UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

The molecular mechanism for vitamin D-mediated changes in global DNA methylation in MCF-7 breast cancer cells

by

Chajinsi Allegra Gaza

(835333)

Dissertation

Submitted in fulfilment of the requirements for the degree

Master of Science

in

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Dr Vanessa Meyer

June 2020

Declaration

I, Chajinsi Allegra Gaza (835333), am a student registered for the degree of Master of Science in the academic year 2020.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where explicitly indicated otherwise and acknowledged. In this context, I understand that the use of editing services is considered aided work and must be declared.
- I have not submitted this work before for any other degree or examination at this or any other University.
- The information used in the Dissertation HAS NOT been obtained by me while employed by, or working under the aegis of, any person or organisation other than the University.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

Chajinsi Allegra Gaza 30 June 2020

Abstract

Vitamin D is a fat-soluble hormone that is principally obtained from the sun, but also from the diet. The biologically active metabolite, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), synthesized within the body upon UVB exposure, plays a central role in cell growth and differentiation, and has been reported to be essential in maintaining a healthy epigenome, with reported effects on histone modification and DNA methylation. DNA methylation refers to the addition of a methyl group on the 5^{th} carbon of a cytosine ring, generally in the sequence context of a CpG dinucleotide. Healthy cells are known to have hypomethylated CpG islands in regulatory regions of tumour suppressor genes, while cancer cells are characterised by global hypomethylation and hypermethylated regulatory regions of tumour suppressor genes. This global hypomethylated state results in dysregulated gene expression and chromosomal instability, while promoter hypermethylation in tumour suppressor genes inhibits expression. Methylation patterns are established and maintained by DNA methyltransferase (DNMT) 3A and 3B, and DNMT1, respectively. The demethylation process involves the ten-eleven translocation enzymes (TETs), including TET1, TET2 and TET3. The interplay between DNMTs and TETs are essential in establishing and maintaining methylation patterns. Notably, vitamin D deficiency (< 20 ng/mL) has been associated with breast cancer susceptibility and fatality. Vitamin D supplementation has also been associated with increased global methylation in leukocytes and has been reported to interact with DNA methylation to influence breast cancer risk. However, the detailed molecular mechanism linking vitamin D to DNA methylation is still unclear and *in vitro* evidence supporting the observed cohort correlations is lacking. Given the genomic effects of vitamin D, it was hypothesized that vitamin D increase DNA methylation by increasing *DNMT* expression or inhibiting *TET* expression. The aim of this study was to assess the molecular mechanism governing vitamin D-induced changes in global DNA methylation in MCF-7 breast cancer cells. In vitro cell culture models for MCF-7 cells and embryonic kidney cells (HEK293 – serving as a control) were supplemented with or without 10 nM or 100 nM $1,25(OH)_2D_3$. After 18 h, DNA

was extracted from the cells for methylation and hydroxymethylation analysis, and RNA for the gene expression study. 10 nM $1,25(OH)_2D_3$ significantly induced *DNMT3B* expression level ($P < 0.010$), while decreasing *TET3* expression ($P < 0.010$), to significantly increase global methylation level in MCF-7 cells ($P < 0.050$). Additionally, 10 nM $1,25(OH)_2D_3$ significantly increased the tumour suppressor-targeting *TET1* and *TET2* expression in MCF-7 cells, suggesting that the reported anticancer effects of vitamin D may be related to demethylation of tumour suppressor genes as a results of vitamin D induced *TET* expression Therefore, the results suggest that vitamin D increases global DNA methylation in MCF-7 breast cancer cells by decreasing *TET3* and increasing *DNMT3B* expression to increase *de novo* methylation, thereby increasing genome stability, while decreasing promoter methylation of tumour suppressor genes by enhancing *TET1* and *TET2* expression. Importantly, vitamin D had minimal effects on the global methylation levels in the embryonic kidney cells, with no change in *TET2* and *TET3* expression, or *DNMT3* level, suggesting that vitamin D acts specifically to correct aberrant hypomethylation. Thus maintaining a sufficient vitamin D status could be key in promoting good health and well-being.

Pour Pappy, Mammy et Ya Brigitte

Acknowledgements

I would like to thank the National Research Foundation (NRF Thuthuka to V Meyer: grant number 118133) for funding the study.

To Dr Vanessa Meyer, my dearest Dr V. Where do I even begin? Words cannot begin to describe my gratitude for you and what this postgraduate journey has meant to me. I still remember the first time you presented your work on vitamin D in front of us, new and eager honour students. Your research was like a breath of fresh air, and when you picked me to be your first postgraduate student, I couldn't have been happier. Not only was the research fresh and exciting, but I had a supervisor with the same character to match. Thank you for allowing me to take part in making your "crazy" research ideas come to life. Thanks to you, vitamin D and methylation has a special place in my heart as my first passion in science research. I would like to express my outmost thanks to you, for believing in me even when my belief in myself was dwindling and for the coffee talks at WAM to cheer me up. For your constructive criticism and your outright honesty when it was truly essential, I thank you for it has helped me to grow, learn and do better over the years. Throughout these tough years, you have not stopped motivating me and have encouraged me time and time again not to give up. You were stern, and for good reasons, but at the same time you are one of the kindest hearts I have ever met. Like how you changed the 'Track changes" colour blue instead of red for me, so I don't freak out as much by the changes I had to make to my writing (bless your heart!). Not only did you care about my academic work, but you also cared about how I was doing personally, emotionally and mentally. Your bubbly personality has lit up the most stressful times and your patience with me, I can't even fathom. You have been truly God-sent and I could never have asked for a better supervisor, because I received the best one for me. The awe and respect I have for you is immense and I know you are and will be just as much of an inspiration to many more students like you are to me. Thank you for all the lessons you have taught me, I am gratefully taking them all with me to the next journey of my life and professional career.

Thank you to my cool, calm and collected advisor, Dr Nikki Gentle. Thank you for your constant push to help me think outside of the box, for your brutal yet well needed honesty, for asking all the tough questions and for motivating me to always question everything I read that is science-related, instead of just blindly believing every word.

To my awesome colleagues in GH514. Lidija, thank you for all your help with the bioinformatics work I struggled with, you eagerness to help has never ceased to amaze me. Thank you as well for all the light banters we had in the lab and all the times we've stressed and laughed together. To my dear Kuda, whom I've known even before being lab colleagues, thank you for how much your dry humour has always made me laugh. Thank you for being that familiar face I had by my side, for the calm energy you exuberated in the lab and for all your warm hugs. I appreciate you so much bro! To Daniesha and Shuaib, I am so thankful to have worked with you two and to have seen you grow in your own respective fields of research. Your presence in the lab was always a pleasure and brought so much life to the lab.

Thank you to my family and friends, for your constant prayers and all the motivation you have given me. For all the times you've called to check up on me and for cheering me up when I was going through a tough time.

Finally, to my heavenly Father, for redirecting my life to places I could never have imagined. For opening up my mind and heart to new passions and skills and for giving me the strength and comfort to endure all the adversities. As well as for all the happy memories and all the wonderful people I encountered on this unforgettable journey. Gloria, laus et honor tibi, Domine!

