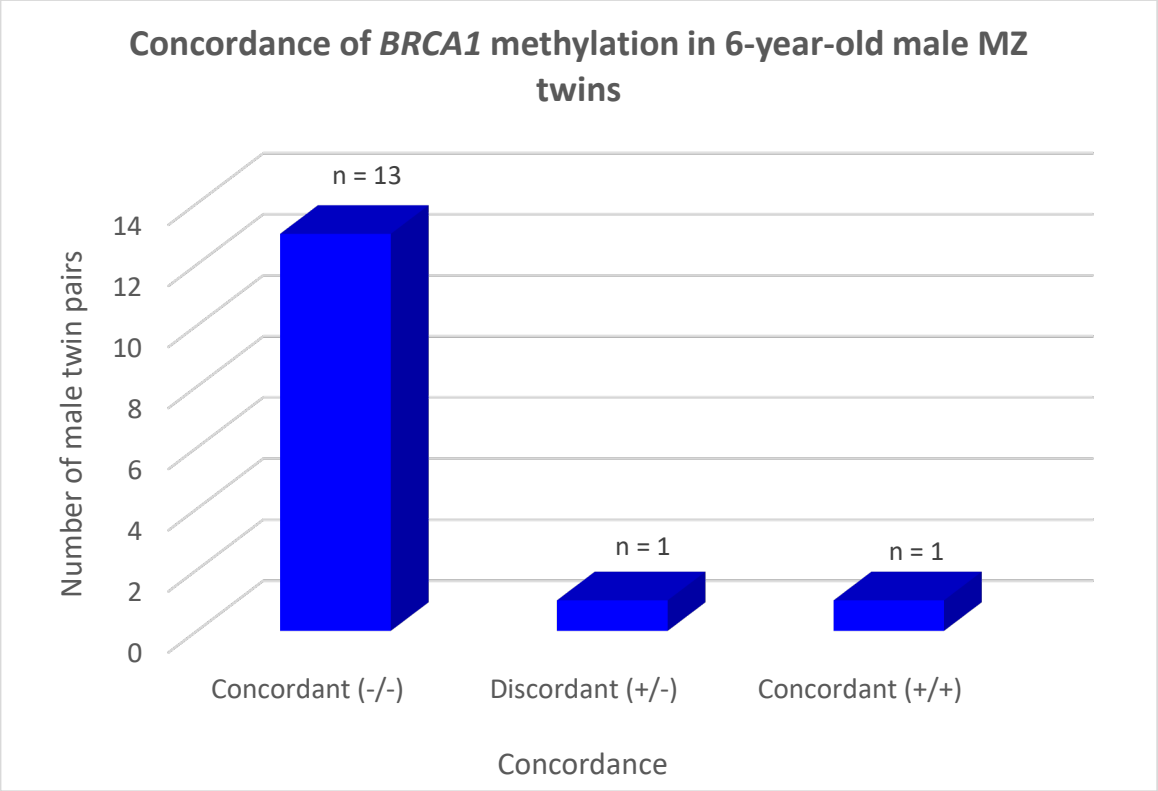


Figure 5-5. Histogram representation of *BRCA1* methylation rates detected in buccal mucosa of 6-year-old male MZ twins. Constitutional *BRCA1* methylation was assessed in buccal mucosa DNA of 15 identical male twin pairs. Most twin pairs had no detectable *BRCA1* methylation in buccal DNA (n = 13), and were classified as concordant negative. One MZ twin pair was discordant for *BRCA1* methylation, and 1 MZ twin pair was concordant positive.

Table 5-5. *BRCA1* methylation frequencies in buccal mucosa DNA of 6-year-old male MZ twin pairs (n = 15 pairs). Values represent the number of MZ twins that are methylated. Percentage values represent the *BRCA1* methylation frequencies. Twin pairs who presented with methylation were categorised into methylation <1% or between 1-3%.

	***BRCA1* methylated (total pairs)**	***BRCA1* methylated (<1%)**	***BRCA1* methylated (1-3%)**
Concordant (+/+)	1/15 (6.6%)	1/1	0
Concordant (-/-)	13/15 (86.6%)	N/A	N/A
Discordant (+/-)	1/15 (6.6%)	0	1/1

5.3. Discussion

The results presented in Chapter 5 reveal complex but consistent methylation patterns in DNA from healthy monozygotic twins. The twin study design was exploited to explore factors that influence variations in DNA methylation of the *BRCA1* gene between individuals. The present study revealed that the unshared intrauterine environmental factors may contribute to the inter-individual variation in *BRCA1* methylation observed within MZ twin pairs.

The initial segment of this study assessed constitutional *BRCA1* methylation in buccal mucosa DNA of identical twin girls sampled at birth, to control for environmental and lifestyle factors that may impact an individual's methylation profile (section 5.2.1). We observed poor concordance of *BRCA1* methylation between MZ twin pairs, given that only discordant *BRCA1* methylation was identified in three twin pairs. The absence of concordant methylation observed in twin pairs suggests that genetics has little influence on *BRCA1* methylation. These findings are supported by previous studies showing discordant *BRCA1* promoter methylation in MZ twins (Heyn *et al.,* 2012; Galetzka *et al.,* 2012).

The exclusion criteria outlined in Table 5-1 limited the accurate attainment of *BRCA1* methylation frequency in the twin population (section 5.2.1). For this reason, all healthy newborn females with available data were analysed for *BRCA1* methylation, irrespective of the availability of their co-twin data (n = 94 newborns) (section 5.2.1.1). *BRCA1* methylation was identified among 7 of the 94 newborns (7.4%), consistent with recently published data by Lonning *et al.* (2018), who identified a methylation frequency of 7% in newborns. Similarly, Al-Moghrabi *et al.* (2018) found *BRCA1* methylation frequencies of 9.9% in 295 newborn females. Interestingly, the *BRCA1* methylation frequencies we and others have observed in newborns is significantly higher than the *BRCA1* methylation

frequency reported in healthy adult women (Lonning *et al.,* 2018), suggesting that the high rates of *BRCA1* methylation may be attributed to sporadic methylating events during early development, which stabilise later in life.

When *BRCA1* methylation was assessed in WBC DNA of the same cohort of twins (section 5.2.2), only one twin pair showed concordant methylation, while two twin pairs showed discordant methylation (n = 4 methylated individuals in total). Interestingly, the discordant twins showing methylation were also methylated in buccal DNA. However, the twin pair concordantly methylated in WBC DNA was unmethylated in buccal mucosa (Table 5-3). When analysing whether *BRCA1* methylation persisted across various tissue types over time, only one twin was found to maintain methylation in buccal DNA at birth and 6 years, and WBC DNA (twin Pair 2, Twin B, Table 5-4). Again, the methylation frequency was relatively high (11.1%), conflicting with previous findings of *BRCA1* methylation in healthy adult women. *BRCA1* methylation detected in buccal DNA was not detected in WBC DNA of most MZ twins. Two theories behind this observation may be that constitutional *BRCA1* methylation in particular may be tissue-specific to a certain degree, or occur as a sporadic methylating event in early life. Although DNA methylation has been reported as the most stable epigenetic mark (Kim and Costello 2017), our findings highlight the erratic nature of DNA methylation of the *BRCA1* promoter during early life.

BRCA1 methylation has previously been detected in WBC DNA among adults and newborns (Lonning *et al.,* 2018) indicating that constitutional *BRCA1* methylation may occur as a prenatal event. Such findings will provide a greater understanding into healthy tissue methylation and the implications on cancer predisposition later in life.