Table of contents

Chapter 1: Literature Review 21

1.1. Breast cancer 21

1.1.1 Prevalence and mortality 22

1.1.2. Risk factors 23

1.1.3. Treatment and diagnosis 25

1.2. DNA methylation 27

1.2.1. Biomarker capability of DNA methylation 28

1.2.2. Regulation of DNA methylation patterns 29

1.2.3. Environmental impact on DNA methylation 32

1.3. Vitamin D 32

1.3.1. Vitamin D metabolism 32

1.3.2. VDR and vitamin D function 36

1.3.3. Vitamin D in cancer 38

1.3.4. Vitamin D in breast cancer 39

1.3.5. Vitamin D and the epigenome 44

1.4. Hypothesis 46

1.5. Aim 46

1.6. Objectives: 46

Chapter 2: Methodology 48

2.1. Cell culture and treatment 48

2.1.1. Trypan blue assay 48

2.1.2. MTT assay 49

2.2. Genomic DNA (gDNA) extraction 50

2.3. RNA extraction 51

2.4. Quality control 52

2.4.1. DNA and RNA purity 52

2.4.2. DNA and RNA integrity 53

2.5. Global DNA methylation analysis 54

2.6. DNA hydroxymethylation analysis 56

2.7. Quantitative reverse transcriptase PCR (RT-qPCR) 57

2.8. Bioinformatics 61

2.9. Statistical analysis 61

Chapter 3: Results 62

3. Results 62

3.1 Quality Control 62

3.1.1. Nucleic acids used in the study were intact and pure 62

3.1.2. Primers specifically amplified the gene of interest after cDNA synthesis 63

3.1.3. Reference gene stability 64

3.2. 1,25$(OH)_2D_3$ induced no significant change in cell viability of MCF-7 or HEK293 cells 65

3.3. 1,25$(OH)_2D_3$ increased methylation in MCF-7 cells but had no effect on HEK293 cells 66

3.4. 1,25$(OH)_2D_3$ induce *de novo DNMT3B* expression in MCF-7 cells and maintenance *DNMT1* expression in HEK293 cells 67

3.5. 1,25$(OH)_2D_3$ downregulates *TET3,* while inducing *TET2* expression in MCF-7 cells 69

3.6. 1,25$(OH)_2D_3$ increased hydroxymethylation in MCF-7 cells, while decreasing hydroxymethylation in HEK293 cells 70

3.7. 1,25$(OH)_2D_3$ supplementation induced *VDR* expression in MCF-7, cells while decreasing *VDR* in HEK293 cells 72

3.8. *DNMT3B* genes showed a positive correlation with *VDR* in response to $1,25(OH)_2D_3$ in MCF-7 cells, but not in HEK293 cells 72

3.9. $1,25(OH)_2D_3$ induced no significant change in cell proliferation of MCF-7 and HEK293 cells 74

3.10. All six genes contained VDR and RXR transcription factor binding sites but also had additional TF binding sites 75

Chapter 4: Discussion 78

4.1. Quality Control 78

4.1.1. Nucleic acids used in the study was intact and pure 78

4.1.2. Primers specifically amplified the gene of interest after cDNA synthesis 78

4.1.3. Reference gene stability 79

4.2. $1,25(OH)_2D_3$ induced no significant change in cell viability of MCF-7 or HEK293 cells 79

4.3. $1,25(OH)_2D_3$ increased methylation in MCF-7 cells but had no effect on HEK293 cells 80

4.4. $1,25(OH)_2D_3$ induce *de novo DNMT3B* expression in MCF-7 cells and maintenance *DNMT1* expression in HEK293 cells 81

4.5. 1,25$(OH)_2D_3$ downregulates *TET3,* while inducing *TET2* expression in MCF-7 cells 82

4.6. 1,25$(OH)_2D_3$ increased hydroxymethylation in MCF-7 cells, while decreasing hydroxymethylation in HEK293 cells 82

4.7. 1,25$(OH)_2D_3$ supplementation induced *VDR* expression in MCF-7, cells while decreasing *VDR* in HEK293 cells 84

4.8. *DNMT3B* genes showed a positive correlation with *VDR* in response to 1,25$(OH)_2D_3$ in MCF-7 cells, but not in HEK293 cells 85

4.9. 1,25$(OH)_2D_3$ induced no significant change in cell proliferation of MCF-7 and HEK293 cells 85

4.10. All six genes contained VDR and RXR transcription factor binding sites but also had additional TF binding sites 86

Chapter 5: Conclusion 89

5.1. Rationale of the study and summary of the findings 89

5.2. Implications of the study 89

5.3. Challenges and limitations 90

5.4. Future direction 90

References 91

List of Figures

Figure 1. 1: Macroscopic presentation of breast cancer 21

Figure 1. 2: Breast cancer was the leading single cancer cause of death worldwide in 2018..... 22

Figure 1. 3: Pathways of oestrogen carcinogenesis 24

Figure 1. 4: Direct and indirect gene silencing by DNA methylation..... 28

Figure 1. 5: The DNA demethylation pathway..... 31

Figure 1. 6: The production and metabolism of D_2 and D_3..... 33

Figure 1. 7: Vitamin D metabolism 35

Figure 1. 8: The genomic mechanism of vitamin D and VDR..... 37

Figure 1. 9: Illustration of a two-step co-regulator model..... 45

Figure 3. 1: The gels show intact DNA and RNA 62

Figure 3. 2: Primers specifically amplified the gene of interest after cDNA synthesis..... 63

Figure 3. 3: Single melt peaks supports the specificity of primers used for RT-qPCR in the study..... 64

Figure 3. 4: $1,25(OH)_2D_3$ supplementation did not significantly influence cell viability 65

Figure 3. 5: 1,25$(OH)_2D_3$ significantly increased global methylation in MCF-7, but not HEK293 cells..... 66

Figure 3. 6: MCF-7 cells have higher global methylation levels than HEK293 cells, irrespective of treatment..... 67

Figure 3. 7: 1,25$(OH)_2D_3$ induced *DNMT3B* expression, but had no effect on *DNMT1* and *DNMT3A* in MCF-7 cells 68

Figure 3. 8: 1,25$(OH)_2D_3$ supplementation induced *DNMT1* expression, but not *DNMT3A* or *DNMT3B* in HEK293 cells..... 68

Figure 3. 9: 1,25$(OH)_2D_3$ significantly induced *TET2*, while downregulating *TET3* expression in MCF-7 cells 69

Figure 3. 10: 1,25$(OH)_2D_3$ significantly induced *TET1* expression, but not *TET2* and *TET3* expression in HEK293 cells 70

Figure 3. 11: 1,25$(OH)_2D_3$ significantly increased hydroxymethylation level in MCF-7 cells, while decreasing hydroxymethylation in HEK293 cells 71

Figure 3. 12: The level of demethylation events in response to 1,25$(OH)_2D_3$ supplementation is cell-type specific and concentration dependent. 71

Figure 3. 13: 1,25$(OH)_2D_3$-mediated *VDR* autoregulation appears to be cell-type specific 72

Figure 3. 14: The cross-correlation between *VDR* and the target genes in MCF-7 cells..... 73

Figure 3. 15: The cross-correlation between *VDR* and the target genes in HEK293 cells..... 74