We observed a considerably high *BRCA1* methylation frequency of 27% in healthy MZ female twins at 6 years, significantly contrasting to reported methylation frequencies in

adult females (Wong *et al.,* 2011). The differences we observed in *BRCA1* methylation frequencies compared to the published literature can partly be explained by the methodologies used to quantify methylation. An abundance of literature exists outlining the high analytical sensitivity and superiority of ddPCR in comparison to alternative methodologies (Hindson *et al.,* 2013; Wiencke *et al.,* 2014; Demuth *et al.,* 2018), particularly in samples with minimal amounts of starting material or poor quality DNA (Taylor *et al.,* 2017). It is also possible that external environmental factors including physical activity and diet, have influenced the methylation patterns and frequencies we observed (Fraga *et al.,* 2005). Recent studies have also shown that childhood stress related to school-aged children leads to DNA methylation changes that resemble those of biological aging (Natt *et al.,* 2015).

Research has shown that MZ twin pairs share many epigenetic similarities at birth and eventually diverge with age (Pal and Tyler 2016; Jones *et al.,* 2015); however, conflicting evidence exists (Martino *et al.,* 2013; Gordon *et al.,* 2012). Interestingly, we observed high rates of discordant *BRCA1* methylation at birth and at 6 years in all tissues of MZ females. For this reason, we assessed *BRCA1* methylation in buccal DNA of 6-year-old identical twin males. We found a methylation frequency of 10% in MZ males (section 5.2.4), and only one male twin pair showed concordant *BRCA1* methylation (Table 5-5). Additionally, methylation frequencies in females at 6 years compared to males was much higher (27% and 10% respectively).

A more recent study analysing epimutations in 700 MZ twin pairs found that 30% of epimutations showed discordant methylation patterns between identical twins (Sharp *et al.,* 2019). These findings indicate that some epimutations can occur sporadically or post-zygotically. Additionally, two studies of MZ twins who were discordant for cancer have provided interesting findings into variable DNA methylation patterns within twin pairs

(Heyn *et al.,* 2015; Galetzka *et al.,* 2012). Using 450k array data, Heyn *et al.* (2015) revealed that MZ twins discordant for breast cancer were also discordant for DOK7 hypermethylation detected in the blood, and that this epigenetic biomarker was detectable years before cancer diagnosis. Collectively, these findings indicate constitutional methylation of some disease-associated genes can play an important role in predicting disease risk, and potentially the opportunity for disease intervention.

The high discordance rates presented in our findings suggest that methylation differences observed within twin pairs is not likely due to genetic differences, but rather environmental epigenetic influence. Discordant *BRCA1* methylation in MZ twins at birth may be attributed to the non-shared intrauterine environment (Gordon *et al.,* 2012). However, due to various and unexpected sample limitations, only limited observations and conclusions could be made from the birth and 6-year data. Section 5.4 will discuss the limitations of this study and suggestions for future research.

5.4. Limitations of the study and suggested improvements

The methodology and assay used to study constitutional *BRCA1* methylation in this chapter is robust. However, the *BRCA1* methylation assay used in section 5.2.1, 5.2.2 and 5.2.3 has since been discontinued. Due to limited amounts of DNA, these samples could not be repeated on the latest assay (Chapter 2, section 2.5.1.1). Although some samples outlined in Chapter 3 and Chapter 5 have been tested on both *BRCA1* methylation assays and produced comparable results, it would also be ideal to run the twin samples on the current *BRCA1* methylation to ensure results are replicated.

A major limitation to this study was the need to exclude 41 of the 73 MZ twin pairs at birth (section 5.2.1) due to missing data. The exclusion of 56% of the sample size significantly reduced our ability to assess *BRCA1* methylation concordance rates in buccal mucosa DNA

in a larger cohort, given that some twins with adequate DNA and methylation present had to be excluded due to their co-twin lacking DNA or having extremely poor quality DNA that was non-amplifiable.

Additionally, when assessing whether *BRCA1* methylation detected in buccal DNA at birth is sustained overtime, and also across an alternative tissue (i.e. WBC DNA), we were again limited in obtaining matched WBC DNA samples for all MZ twins assessed at birth. For this reason, we could not accurately determine whether *BRCA1* methylation was maintained across different tissues, as we could not obtain matched buccal DNA and WBC DNA for all twins assessed for methylation at birth. Due to the missing data across birth samples, matched 6-year-old samples and WBC samples, statistical analyses comparing the three sub-studies could not be achieved.

Although the DNA obtained from male MZ twin males was of high quality (section 5.2.4), DNA from buccal mucosa was only available for 6-year-olds. Future studies should include matched birth samples to enable observation of *BRCA1* methylation patterns in male MZ twins overtime.

Finally, the type of twin pregnancy for each of the twin pairs analysed in this study was unknown. Given that the shared uterine factors vary depending on the type of twin pregnancy (Figure 5-1), this may have influenced the observed concordance or discordance of constitutional methylation within twin pairs. Prospective studies should control for these environmental factors by analysing constitutional *BRCA1* methylation in a cohort of monochorionic-monoamniotic twins, as this subtype of MZ twins share a placenta and amniotic sac.

Priorities for future research would involve looking at constitutional *BRCA1* methylation in multiple tissues including buccal mucosa, WBC, and peripheral blood DNA from the

entire twin cohort at all time points. Moreover, increasing the MZ twin sample size for both females and males and various time points across the lifespan would aid in identifying trends in *BRCA1* methylation that may be occurring. Such trends may be observed at the population level, within twin pairs, or at an inter-individual level. Finally, longitudinal studies will be necessary to determine the timing of *BRCA1* methylation in relation to disease onset, and to determine its role in cancer predisposition and progression.

5.5. Summary

To our knowledge, this is the first study to quantify constitutional *BRCA1* methylation in identical twins by ddPCR. We found that *BRCA1* methylation was detected in buccal mucosa and WBC DNA of newborn MZ twins and 6-year-old twins. We also observed high discordance rates of constitutional *BRCA1* methylation across twin pairs at birth and at 6 years, and also across the two tissue types. Collectively, our data suggests that healthy tissue methylation of the *BRCA1* gene most likely occurs as an early embryonic event that may inform the risk of developing breast cancer later in life. However, extensive research is needed to understand the molecular mechanisms underlying phenotypic disease discordance in MZ twins, which may lead to novel theoretical and experimental opportunities in health and disease. Finally, understanding the interaction between genetics and environment, and its influence on disease may inform clinical practice and lead to new approaches to defining breast cancer risk.

CHAPTER 6.

DETETCTION OF *RASSF1A* AND *RARβ* METHYLATION IN TUMOUR AND PERIPHERAL BLOOD OF MELANOMA PATIENTS.

6.1. Introduction

Aberrant DNA methylation has been commonly observed in melanoma, and is recognised as a crucial component in tumour initiation and progression (Schinke *et al.,* 2010). Melanoma is the most common cancer affecting young Australian men and women aged between 15 and 39 years (AIHW 2019), and is often associated with poor prognosis due to a lack of effective targeted therapies for those without common driver mutations such as *BRAF* V600E. This has led to the exploration of alternative mechanisms that underlie the disease.

A number of studies have described hypermethylation of tumour suppressor genes in melanoma. In 2009, Koga *et al.* assessed promoter methylation profiles of eight human melanoma cell lines and compared them to newborn and adult melanocytes. They reported 76 DNA methylation markers, 68 of which were hypermethylated, and eight were hypomethylated. More recently, a meta-analysis identified 50 hypermethylated genes that are associated with melanoma, including *CLDN1, MGMT, RASSF1A* and *RARβ* (Guo *et al.,* 2018). Taken together, these findings indicate that certain genes may be implicated in an increased melanoma risk.