Figure 3. 16: $1,25(OH)_2D_3$ had no significant effect on cell proliferation in MCF7- or HEK293 cells..... 75

Figure 3. 17: Putative transcription factor binding sites in the target genes 77

List of Tables

Table 1: Efficacy of vitamin D supplementation as adjunct therapy in female breast cancer patients. 42

Table 2. 1: Reaction components for the methylated and unmethylated DNA standards..... 55

Table 2. 2: Reaction components for the antibody mix used to quantify global DNA methylation level. 55

Table 2. 3: Concentration 5-hydroxymethylation of the standards included in the analysis. 57

Table 2. 4: Reaction components of the cDNA synthesis master mix. 58

Table 2. 5: Reaction components of the qPCR reaction. 58

Table 2. 6: The PCR reaction done in a 3-step cycle. 59

Table 2. 7: Primer sequences used to amplify the target and reference genes… 60

Table 3. 1: The M-values for the reference genes in MCF-7 and HEK293 cells..655

Abbreviations

$1,25(OH)_2D_3$	1alpha,25-dihydroxyvitamin D_3
5-hmC	5-hydroxymethylcytosine
5-mC	5-methyl cytosine
ACTB	Actin Beta
ANKRD17	Ankyrin repeat domain-containing protein 17
B2M	Beta-2-Microglobulin
BRCA1	DNA repair associated gene 1
BRCA2	DNA repair associated gene 2
CCSER2	Serine-rich coiled-coil domain-containing protein 2
cDNA	complementary DNA
CEBP:	CCAAT/enhancer-binding protein beta
CpG	5'—C—phosphate—G—3'
DIMT1	Probable dimethyladenosine transferase
DNA	Deoxyribonucleic Acid
DNMT1	DNA methyltransferase 1
DNMT2	DNA methyltransferase 2
DNMT3A	DNA Methyltransferase 3 Alpha
DNMT3B	DNA Methyltransferase 3 Beta
DNMT3L	DNA methyltransferase 3-Like
E2	Oestadiol
ELISA	Enzyme-linked Immune Sorbent Assay
ERα	Oestrogen Receptor alpha
FBS	Fetal Bovine Serum
FP	Forward Primer
GABPA	GA Binding Protein Transcription Factor Subunit Alpha
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
HCl	Hydrogen Chloride

HCT116	Human Colorectal Carcinoma 116 cell
HEK293	Human Embryonic Kidney 293
HRP	Horse Radish Peroxidase
MCF-7	Michigan Cancer Foundation-7
MDAMB231	M.D. Anderson and Metastasis Breast cancer
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromid
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NaOH	Sodium hydroxide
PGK1	Phosphoglycerate Kinase 1
PU.1	Putative Oncogene Spi-1
RNA	Ribonucleic Acid
RP	Reverse Primer
RPL13A	Ribosomal Protein L13a
rRNA	ribosomal Ribonucleic Acid
RT-qPCR	Quantitative reverse transcription PCR
$RUNX_2$	Runt-related transcription factor 2
RXR	Retinoid X receptor
SDS	Sodium Dodecyl Sulfate
SYMPK	Symplekin
TBE	Tris Boric EDTA
TCF_4	Transcription Factor 4
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TET2	Tet methylcytosine dioxygenase 2
TET3	Tet Methylcytosine Dioxygenase 3
VDR	Vitamin D receptor

Table i: Techniques performed by Chajinsi Gaza

Technique	**Done by**
MCF-7 and HEK293 culture and vitamin D treatment	Chajinsi Gaza
Trypan blue and MTT assays	Chajinsi Gaza
DNA extraction and methylation and hydroxymethylation analysis	Chajinsi Gaza
RNA extraction and RT-qPCR	Chajinsi Gaza
Primer design for DNMT3A, DNMT3B, TET1, TET2 and TET3	Chajinsi Gaza
Bioinformatics analysis	Chajinsi Gaza with the help of Lidija Damjanović
Genomatix analysis of the *GABPA* gene	Kudakwashe Nyamupangedengu

Presentation arising from research

Gaza CA and Meyer V., 2017. *The relationship between vitamin D and DNA methyltransferases 1 (DNMT1) activity*. Molecular Biosciences Research Thrust (MBRT) Postgraduate Research Day - University of the Witwatersrand – Johannesburg, Gauteng, South Africa, Poster presentation by Chajinsi Gaza.

Gaza CA and Meyer V., 2018. *Vitamin D: A master regulator of DNA methylation?* South African Society for Bioinformatics (SASBi)/South African Genetics Society (SAGS) Joint Congress – Golden Gate Highlands National Park, Free State, South Africa, Oral presentation by Chajinsi Gaza.

Gaza CA and Meyer V., 2017. *Vitamin D: A master regulator of DNA methylation?* Molecular Biosciences Research Thrust (MBRT) Postgraduate Research Day - University of the Witwatersrand – Johannesburg, Gauteng, South Africa, Poster presentation by Chajinsi Gaza.

Publication in preparation

Damjanović, L., Govender, D., Gaza, C. A. and Meyer, V., 2020. Vitamin D decreases silencer methylation to downregulate renin gene expression. To be submitted to: *International Journal of Endocrinology*.

1.1. Breast cancer

Breast cancer (BCa) can be defined as atypical hyperplasia which is the build-up of abnormal cells in the breast (Myers and Walls, 2018). The breast is made of two main types of tissues: glandular and stromal tissue. Glandular tissues contain the milk-producing lobules and ducts, while stromal tissues house the fatty and fibrous connective tissues (Khuwaja and Abu-Rezq, 2004). BCa commonly originates from the inner lining of the milk ducts, but can also originate from cells lining the lobules or metastasize from other tissues (Sharma et al., 2010).

BCa may present with lumps, skin dimpling, changes in the skin colour and texture, inversion of the nipple and discharge of blood or fluid (Fig. 1.1; Bisen, 2013)

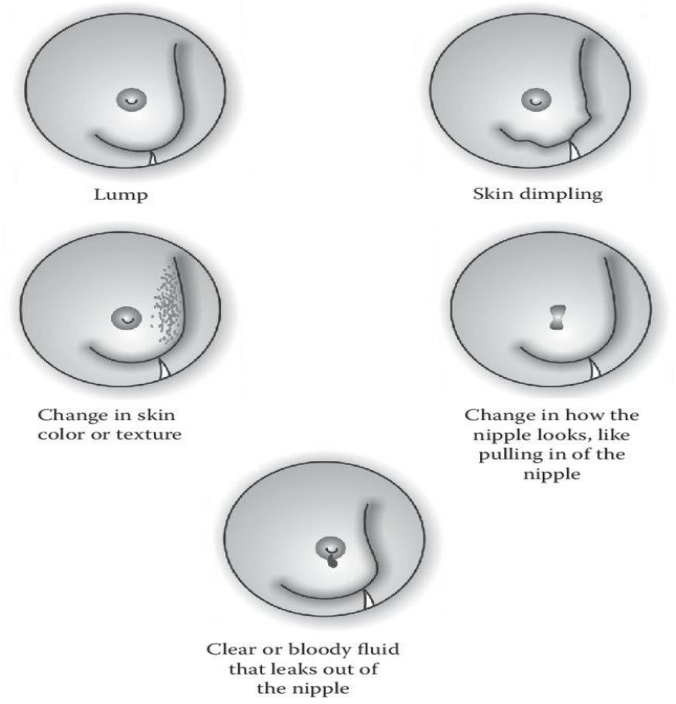

Figure 1. 1: Macroscopic presentation of breast cancer. Breast cancer may present with lumps, skin dimpling, changes in the skin colour and texture, inversion of the nipple and discharge of blood or fluid. Taken from Bisen (2013).

1.1.1. Prevalence and mortality

In 2015, BCa caused 571 000 deaths globally (World Health Organisation, 2016). In 2018, the number of BCa deaths increased to 626 679 (Fig. 1.2A; GLOBOCAN 2018). Over 2 million new BCa cases have been recorded in 2018 and it still remains the second most common cancer globally (Fig. 1.2B; GLOBOCAN, 2018). Developing countries account for roughly 50% of BCa cases and 58% of global mortalities rates (GLOBOCAN, 2008). The number of women at risk of BCa stand at roughly 19.4 million from the age of 15 years and older.

In South Africa, BCa accounted for 0.7% of all deaths in 2013 (CANSA, 2018) and is the most common cancer type among women. All races combined, 21% of women were diagnosed with BCa in 2014 (National Cancer Registry; NCR 2014). Although BCa is 100 times more common in women, men can acquire it too. Men generally have a poorer prognosis due to delays in diagnosis (National Cancer Institute, 2006; American Cancer Society, 2007). The low survival rate in developing countries such as South Africa is due to the lack of appropriate diagnosis, treatment facilities and the lack of early detection programmes, which result in a large number of women presenting with late-stage disease (WHO, 2013).

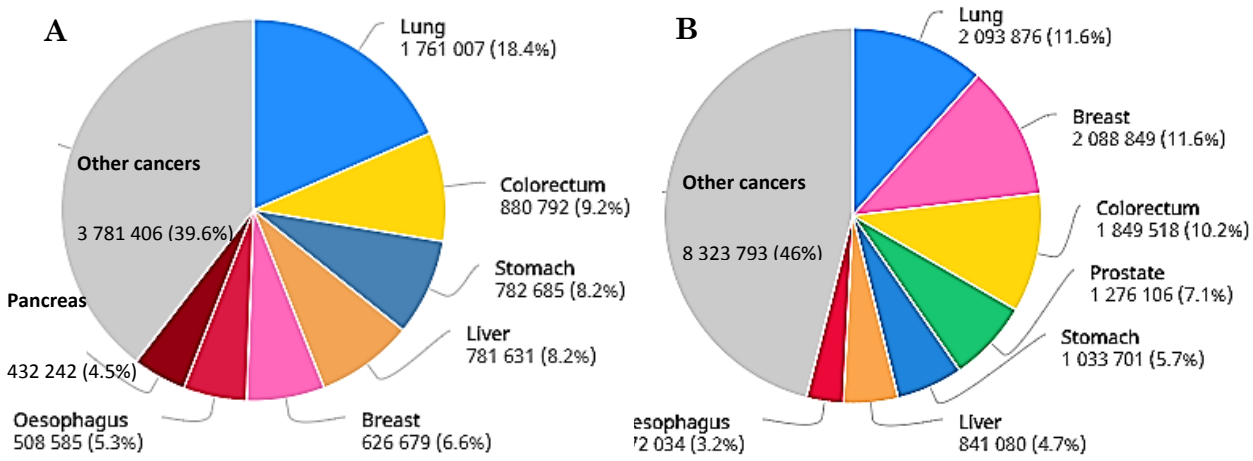

Figure 1. 2: Breast cancer was the leading single cancer cause of death worldwide in 2018. The graph shows the global estimated mortality rate in 2018 (A) and number of new cases in 2018 (B) in all cancers irrespective of sex and age. Taken from GLOBOCAN (2018).

1.1.2. Risk factors

It is estimated that about 27% of diagnosed BCa are due to genetic mutations (Lichtenstein et al., 2000). Mutations in tumour suppressor genes such as *p53*, DNA repair associated gene 1 (*BRCA1*) and DNA repair associated gene 2 (*BRCA2*) increase the risk of BCa (Petrucelli et al., 2016). Women who carry mutations in *p53*, have an 85% chance of developing BCa by the age of 60 (Schon and Tischkowitz, 2017). Mutations in *BRCA1* or *BRCA2* increases BCa risk by 60-85% (Juwle and Saranath, 2012). However, these mutations rarely occur and only account for about 5% of the total number of BCa cases (Calderon-Garciduenas et al., 2005). Thus, while these genetic mutations contribute to BCa risk, it is not the sole contributor. Lifestyle, environmental and hormonal factors also influence disease risk. In less developed countries, non-genetic risk factors such as obesity, being overweight, excessive alcohol consumption and lack of physical activity, accounts for ~18% of the cases; with lack of physical activity making the largest contribution to risk (10%; Danaei et al., 2005). In Africa, BCa was the top cancer among females attributed to excess body mass index in 2012 (GLOBOCAN, 2018). Chemicals including diethylstilboestrol (DES, Veurink et al., 2005) and ethylene oxide (Steenland et al., 2003), age (Dana-Farber, 2017), and increased oestrogen exposure resulting from early menarche and late onset menopause (after 55 years of age, Eaton, 2002) are all associated with BCa development.

The molecular mechanism by which oestrogen affects BCa is poorly characterized. Nonetheless, experimental evidence suggests that oestadiol (E2) along with its oestrogen receptor alpha (ERα), stimulates cell proliferation and induce mutations that form due to errors during DNA replication (Fig 1.3; Preston-Martin et al., 1993; Preston-Martin et al., 1990). This effect of E2 then reinforces the growth of cells bearing mutations, which accumulate until cancer develops. From a metabolic point of view, oestrogen molecules are converted to quinone metabolites that directly bind to DNA, forming adducts, resulting in DNA damage (Yager and Davidson, 2006). Moreover, catechol oestrogen metabolites go through redox cycling, generating oxygen free radicals that damage DNA-bound guanine to form

8-OXO-guanine bases which are unstable and get deleted from the affected DNA fragments through depurination. Error-prone DNA repair mechanisms employed may then result in mutation formation at the depurinated sites (Cavalieri et al., 2006). Accumulation of mutations may contribute to BCa development.