Methylation markers in various cancer types have been implicated in predicting prognosis and response to treatment (Mikeska and Craig, 2014). Further studies have also described the value of methylated genes in predicting survival of melanoma patients. In 2010, Lahtz *et al.* reported that melanoma patients with somatic *PTEN* gene silencing by DNA methylation have a significantly poorer survival rate. These findings highlight the clinical importance of identifying loci that have a tendency to be methylated in melanoma.

RASSF1A and *RARβ* methylation have also been observed in melanomas, particularly in the metastatic setting (Hoon *et al.,* 2004). Most research has assessed methylation of these loci in tumour or in cell line DNA. While important and informative, the limitations of these studies are two-fold: i) reliance on cell lines is poor, as cell line DNA is known to have an altered DNA methylation landscape (Maitra *et al.,* 2005), and ii) assessment of tumours alone provides no information that can be used for predisposition and early detection.

Given that epigenetic changes are influenced by environmental factors including UV exposure, it is particularly important to assess DNA methylation in both the primary tumour and healthy tissue. For this reason, this chapter will explore the methylation frequencies of *RASSF1A* and *RARβ* in primary melanomas, as well as in the corresponding peripheral blood DNA from each patient.

6.2. Results

All results presented in this chapter were generated using methylation sensitive high resolution melting (MS-HRM), with the assays outlined in Chapter 2, sections 2.6.2 and 2.6.3. All bio-specimens used in this study were obtained from patients who are enrolled in the Melanoma Research Victoria (MRV) study. Formalin-fixed paraffin-embedded (FFPE) tissue of primary melanomas and blood cell pellets were provided by MRV to the Translational Genomics and Epigenomics Laboratory, for 25 melanoma patients.

6.2.1. Temperature optimisation of *RASSF1A* assay for methylation detection in peripheral blood.

To enable the detection of low-level DNA methylation, annealing temperatures are often increased to boost sensitivity of the PCR assay (Hecker and Roux 1996). Hence, a temperature gradient was performed on control DNA to determine optimum conditions for

RASSF1A methylation detection. Given that sensitivity of the assay was being assessed, a methylation dilution series of 100%, 50% 10%, 3% and 0% was created using a bisulfite modified negative methylation control derived from healthy donor peripheral blood mononuclear cells (PBMC), combined with CpGenome as the fully methylated control (100%).

The temperature optimisation for the *RASSF1A* methylation assay was two-fold. The first stage involved assessing the methylation status and amplification efficiency of all dilutions at an annealing temperature of 58ºC (Figure 6-1, A and B). The second stage assessed the ability of the assay to detect lower levels of methylation (i.e. 10% and 3%) by increasing the annealing temperature in the PCR reaction to 60ºC (Figure 6-1, C and D).

Interestingly 100% PCR efficiency was achieved at an annealing temperature of 58ºC compared to 60ºC (approximately 65%) (Figure 6-1, B and D respectively) for the 10% and 3% dilutions, and no amplification of the NTC was observed at either temperature. Given these findings, an annealing temperature of 58ºC was used for measuring *RASSF1A* methylation in tumour and blood DNA of melanoma patients.

Figure 6-1.

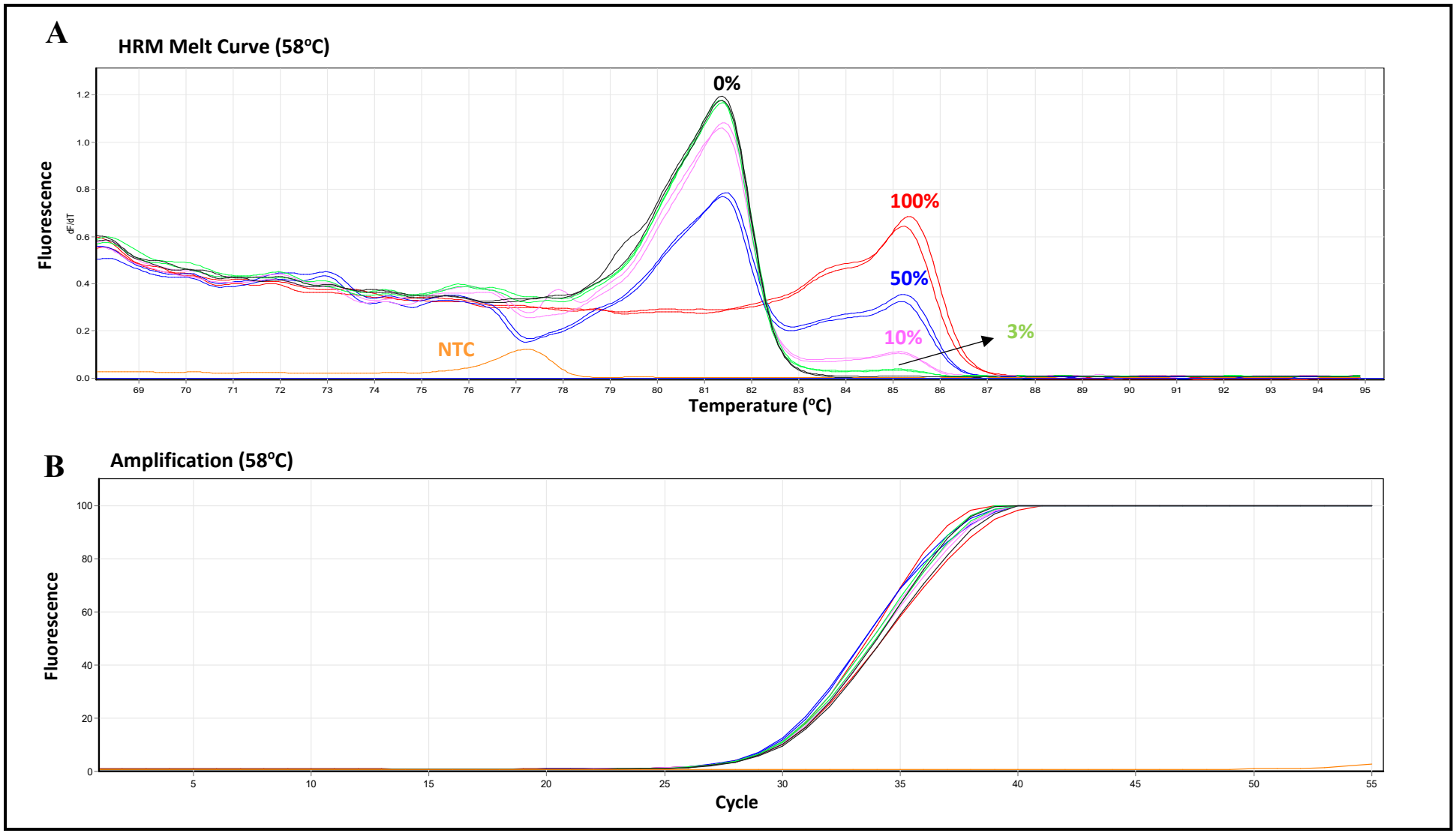

A
HRM Melt Curve (58°C)
Fluorescence
dF/dT
0%
100%
50%
10%
3%
NTC
Temperature (°C)
B
Amplification (58°C)
Fluorescence
Cycle