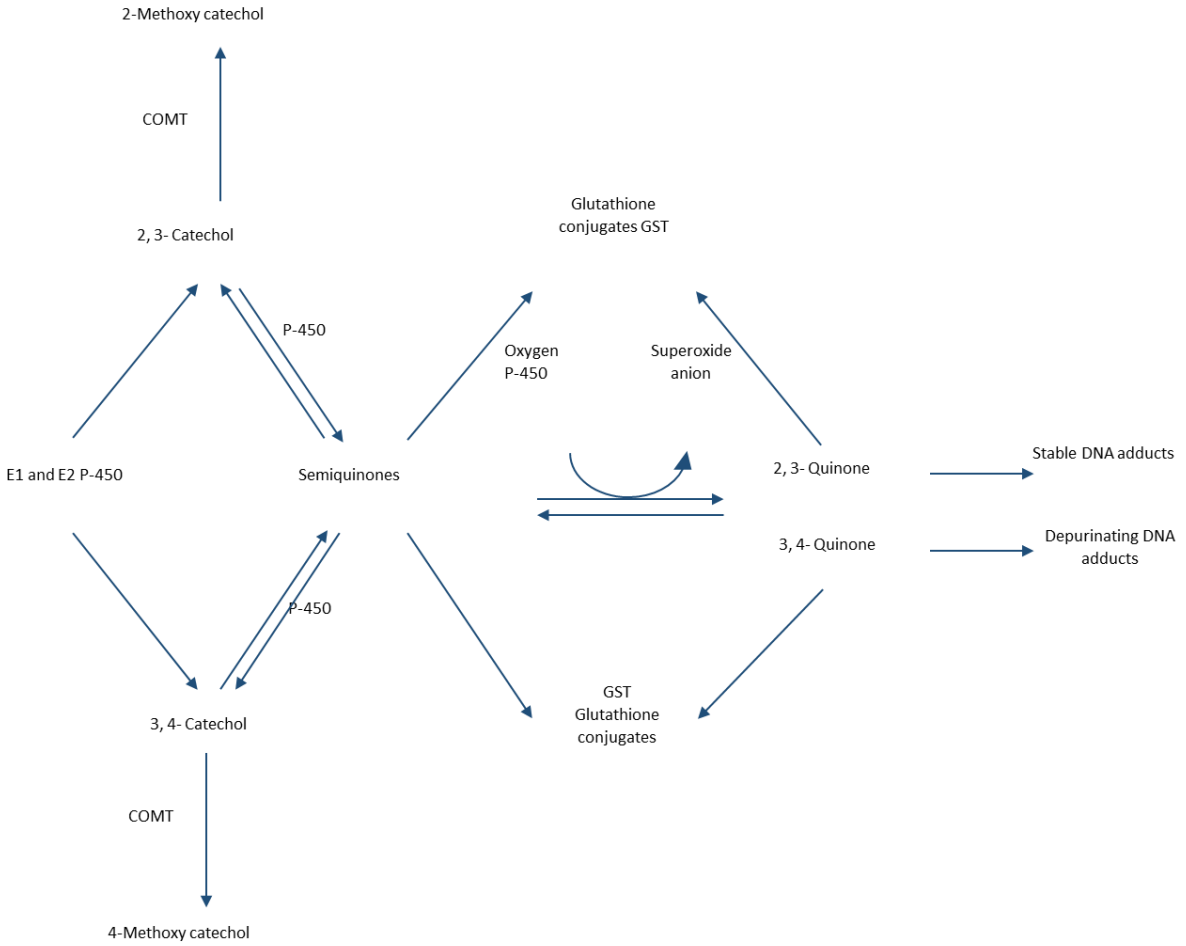

Figure 1. 3: Pathways of oestrogen carcinogenesis. The two different yet complementary pathways may contribute to oestrogen carcinogenesis and to the initiation, promotion and progression of breast cancer. E_1 denotes estrone, E_2 estradiol, 2-OH-E_1 2-hydroxyestrone, 2-OH-E_2 2-hydroxyestradiol, 4-OH-E_1 4-hydroxyestrone, 4-OH-E_2 4-hydroxyestradiol, and 16α-OH-E_1, 16α-hydroxyestrone. Taken from Yager and Davidson (2006).

1.1.3. Treatment and diagnosis

Current treatments for BCa include breast biopsy, which is the surgical removal of breast tissue, followed by chemotherapy. Chemotherapy is the use of drugs to stop the ability of cancer cells to grow and divide (Shewach and Kuchta, 2009). Ways to administer chemotherapy comprise of intravenous tubes, subcutaneous injections, intramuscular injections or oral administration. Radiation can also be used after the tumour has been removed in order to kill or shrink any surviving BCa cells. Chemotherapy can be given before surgery to shrink a big tumour, making the surgery easier (neoadjuvant chemotherapy), or after the surgery to decrease recurrence risks (adjuvant chemotherapy; Kufe et al., 2003). Chemotherapy can be given through different schedules which depends on what functioned best in the clinical trials of the specific drug. It can be given once a week or once every 4 weeks. Some of the common drugs used in chemotherapy are Capecitabine, Carboplatin, Cisplatin, Cyclophosphamide, Docetaxel, Doxorubicin, Tamoxifen, Pegylated liposomal doxorubicin, Epirubicin and Fluorouracil (Mohamed et al., 2015).

Early detection and treatment increase the prognosis and chance of survival. The most reliable test for BCa detection is the mammogram. This is an X-ray test of the breast that can detect BCa up to 2 years prior to tumour formation. Although this is a widely used method, it lacks sensitivity and specificity, and is considered invasive (Brekelmans et al., 2001). Consequently, there is a need for the identification and validation of non-invasive tumour biomarkers that will facilitate early detection. Several biomarkers have been proposed encompassing a host of biomolecules including lipids (Gönenç et al., 2006; Sharipov et al., 2003), carbohydrates (Kurebayashi et al., 2006; Jeschke et al., 2005), polyamines (N1, N^{12}–diacetylspermine; Hiramatsu et al., 2005), proteins (Duffy, 2006), DNA (i.e. aberrant methylation in the *p16INK4a* gene; Silva et al., 1999; Chen et al., 1999) and RNA (Silva et al., 2002).

One of the first identified tumour antigens and prospective biomarkers for BCa was the circulating glycoprotein carcinoembryonic antigen (CEA; Gold and

Freedman, 1965). An increase in CEA levels, quantified by enzyme-linked immunosorbent assay or radioimmunoassay in BCa differentiates between ductal carcinomas and lobular carcinomas (Thompson, 1995, Kuhajda et al., 1983). Additionally, cytokeratin 8, 18 and 19 (Duffy, 2006; Giovanella et al., 2002) have been suggested as cancer biomarkers for early stage of BCa (Eskelinen et al., 1994); despite their low sensitivity. Another class of early-stage markers are cancer-specific autoantibodies (CSA; Casiano et al., 2006), with the added advantage of high throughput analysis. Since the immune system rapidly responds to emerging tumours, CSAs can be detected in the serum of patients earlier than any other cancer biomarkers (Wingren and Borrebaeck, 2006). The extracellular protein kinase A (ECPKA) is an example of an autoantibody that is upregulated in the serum of cancer patients (Cho et al., 2000; Cvijic et al., 2000). However, it lacks specificity and is elevated in a large number of cancers including bladder, cervical, lung, colon, liver, prostate and BCa (reviewed by Zaenker and Ziman, 2013).

Cancers are formed as a result of numerous effects and many cancers are associated with epigenetic changes resulting in dysfunctional regulation and concurrent changes in the transcriptome (Cheung et al., 2009). One of the earliest changes commonly observed in cancer development is aberrant DNA methylation (Moulton et al., 1994). Changes in DNA methylation occurs in genes that regulate apoptosis, the cell cycle, proliferation, drug resistance, intracellular signalling and metastasis (Sever and Brugge, 2015). Since aberrant DNA methylation occurs early in cancer development, it can be considered a hallmark for early detection of BCa (Moulton et al., 1994).

1.2. DNA methylation

DNA methylation is an epigenetic modification of DNA which plays a crucial role in regulating processes like chromatin structure, transcription, X chromosome inactivation, genomic instability, embryonic development, genomic imprinting and carcinogenesis (reviewed by Dor and Cedar, 2018). In mammals DNA methylation mostly occurs on the 5th carbon of the cytosine ring in the sequence context of a CpG dinucleotide, but can also occur in the sequence context of CHH or CHG (where H = A/ T/ G). However, non-CpG methylation has only been observed in embryonic stem cells, glial cells, oocytes and neuronal cells (reviewed by Hyun et al., 2017). In healthy cells, 70-80% of the human genome is methylated. This global, hypermethylated state is essential to maintain chromatin organisation, regulate tissue-specific expression and prevent the expression of repetitive elements (Ehrlich et al., 1982). Moreover, healthy cells have hypomethylated CpG islands in the regulatory regions of tumour suppressor genes. In contrast, cancer cells are characterized by a global hypomethylation and hypermethylated CpG islands in the regulatory regions of tumour suppressor genes (reviewed by Pfeifer, 2018). This drop in global methylation results in a dysregulated gene expression and chromosomal instability caused by the decrease in the relative proportion of genomic heterochromatin in the cancer cells, while promoter methylation of tumour suppressor genes inhibit their expression (Cheung et al., 2009).

DNA methylation can silence gene expression by directly hindering the binding of transcriptional factors to the gene (Fig. 1.4A) and indirectly by binding methyl-CpG-binding domain proteins (MBDs and MeCP2). MBDs and MeCP2 then recruit proteins like histone deacetylases (HDACs) and other chromatin remodelling

proteins that modify the histones by forming compact, inactive chromatin, inhibiting the expression of the target genes (Fig. 1.4B, Szyf, 2006).

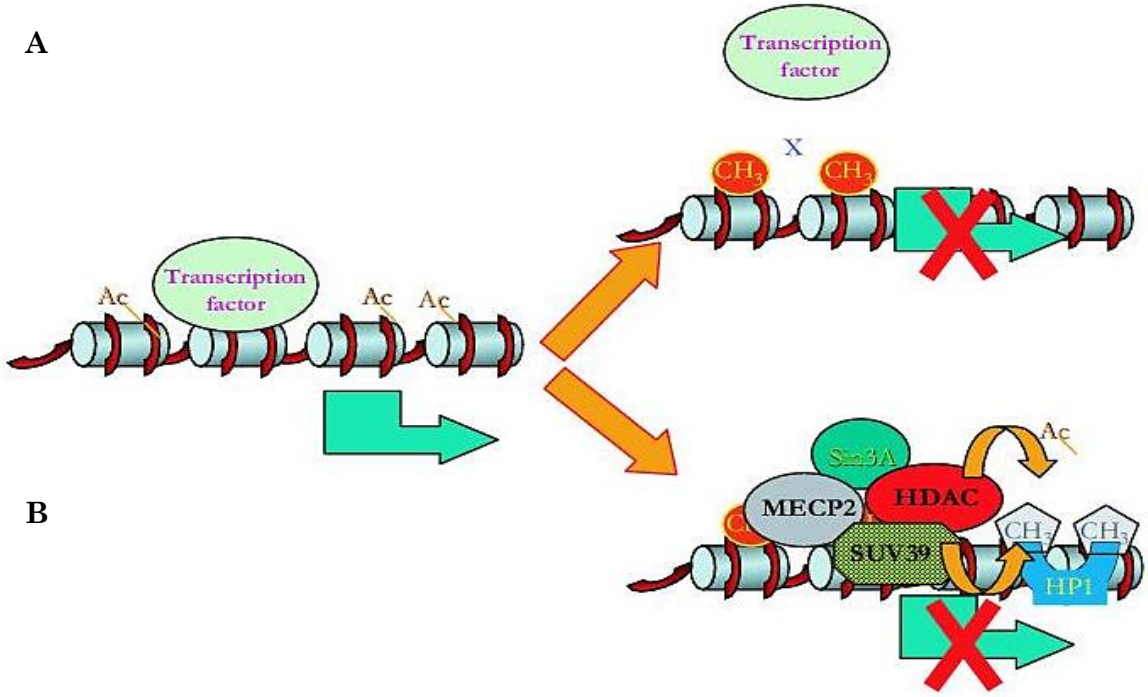

Figure 1. 4: Direct and indirect gene silencing by DNA methylation. (A) DNA methylation can silence gene expression by directly hindering the binding of transcriptional factors to the gene. (B) Indirect gene silencing involves the binding of methyl-CpG-binding domain proteins (MBDs and MeCP2) which recruit proteins such as histone deacetylases (i.e. HDAC2) and other chromatin remodelling proteins to inhibit gene expression. Taken from Szyf (2006).

1.2.1. Biomarker capability of DNA methylation

Using blood samples and a genomic approach, a number of successful DNA methylation markers for early cancer detection has been discovered. For example, Guerrero-Preston et al. (2014) showed that the promoter of the kinesin family member 1A gene (*KIF1A*) encoding the motor protein KIF1A, responsible for the transportation of membranous organelles on axonal microtubules, was hypermethylated in BCa patients, but not in controls. This suggests that methylation level of *KIF1A* can be a biomarker for early BCa detection (Guerrero-Preston et al., 2014). In another study on monozygotic twin patients, it was shown that the promoter of the docking protein 7 gene (*DOK7*), which is essential for

forming connections between nerve and muscle cells in the neuromuscular junction was hypermethylated in the blood of the affected patient but not in the unaffected twin (Heyn et al., 2013). Of note, this hypermethylation was present several years prior to diagnosis, suggesting that changes in *DOK7* promoter methylation could be a biomarker for early BCa detection (Heyn et al., 2013).

Recently, liquid biopsy has gained a lot of attention as a non-invasive, non-injurious, highly sensitive diagnostic tool (review by Wang et al., 2017). During liquid biopsies, circulating cell-free DNA, originating from both normal and tumour cells, is used to detect tumour-specific mutations, loss of heterozygosity, genetic polymorphism and changes in DNA methylation (Schwarzenbach et al., 2011). Combining DNA methylation markers, circulating tumour DNA concentrations and other genetic variations like DNA integrity might be much more informative with regard to cancer diagnosis, than using protein markers alone (review by Wang et al., 2017). Circulating methylated DNA has been regarded as a potential biomarker for detecting several cancers including lung cancer, colorectal cancer, pancreatic cancer and BCa (Pixberg et al., 2015). DNA methylation-based biomarkers are currently used in the clinic. For example, DNA methylation levels of *MLH1* is used to diagnose Lynch Syndrome (hereditary non-polyposis colorectal cancer; Church et al., 2014).