Figure 6-1. *Continued*

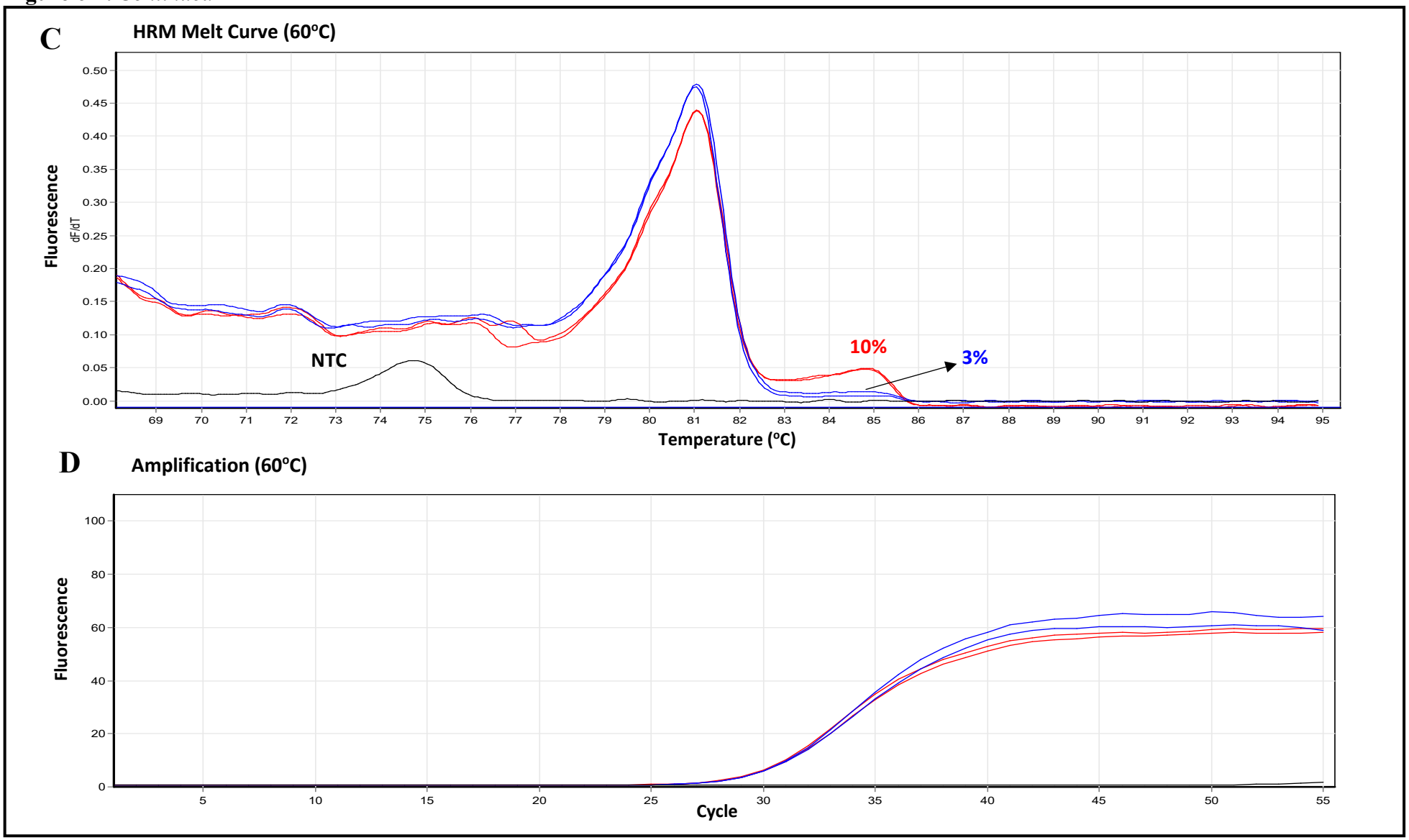


Figure 6-1. *RASSF1A* methylation detection by MS-HRM in a dilution series at 58ºC and 60ºC. (A) HRM melt curve of *RASSF1A* methylation in a dilution series at an annealing temperature of 58ºC. Each dilution is colour-coded and presented as duplicate peaks; 100% (blue), 50% (red), 10% (pink), 3% (green), 0% (black) and NTC (orange). (B) Amplification efficiency curve of each dilution at 60ºC. (C) HRM melt curve of *RASSF1A* methylation in dilutions of 10% (red) and 3% (blue), performed in duplicate. NTC is presented as a black peak. (D) Amplification efficiency curve of each dilution and NTC at 58ºC.

6.2.2. Measuring *RASSF1A* methylation in tumours and peripheral blood of melanoma patients by HRM.

RASSF1A methylation was assessed in primary melanoma tumours and matched peripheral blood samples of 25 melanoma patients. Three of the 25 tumours were methylated for *RASSF1A* (12%). High levels of methylation were observed in tumours of Patients 1 and 2 (100% and 50% respectively); however no peripheral blood methylation was detected. These findings indicate that highly methylated primary tumours do not indicate constitutional methylation of *RASSF1A* but a somatic event.

Interestingly, Patients 16 and 24 had unusual melt patterns detected in their blood (Figure 6-2) which may indicate a single nucleotide polymorphism (SNP) in the *RASSF1A* promoter region. Given that the melt curve of the putative SNP has two peaks produced at an equivalent frequency, this demonstrates that the SNP is likely a germline alteration that is inherited from a single parent.

Table 6-1. *RASSF1A* methylation status in tumours and blood of melanoma patients. *RASSF1A* methylation assessed in tumour and blood is presented for 25 melanoma patients. 3 of 25 patients had detectable *RASSF1A* methylation in their primary tumours. No methylation was detected in blood of any melanoma cases. Patients with detectable SNPs are highlighted in orange.

Patient no.	Tumour methylation (%)	Tumour purity
1	100	70-80%
2	50	20-30%
3	0	60%
4	0	50%
5	0	90%
6	0	10%
7	10	10%
8	0	20%
9	0	5%
10	0	5%
11	0	20%
12	0	5%
13	0	30%
14	0	70%
15	0	30%
16	0	10%
17	0	20%
18	0	80%
19	0	20%
20	0	10%
21	0	40%
22	0	10%
23	0	5%
24	0	20-30%
25	0	20%

Figure 6-2.

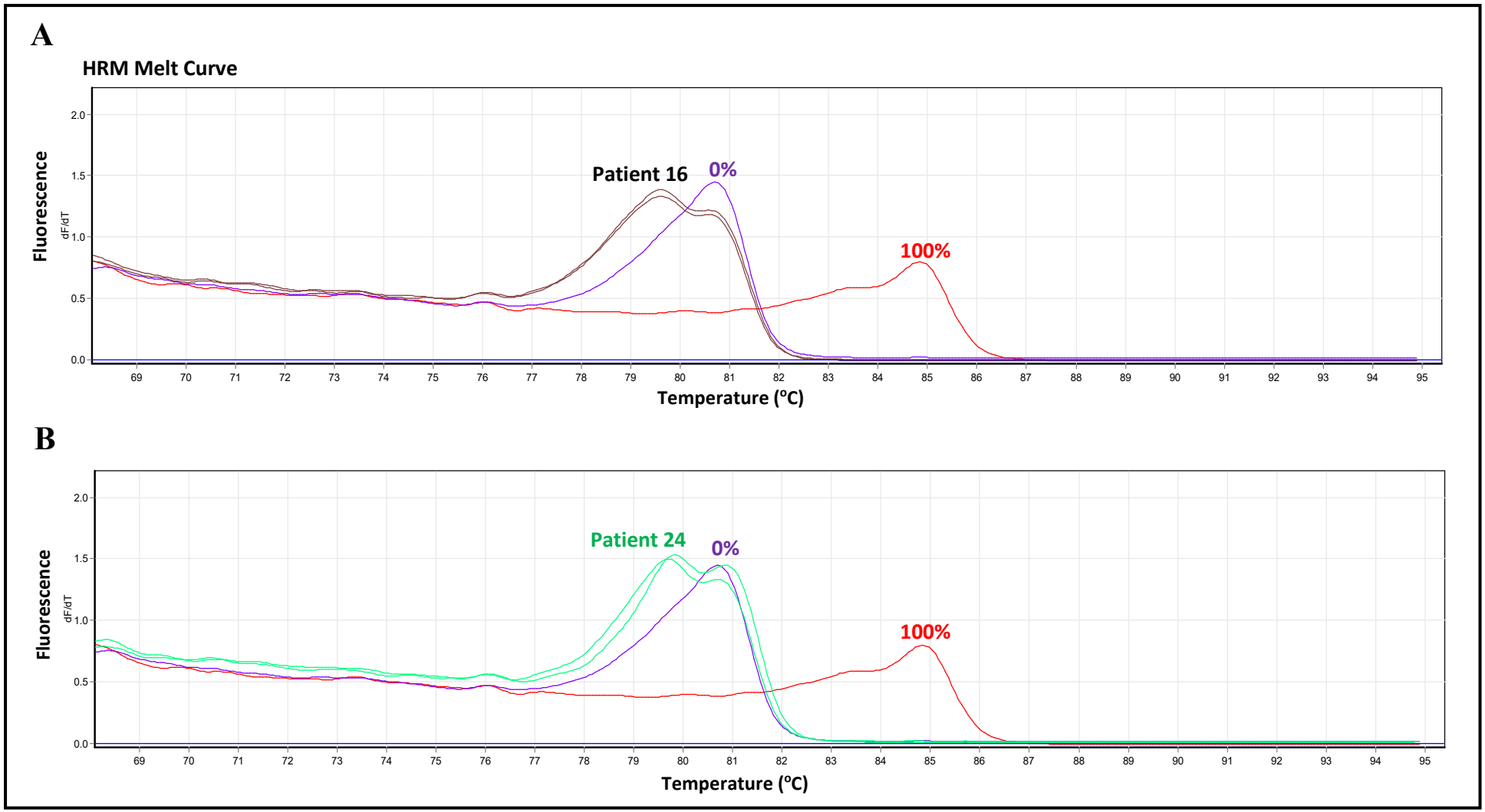

A
HRM Melt Curve
Fluorescence
dF/dT
Patient 16
0%
100%
Temperature (°C)
B
Fluorescence
dF/dT
Patient 24
0%
100%
Temperature (°C)

Figure 6-2. HRM SNP identification in peripheral blood of two melanoma patients at the *RASSF1A* locus. *RASSF1A* HRM melt curves are presented for two melanoma patients. **(A)** Melt curve for Patient 16 showing detection of a SNP identified in blood pellet DNA. The patient sample was run in duplicate and is highlighted by the two black overlapping melt curves. 0% and 100% methylated control DNA (purple and red respectively) are also included for reference. **(B)** Melt curve for Patient 24 showing a SNP detected in blood pellet DNA. The duplicate patient sample is demonstrated by the two green overlapping melt curves.

6.2.3. Measuring *RARβ* methylation in tumours and peripheral blood of melanoma patients by HRM.

RARβ methylation was assessed in primary melanoma tumours and matched peripheral blood samples of 25 melanoma patients. 19 of 25 patients had detectable *RARβ* methylation in their tumours (76%), whereas no methylation was detected in blood (Figure 6-3). High levels of methylation (>50%) were observed in all patients with detectable tumour methylation, however no methylation was detected in peripheral blood.

Findings showed that one patient (Patient 24) had a SNP in the *RARβ* detected in their tumour DNA, despite no methylation being detected.

Table 6-2. *RARβ* methylation status in tumours and blood of melanoma patients. *RARβ* methylation assessed in tumour and blood is presented for 25 melanoma patients. 19 of 25 patients had detectable *RARβ* methylation in their primary tumours, whereas no methylation was detected in blood. One patient had a detectable SNP and is highlighted in orange.

Patient no.	Tumour methylation (%)	Tumour purity	Blood methylation (%)
1	100%	70-80%	0
2	100%	20-30%	0
3	50%	60%	0
4	0	50%	0
5	0	90%	0
6	50%	10%	0
7	100%	10%	0
8	10%	20%	0
9	50%	5%	0
10	50%	5%	0
11	50%	20%	0
12	0	5%	0
13	100%	30%	0
14	50%	70%	0
15	0	30%	0
16	50%	10%	0
17	50%	20%	0
18	50%	80%	0
19	100%	20%	0
20	50%	10%	0
21	50%	40%	0
22	100%	10%	0
23	50%	5%	0
24	0 - SNP	20-30%	0
25	0	20%	0

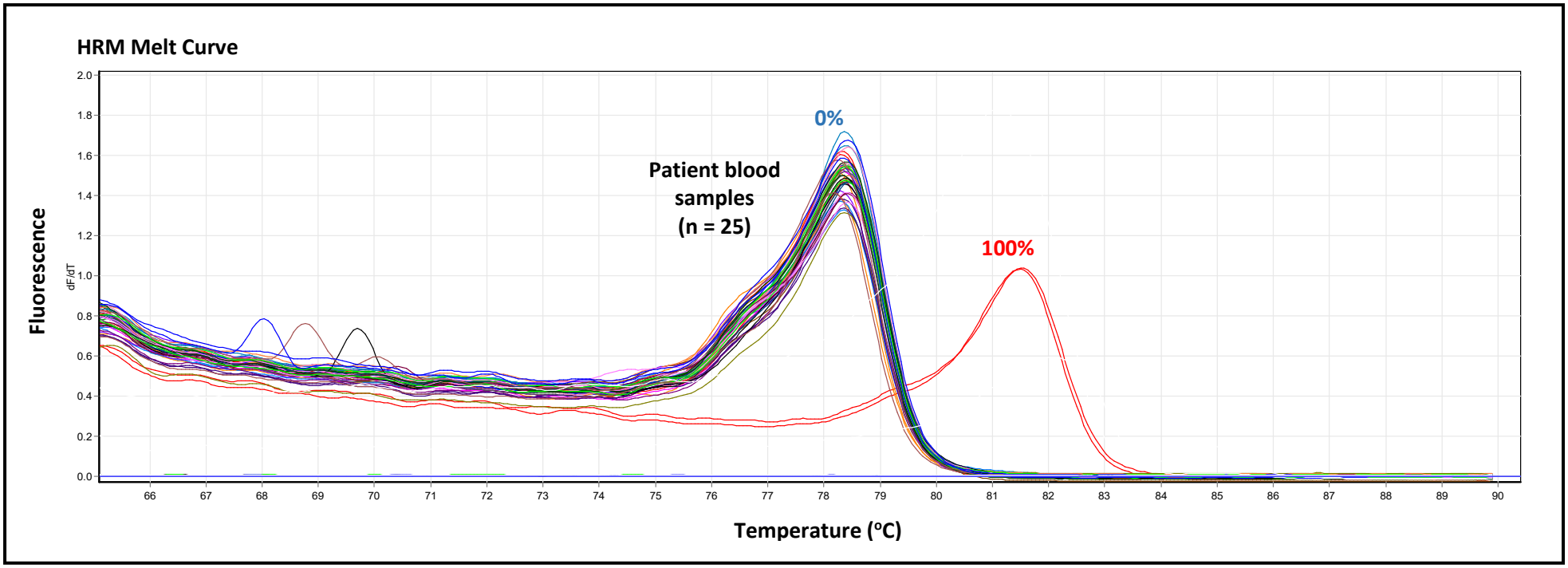


Figure 6-3. HRM methylation analysis of *RARβ* in blood pellets of melanoma patients. *RARβ* methylation was assessed in blood pellets of 25 melanoma patients. The left coloured peaks represent 25 superimposed melanoma patient samples, including the unmethylated (0%) control DNA (i.e. healthy donor DNA). All patient samples are unmethylated. The 100% methylated control DNA (i.e. CpGenome) is represented by the red peak. All samples were run in duplicate.