1.2.2. Regulation of DNA methylation patterns

In the human genome, DNA methylation patterns are established and maintained by a class of transferase enzymes known as DNA methyltransferases (DNMTs) including DNMT1, DNMT2, DNMT3A, DNMTT3B and DNMT3L (Hervouet et al., 2018). DNMT1 functions as the maintenance methyltransferase (Negishi et al., 2009) and DNMT3A and DNMT3B regulate *de novo* methylation (Okano et al., 1999). DNMT2 methylates tRNA contributing to translational control (Goll et al., 2006), while DNMT3L lacks catalytic activity and its function remains poorly defined. However, DNMT3L is known to stimulate *de novo* methylation through

DNMT3A/DNMT3B and is also thought to be required for establishing maternal genomic imprints (Hervouet et al., 2018). In essence, during early embryonic development, DNA undergoes *de novo* methylation by DNMT3. Replication of the methylated DNA results in hemimethylated DNA where the parent strand is methylated and the daughter strand is not. DNMT1 is recruited to the hemimethylated DNA molecules where it catalyses methylation of the daughter strand based on the parental template, thereby ensuring that previously established methylation patterns are maintained at the end of each round of DNA replication. In the absence of DNMT1, replication itself would produce unmethylated daughter strands resulting in passive demethylation of the genome (Goll et al., 2006). In a study on the HCT116 colorectal cancer cell line, a co-operating function between DNMT3B and DNMT1 was important for establishing nearly all methylation in the cells (Rhee et al., 2002). Minimal effect on genomic methylation was observed when either DNMT1 or DNMT3B was knocked out, but there was a major loss of methylation after a double knockout of the genes. This suggests that both maintenance and *de novo* enzymes are required to co-operate in order to maintain methylation in mammalian genomes (Kim et al., 2002).

The demethylation process involves the ten-eleven translocation enzymes (TETs) including TET1, TET2 and TET3 (Fig. 1.5). These proteins oxidise 5-methylcytosine (5-mC) to 5-hydroxy-methylcytosine (5-hmC; Tahiliani et al., 2009; Koh et al., 2011). 5-hmC can be further oxidised to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC; Ito et al., 2011). DNA demethylation can occur actively or passively. On the one hand, active demethylation occurs through an enzymatic process that removes or modifies the methyl group from 5-mC to its other oxidised forms. On the other hand, passive demethylation involves the loss of 5-mC during successive replication rounds in the absence of functional DNMT1 activity. 5-hmC is the central key to demethylation where it can either go through passive depletion by DNA replication or active reversion to cytosine through repetitive oxidation and thymine DNA glycosylase (TDG)-mediated base excision repair (BER). Active demethylation can be further categorised into two pathways; the pathway that involves active modification and passive dilution (AM-PD) and the

pathway involving active modification and active restoration (AM-AR). The AM-PD pathway involves the conversion of 5-mC to 5-hmC and a replication-dependent dilution of the modified cytosine base back to the unmodified cytosine. In the AM-AR pathway, there is repetitive oxidation by TET that yields 5-fC or 5-caC. These can be removed by TDG to create abasic sites as part of the DNA repair mechanism and finally regenerate the unmodified cytosine (reviewed by Kohli and Zhang, 2013). The interplay between *DNMTs* and *TETs* are therefore essential in establishing and maintaining methylation patterns.

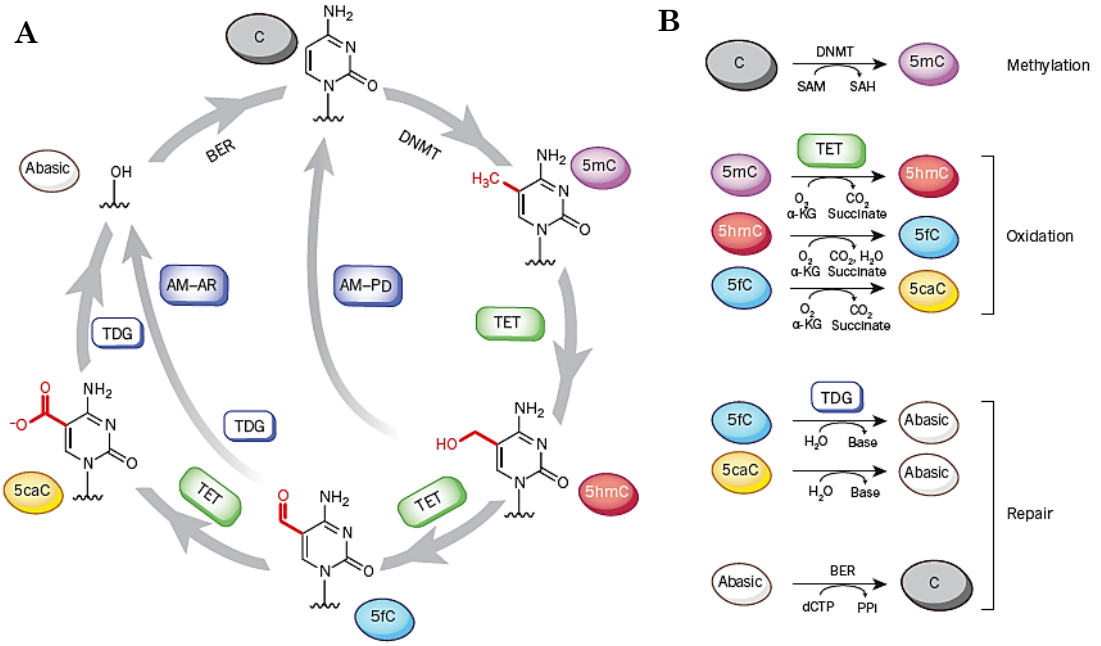

Figure 1. 5: The DNA demethylation pathway. The image summarizes the pathway for modification of the cytosine ring within DNA molecules. (A) 5-methyl cytosine (5-mC) bases introduced by DNA methyl transferase (DNMT) enzymes can be oxidised to 5-hydroxymethyl cytosine (5-hmC), 5-formyl cytosine (5-fC) and 5-carboxy cytosine (5-caC). In the active modification and passive dilution (AM-PD) pathway, 5-hmC is diluted in a replication-dependent manner to regenerate the unmodified cytosine. In the active modification and active restoration (AM-AR) pathway, 5-fC or 5-caC is removed by thymine DNA glycosylase (TDG) producing an abasic site as part of the base excision repair (BER) process that regenerates the unmodified cytosine. (B) The BER pathway involves excision of the abasic site, replacement of the nucleotide using unmodified deoxycytidine triphosphate (dCTP) by a DNA polymerase and ligation to repair the nick. Taken from Kohli and Zhang (2013).

1.2.3. Environmental impact on DNA methylation

Environmental factors such as asbestos (Kettunen et al., 2017), sunlight (Aslibekyan et al., 2014), and vitamin D (review by Fetahu et al., 2014), have also been associated with changes in DNA methylation. For examples, Zhu et al. (2016) reported an increase in global DNA methylation in leukocytes of African-Americans after vitamin D supplementation, while Lopes et al. (2012) showed reduced DNA methylation in the e-cadherin promoter of the triple negative BCa cell line MDA-MB-231 after treatment with $1,25(OH)_2D_3$. Combined, these studies suggest that vitamin D increase global methylation while decreasing promoter methylation – thus favouring gene expression. However, the molecular mechanism through which vitamin D alters DNA methylation is unknown.