6.3. Discussion

The current study investigated the relationship between tumour methylation and healthy tissue methylation (i.e. constitutional methylation) of *RASSF1A* and *RARβ* in a cohort of melanoma patients (n = 25). This study revealed a significantly higher methylation frequency of *RARβ* in primary melanomas when compared to methylation detected of *RASSF1A.* However, both loci were unmethylated in matched peripheral blood DNA of these patients.

Epigenetic silencing of tumour suppressor genes has been implicated in the tumorigenesis of melanoma (Herman and Baylin 2003). The present study detected *RARβ* methylation at significantly higher rates in melanoma samples compared to *RASSF1A* (76% versus 12% respectively). These findings support previous data demonstrating that *RARβ* methylation was observed at rates of up to 70% in primary melanoma, whereas *RASSF1A* methylation in primary tumours was only detected at a frequency of 15% (Hoon *et al.,* 2004). Earlier work has also reported that *RASSF1A* methylation is significantly higher in metastatic melanomas compared to primary lesions (Hoon *et al.,* 2004), which may explain the low *RASSF1A* methylation frequency observed in the present study.

Most detectable *RASSF1A* and *RARβ* methylation levels were between 50% and 100% in tumours. Despite the high levels of methylation detected in the tumours, these methylation levels were not reflected in blood cell pellets from the same patients, suggesting that a methylated primary tumour is not indicative of constitutional methylation. Determining the methylation status at particular loci in tumours alone is key in certain cancer types (Dejeux *et al.,* 2010; Connolly *et al.,* 2018). For example, *MGMT* methylation in glioblastoma is significantly associated with improved survival and overall response rate in patients treated with temozolomide compared to patients without *MGMT* methylation in their tumours (51.8% versus 17.7% respectively) (Campana *et al.,* 2018). Thus, identifying tumour

methylation in relevant genes can play a vital role in determining treatment regimens for patients.

Given that methylation was only detected in primary tumours suggests that *RASSF1A* and *RARβ* methylation may be tumour specific in early-stage melanoma. However, this does not preclude constitutional methylation of these loci from being a rare epigenetic phenomenon. Despite the absence of methylation in blood pellet DNA, putative SNPs were identified in two patient samples when assessed for *RASSF1A* methylation (Figure 6-2). Earlier work has shown that SNPs may predispose individuals to somatic methylation of certain genes (Candiloro and Dobrovic 2009), and one such gene is *MGMT*. Candiloro and Dobrovic (2009) reported that colorectal cancer patients who possess the T allele of the rs16906252 SNP in their tumours were strongly predisposed to developing *MGMT*-methylated colorectal tumours. In some cases, SNPs in many genes including *BRCA1/2, TP53, MLH1* and *RAD51C* have been shown to be hereditary and are associated with increased cancer risk (Mahdi *et al.,* 2013; Cybulski *et al.,* 2011), and thus present an opportunity for screening in high-risk populations. The implications of the SNPs detected in *RASSF1A* in the blood of melanoma patients throughout this study remain unknown, and the significance of crosstalk among SNPs and methylation patterns requires further investigation.

Constitutional methylation of both *RASSF1A* and *RARβ* was not detected in the blood of any patient, indicating that methylation of these genes is unlikely to predispose to the development of melanoma, and that their methylation is not involved in the cancer initiation process given that methylation was absent in the blood of all early-stage melanoma patients. In patients who showed tumour methylation, it is unknown whether methylation was present in adjacent normal tissue. Limitations of this study and suggestions for future research are addressed in section 6.4.

6.4. Limitations of the study and suggested improvements

The DNA extracted from FFPE tissue and blood cell pellets were of high quality for all melanoma patients. However, prospective improvements to this study relating to tissue types and sample size could be considered. In this section, limitations encountered during this study will be reviewed.

As outlined, this study revealed no detectable methylation of *RASSF1A* and *RARβ* in the blood of any melanoma patient, though tumour methylation of these loci has been detected in metastatic melanoma lesions in previously published literature. Given this knowledge, it would be informative to obtain metastatic tumour tissue or tumour DNA samples from patients in this cohort who have progressed to advanced disease to better understand the role of *RASSF1A* and *RARβ* methylation in the progression of melanoma. Assessing methylation in healthy tissue including peripheral blood DNA and adjacent normal tissue of these patients is also essential in gaining a greater understanding of the association between tumour methylation and constitutional methylation of these loci in melanoma. The use of peripheral blood can be powerful as it is relatively non-invasive to attain and blood-based methylation markers can be identified, validated and applied in the clinical setting for early detection, prognosis and disease monitoring, as well as determining treatment regimens in some cases. Furthermore, observing progression-free survival and overall survival in patients where methylation of *RASSF1A* and *RARβ* was identified in primary melanomas may identify trends in treatment response relative to the patient's methylation status.

Although the present study focused on two loci as putative methylation markers of predisposition in melanoma, larger combinations of DNA methylation markers may achieve increased precision in predicting clinical relevance based on the methylation status of these loci in melanoma patients.

6.5. Summary

This study identified tumour methylation in primary melanoma samples in both *RASSF1A* and *RARβ,* albeit methylation was much more prominent in *RARβ.* The population frequency of tumour methylation in both loci was comparable to published literature (76% in *RARβ* versus 12% in *RASSF1A*). When constitutional methylation of both genes was assessed, methylation of either gene was not detected in the matched blood samples of any melanoma patients. Collectively, this data suggests that hypermethylation of *RASSF1A* and *RARβ* are unlikely to predispose to the development of melanoma, and that constitutional methylation of these genes is not be clinically relevant in identifying individuals who may be predisposed to developing melanoma. Rather, hypermethylation of these loci is more likely acquired as disease progresses given that higher methylation frequencies have been identified in metastatic melanomas (Hoon *et al.,* 2004). Such findings warrant further research to determine the correlation between tumour methylation and constitutional methylation, particularly in advanced disease.

CHAPTER 7.

DISCUSSION AND CONCLUSION

7.1. General discussion

Loss of function of tumour suppressor genes due to promoter hypermethylation is crucial in driving neoplastic processes of various cancer types. The inactivation of tumour suppressor genes by promoter methylation is often observed in genes involved in hereditary cancer many of which are DNA repair genes, however the cause of this aberrant methylation remains largely unknown. In some cancer cases, constitutional methylation of hereditary cancer genes can be detected in disease-free tissue, particularly peripheral blood, and is associated with disease susceptibility (Chan *et al.,* 2006; Hitchins *et al.,* 2007; Dobrovic and Kristensen 2009).

7.1.1. Breast cancer

Despite significant advancements in screening and therapeutic regimens, breast cancer remains the leading cause of cancer-related death in women worldwide, and the second leading cause of cancer death in Australia (AIHW 2017; Global Health Estimates 2016). Given that a proportion of sporadic breast cancers have a *BRCA1* methylated tumour, it is important to determine whether constitutional *BRCA1* methylation may predispose to breast cancer.

Pathogenic *BRCA1* germline mutations are significantly associated with hereditary breast cancer, yet less than 5% of breast cancer cases are attributed to mutations, including cases diagnosed in women before 40 years of age (Southey *et al.,* 1999).

BRCA1 inactivation due to aberrant DNA methylation of the promoter has been linked to some sporadic breast cancers, and a portion of these tumours has a *BRCA1* mutation-associated morphology (Wong *et al.,* 2011; Southey *et al.*, 2011). Constitutional

methylation of the *BRCA1* gene detected primarily in the blood has been associated with an increased risk of breast and ovarian cancer development (Dobrovic *et al.,* 2014; Gupta *et al.,* 2014; Al-Moghrabi *et al.,* 2014; Wong *et al.,* 2011; Iwamoto *et al.,* 2011). It has been considered that this epigenetic phenomenon may identify women with an elevated risk of breast carcinogenesis, however the mechanisms of constitutional methylation remains obscure.

Much of our breast cancer knowledge has emerged from assessing breast tumours that have been resected during surgery or biopsy procedures (Bombonati and Sgroi, 2011). The gold standard for diagnosing breast cancer and determining the correct course of treatment is by analysing the molecular pathology of the tumour (Rezvani *et al.,* 2018). The pathological features of certain breast cancers, for example those that are *BRCA1*-mutated, have unique pathological features, often resembling some sporadic breast tumours that are methylated at the *BRCA1* promoter (Loughrey *et al.,* 2008; Snell *et al.,* 2008).

More recently, Al-Yousef *et al.* (2020) treated *BRCA1*-methylated TNBC cell lines with curcumin in an attempt to re-express hypermethylated *BRCA1*. Curcumin is the active component in turmeric, and is believed to have chemotherapeutic properties (Giordano and Tommonaro 2019). Their findings showed that *BRCA1* expression was restored following curcumin treatment. This highlights the possibility of tailored therapeutic options for treating *BRCA1*-methylated breast cancers, and perhaps even the potential to prevent breast cancer in healthy women harbouring a *BRCA1* methylated gene.

BRCA1 mutation and methylation occur mutually exclusively, however *BRCA1* methylation analysis is not a routine test in breast cancer, despite evidence suggesting that *BRCA1*-methylated tumours are sensitive to treatment by PARP inhibitors (Fong *et al.,* 2010; Island 2010).

Currently, there is insufficient evidence describing constitutional methylation as a robust marker of cancer predisposition. Therefore, unravelling the role of constitutional methylation is paramount. However, investigating constitutional methylation is further complicated by the rare inheritance of DNA methylation patterns (Hitchins *et al.,* 2007). Studies have shown that some women with *BRCA1* methylated breast tumours indeed have detectable *BRCA1* methylation in peripheral blood, albeit at extremely low levels (~1%) (Snell *et al.,* 2008). These findings beg the question, *could constitutional BRCA1 methylation in peripheral blood be used to predict BRCA1 methylated tumours, and more importantly, identify healthy women who are predisposed to developing breast cancer?*

Droplet digital PCR (ddPCR) is a highly sensitive methodology that is able to accurately detect methylation at low levels in many tissue types, including peripheral blood and tumours (Wiencke *et al.,* 2014). Given that constitutional methylation detected at less than 1% can be found in patients whose tumours are fully methylated (Snell *et al.,* 2008; Wong *et al.,* 2011), utilising ddPCR to assess the methylation status of cancer-specific markers is valuable.

7.1.2. Melanoma patients

Melanoma is classified as the most aggressive and deadly form of skin cancer, and accounts for most skin cancer related deaths worldwide (AIHW, 2016). Current treatment include chemotherapy, tumour resection, targeted therapy and immunotherapy. Although the introduction of immunotherapies has improved survival rates in some patients, the efficacy of these therapeutic approaches can be diminished as a result of complex resistance mechanisms (either primary or acquired) (Robert *et al.,* 2019). In cases of metastatic melanoma, the prognosis is particularly poor, hence necessitating the identification of novel markers and mechanisms of early detection, monitoring and predisposition.

Hypermethylation of tumour suppressor genes associated with melanoma have been well documented, particularly in the metastatic setting (Hoon *et al.,* 2004; Mori *et al.,* 2005; Bustos *et al.,* 2008). An increase in DNA methylation is a common somatic event, and can result in soma-wide transcriptional silencing of a gene. Aberrant promoter methylation of tumour suppressor genes such as *RARβ* and *RASSF1A* have been associated with poor overall survival and treatment response (Mori *et al.,* 2005).

In some cases, constitutional methylation has been detected in blood or disease-free tissue, and has been associated with susceptibility to certain cancers (Gupta *et al.,* 2014). Though there is limited research evaluating the significance of constitutional methylation in melanoma, early work describing this phenomenon was often performed using methodologies with limited sensitivity in detecting methylation. Given the introduction and high analytical sensitivity of ddPCR, it is important to assess constitutional methylation in melanoma patients to further explore the prospective utility of blood-based analysis in relation to improving early detection, as well as evaluating the association between tumour methylation and peripheral blood methylation in early-stage melanoma.

7.2. Overall summary

This thesis evaluated the methylation profiles of tumour suppressor genes in various tissues from individuals with either breast cancer, melanoma or healthy individuals. Tumour suppressor genes were selected based on their implications in certain cancer types. Constitutional methylation of putative tumour-specific methylation markers were analysed in DNA from tumour, peripheral blood and buccal mucosa at the population level.

Constitutional methylation detected at levels as low as below 1% have been shown to predispose to a fully methylated breast tumour. For this reason, Chapter 3 focused on optimising a highly sensitive probe-based ddPCR assay for the precise quantification of

BRCA1-methylated alleles. The *BRCA1* ddPCR assay is key for the precise quantification of *BRCA1* methylation outlined throughout this thesis, and also for the identification of poor quality specimens. Ensuring the accurate quantification of *BRCA1* methylation is important when considering *BRCA1* methylation dosage in tumours (Kondrashova *et al.,* 2018), and in exploring the relationship between levels of constitutional methylation and cancer predisposition.

Chapter 4 was a case-control study that sought to establish the frequency of constitutional *BRCA1* methylation in age-matched healthy adult women and women with breast cancer. This chapter also examined the relationship between *BRCA1* methylation detected in the tumour and constitutional *BRCA1* methylation detected in peripheral blood of women with breast cancer. The initial phase of this study involved constitutional *BRCA1* methylation analysis in 300 cases and 327 control women using the optimised *BRCA1* ddPCR assay outlined in Chapter 3. Interestingly, no differences were observed in constitutional *BRCA1* methylation frequencies between cases and controls. However, women with breast cancer had significantly higher levels of *BRCA1* methylation in normal tissues compared to healthy controls, suggesting that the presence of constitutional *BRCA1* methylation alone may not predispose to breast cancer, but rather the levels of detectable methylation may also play a role.

Once age was considered, *BRCA1* methylation frequency in peripheral blood was significantly higher in women under 40 years of age compared to women over 40. When case and control groups were considered independently, age at blood donation was not significantly associated with *BRCA1* methylation frequency due to sample size limitations. However, when the data was pooled from cases and controls, a significant association between age at blood donation and *BRCA1* methylation frequency was achieved.

Breast cancers that are associated with *BRCA1* mutations have distinct morphological features including high mitotic index, high-grade and prominent lymphocytic infiltration (Loughrey *et al.,* 2008; Snell *et al.,* 2008). These histological characteristics have also been observed in sporadic breast cancers with *BRCA1*-methylated tumours (Esteller *et al.,* 2000). Interestingly, sporadic breast cancers with a *BRCA1*-methylated promoter have been reported to be of the triple-negative subtype, defined by the absence of ER and PR hormone expression and no amplification of HER2 (Lips *et al.,* 2013; Stirzaker *et al.,* 2015). Given these findings, hormone receptor (HR) status was considered in Lifepool women with breast cancer. Findings showed that only a small portion of women with detectable *BRCA1* methylation in their peripheral blood and tumour had triple-negative breast cancers; however, most women were positive for at least one hormone receptor or HER2, suggesting that HR status does not play a significant role on the presence of constitutional *BRCA1* methylation.

Chapter 5 explored the extent to which the presence of constitutional methylation of the *BRCA1* gene might be influenced by genotype by exploiting the monozygotic twin model. DNA methylation was assessed in multiple tissues of identical twins at various time points. The first stage of this study involved measuring constitutional *BRCA1* methylation in buccal mucosa DNA of identical twin females at birth. The use of a probe-based ddPCR assay facilitated methylation detection at extremely low levels. Results demonstrated high discordance rates of *BRCA1* methylation across twin pairs at birth, indicating little genetic influence on methylation of the *BRCA1* promoter. Although MZ twins share identical genomic information, the variable DNA methylation patterns can be explained by the significant non-shared environmental factors in-utero, that influence the epigenome (Gordon *et al.,* 2012; McNamara *et al.,* 2016; Craig *et al.,* 2020, *in press*).

To determine whether methylation was present across various tissues, *BRCA1* methylation was analysed in white blood cell (WBC) DNA from cord blood of MZ twins. Findings showed that methylation detected in buccal DNA was not reflective of methylation observed in WBC DNA, given that most twins who were methylated in WBC DNA were unmethylated in buccal mucosa at birth. The observation that constitutional *BRCA1* methylation patterns differ across various tissue types within the same individual can be attributed to the fact that this methylating event is tissue-specific to a certain extent.

An extension of this study involved methylation analysis in buccal mucosa of the same twin cohort at 6 years of age to determine whether methylation detected at birth was sustained overtime, across WBC DNA and buccal mucosa DNA collected at birth. The observed *BRCA1* methylation frequency detected in buccal DNA at 6 years was significantly higher than that observed at birth, and conflicts with methylation frequencies observed in healthy adult women (Lonning *et al.,* 2018). These findings demonstrate the erratic nature of DNA methylation, particularly during early life. They also suggest that *BRCA1* methylation may be occurring as a sporadic methylating event during early development, and stabilise in adulthood.

Finally, chapter 6 explored the possibility of constitutional methylation of *RASSF1A* and *RARβ* in patients with primary melanoma, using MS-HRM. Despite high levels of tumour methylation of both loci, no methylation in peripheral blood (i.e. constitutional methylation) was detected. Although methylation was not detected in the blood of our patient set, these findings do not discount constitutional methylation of *RASSF1A* and *RARβ* from being a rare epigenetic phenomenon.

When the methylation frequencies of both loci were determined in tumours, *RARβ* was methylated at significantly higher rates compared to *RASSF1A*. Earlier work reported that *RASSF1A* methylation of primary tumour lesions occurs at significantly lower frequencies

in primary melanomas compared to metastatic melanomas (Hoon *et al.,* 2004). As such, these findings may explain the low *RASSF1A* methylation frequency observed in the primary tumours used in the present study. Collectively, the results presented in this chapter suggest that *RASSF1A* and *RARβ* methylation may be tumour specific, given that methylation was only detected in the tumour.

7.3. Concluding remarks

The results described in this thesis contribute to the understanding of the role of constitutional methylation of *BRCA1* in breast cancer predisposition using an optimised ddPCR assay, and provides insight into the role of *RASSF1A* and *RARβ* in primary melanoma.

To our knowledge, this is the first study to quantify *BRCA1* methylation in peripheral blood of healthy women and women with breast cancer using the ddPCR technology. The equivalent *BRCA1* methylation frequencies in peripheral blood of cases and controls was unexpected. However, clear distinctions in the levels of constitutional methylation were apparent in cases compared to controls. This study also identified a significant age association, where constitutional *BRCA1* methylation was highly prevalent in women under 40 years of age compared to women over 40. Taken together, these findings suggest that pre-menopausal women with higher levels of constitutional *BRCA1* methylation may be at an increased risk of breast cancer throughout the course of their lifetime.

To elucidate the interaction between genetics and environment on constitutional methylation, the monozygotic twin model was utilised. Our study revealed that constitutional *BRCA1* methylation is likely to occur as a result of environmental exposure rather than genetic influence. *BRCA1* methylation was detected in various tissue types from healthy female twin pairs at birth and at 6 years, however high rates of discordance within

twin pairs were observed. The frequency of constitutional *BRCA1* methylation was also significantly higher in newborns and 6-year-olds compared to *BRCA1* methylation frequencies in healthy adult women, thus highlighting the sporadic nature of DNA methylation during early life.

Determining the extent of genetic influence versus environmental exposure on constitutional *BRCA1* methylation using the twin model emphasised the substantial impact of environmental differences on the presence or absence of methylation. Understanding the interaction between genes and environment and disease implications can lead to new approaches to defining disease risk, as well as informing clinical practice.

Constitutional methylation of *RASSF1A* and *RARβ* was also assessed in the context of melanoma, given that little exists analysing this phenomenon for both loci. In fact, to our knowledge, this is the first study to analyse constitutional methylation of *RARβ* in melanoma, and the first study to assess *RASSF1A* methylation in primary melanoma patients rather than in the metastatic setting. Our results showed that constitutional methylation of either gene was not detected in the blood of melanoma patients. However, a larger cohort of patients may reveal that methylation of these loci is in fact present in primary melanoma patients at low population frequencies.

When methylation from matched tumour DNA samples was analysed, our results revealed significantly higher methylation frequencies of *RARβ* compared to *RASSF1A*, thus supporting published literature (Hoon *et al.,* 2004).

The findings of this thesis highlighted the complex and sporadic nature of constitutional methylation. In relation to breast cancer, our studies revealed that constitutional *BRCA1* methylation alone does not predispose to breast cancer; however, the level of *BRCA1* methylation detected in peripheral blood of an individual may identify women at an increased breast cancer risk throughout their lifetime. In line with earlier work by Wong *et*

al., (2011), constitutional *BRCA1* methylation was also shown to be associated with age in women with breast cancer and healthy women, thus implicating this epigenetic phenomenon in early-onset disease. However, further studies are required to determine the functional significance of widespread somatic *BRCA1* methylation.

Methylation marks detected in peripheral blood DNA have been implicated as putative biomarkers for early detection of breast cancer (Snell *et al.,* 2008; Wong *et al.,* 2011; Li *et al.,* 2017; Joo *et al.,* 2018). So far, DNA methylation markers identified in peripheral blood have been insufficient for early detection of breast cancer, however some have the potential to stratify breast cancer risk (Kazarian *et al.,* 2017).

Research efforts aimed at disease prevention is a promising area of investigation. In particular, identifying women who are at a high risk of breast cancer development can provide opportunities for chemoprevention. Research has shown that tamoxifen reduced the risk of invasive and non-invasive breast cancer in high risk women by 49% (Fisher *et al.,* 1998). Subsequent studies exploring the efficacy of additional chemopreventive agents have since been carried out, and continue to be explored (Cuzick *et al.,* 2003; Masuda *et al.,* 2012; Cuzick *et al.,* 2014).

Constitutional methylation is an underappreciated epigenetic phenomenon that likely influences a significant fraction of cancers. This thesis sought to examine the role of constitutional methylation of tumour suppressor genes in breast cancer and melanoma. Although interesting insights were obtained, further work exploring the impact of constitutional methylation in cancer is required. Using quantitative methodologies, prospective studies should focus on identifying methylation markers or marker-combinations that aid in the stratification of cancer risk. Epigenetic markers of cancer predisposition can be identified using a genome-wide approach, and require autonomous validation to ensure the reliability and utility of these markers.

Analyses of constitutional methylation of candidate markers of disease risk would be valuable in peripheral blood of patients at three time points: prior to cancer diagnosis, post-diagnosis, and during remission (if applicable). Longitudinal studies assessing constitutional methylation in patients with early-stage disease who progress to metastatic cancer can also shed light on epigenetic changes that occur at various stages of disease, and can provide insight into the cause-and-effect relationship between constitutional methylation and cancer. Finally, methylation of candidate genes should also be evaluated in both primary and metastatic tumours (if applicable) of each patient, to determine whether constitutional methylation detected in healthy tissue is in fact reflected in early-stage tumour lesions and persists in advanced disease.

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