1.3. Vitamin D

1.3.1. Vitamin D metabolism

Vitamin D is a fat-soluble hormone that is principally obtained from the sun, but also from the diet. Skin production of cholecalciferol ($25(OH)D_3$) does not occur enzymatically. It is produced from the precursor of vitamin D, 7-dehydrocholesterol (7-DHC) in a 2-step process where the B ring is lysed by UVB light radiation from the sun, producing pre-cholecalciferol (pre-$25(OH)D_3$) that is isomerised to $25(OH)D_3$ a non-catalytic but thermosensitive process (Bikle, 2014; Fig. 1.6A). Vitamin D obtained from the diet comes from dairy products but mostly from fatty fish. Vitamin D found naturally in fish is $25(OH)D_3$, however for fortified fish contains ergocalciferol ($25(OH)D_2$; Bikle, 2014). $25(OH)D_2$ is made as a result of UVB irradiation of ergosterol in fungi and plants. $25(OH)D_2$ differs from $25(OH)D_3$ in having a double bond between C_{22} and C_{23} and a methyl group on C_{24} located in the side chain. $25(OH)D_2$ can be regarded as the first analog of vitamin D (Houghton and Vieth, 2006; Fig. 1.6B).

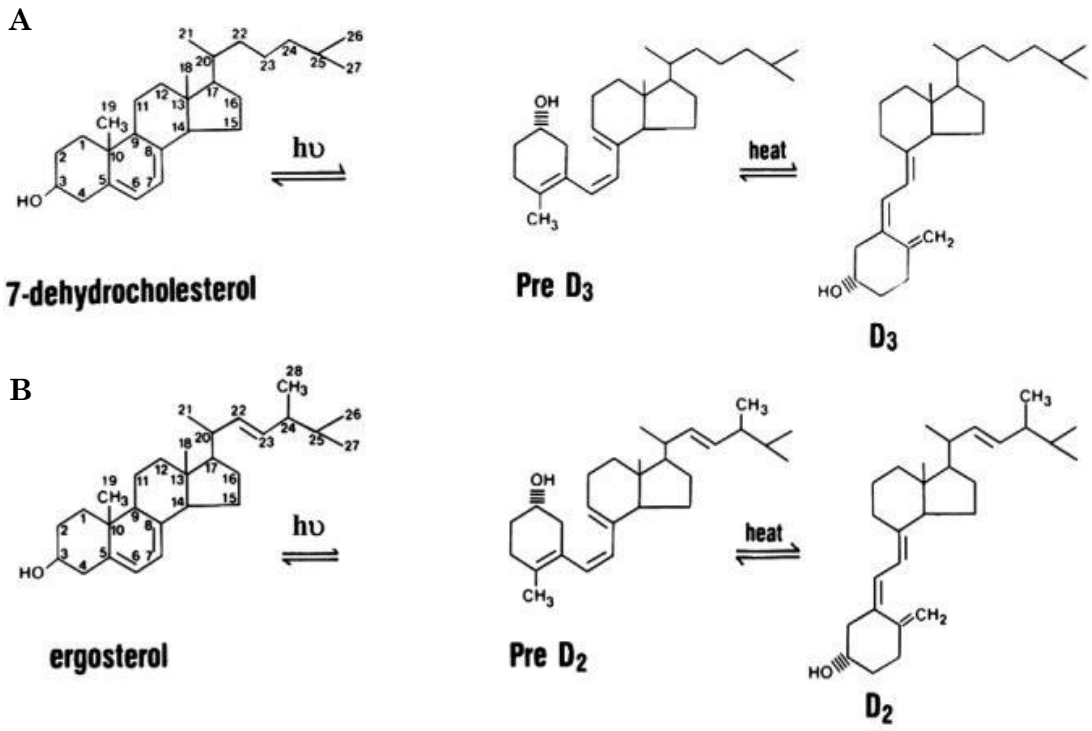

Figure 1. 6: The production and metabolism of D_2 and D_3. (A) 25(OH)D_3 (D_3) is produced in the skin from 7-DHC where the B ring is broken by UVB radiation, and the pre-25(OH)D_3 (pre-D_3) is isomerized to 25(OH)D_3 in a thermo-sensitive process. (B) 25(OH)D_2 (D_2) is considered as the first analog of vitamin D. It is made from UVB irradiation of ergosterol in plants and fungi. Taken from Bikle (2014).

The three principal steps in vitamin D metabolism are 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation, carried out by cytochrome P450 mixed-function oxidases (CYPs). These enzymes are found in the mitochondria (i.e. CYP27A1, CYP27B1, and CYP24A1) or the endoplasmic reticulum (i.e. CYP2R1; Bikle, 2014). The liver is the major if not the main source of 25(OH)D production from 25(OH)D_3. CYP27R1 has been found in microsomal pieces of mouse liver and is known to 25-hydroxylate D_3 and D_2 (Cheng et al., 2013). It is mainly expressed in the liver and testes. CYP2C11 also 25-hydroxylates D_3 and D_2 but is more commonly known to hydroxylate testosterone (Rahmaniyan et al., 2005). CYP3A4, also located in the liver and intestine has 25-hydroxylase activity but prefers 1α(OH)D as a substrate instead of 25(OH)D (Gupta et al., 2004). Another enzyme, CYP2D25, initially taken from pig kidney and liver (Postlind et al., 1997) was seen

to lack 25-hydroxylase activity (Hosseinpour and Wikvall, 2000). Therefore, CYP2R1 seems to be the principle 25-hydroxylase, however other enzymes do have 25-hydroxylase activity affecting 25(OH)D levels within a specific tissue, contributing to its circulating levels (Bikle, 2014).

The kidney is the major if not the main source of circulating $1,25(OH)_2D_3$ levels. Unlike 25-hydroxylation, only one enzyme is known to have 25(OH)D 1α-hydroxylase activity, CYP27B1. This enzyme is homologous to the other enzymes active in vitamin D metabolism; CYP27A1 and CYP24A1 (review by Bikle, 2010). Renal 1α-hydroxylase is modulated by three hormones; the fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH) and the $1,25(OH)_2D_3$ itself. PTH activates while FGF23 and $1,25(OH)_2D_3$ inactivate CYP27B1. Increased calcium levels represses CYP27B1 mainly by suppressing PTH, increased phosphate represses CYP27B1 by activating FGF23, although phosphate ions can directly affect CYP27B1 independently (Bikle and Rasmussen, 1975; Bikle et al., 1975). $1,25(OH)_2D_3$ reduces activity of CYP27B1 through inhibition of PTH and activating FGF23 as well as reducing the levels of $1,25(OH)_2D_3$ by increasing CYP24A1, the catalytic enzyme of vitamin D (Kim et al., 2007).

CYP24A1 is the only well-known 24-hydroxylase that takes part in vitamin D metabolism. It has 24-hydroxylase as well as 23-hydroxylase activity, of which the ratio is species dependent (Jones et al., 2012). The 24-hydroxylase pathway causes biological inactivation of calcitroic acid and the 23-hydroxylase pathway produces biologically active 1,23, 25-26 lactone. The main function of CYP24A1 is known to be the prevention of accumulation of toxic 25(OH)D and $1,25(OH)_2D_3$ levels (Bikle, 2014). The biologically active metabolite, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) produced in the kidneys (Bikle, 2011) is the preferred substrate, with the highest affinity to the vitamin D receptor (VDR).

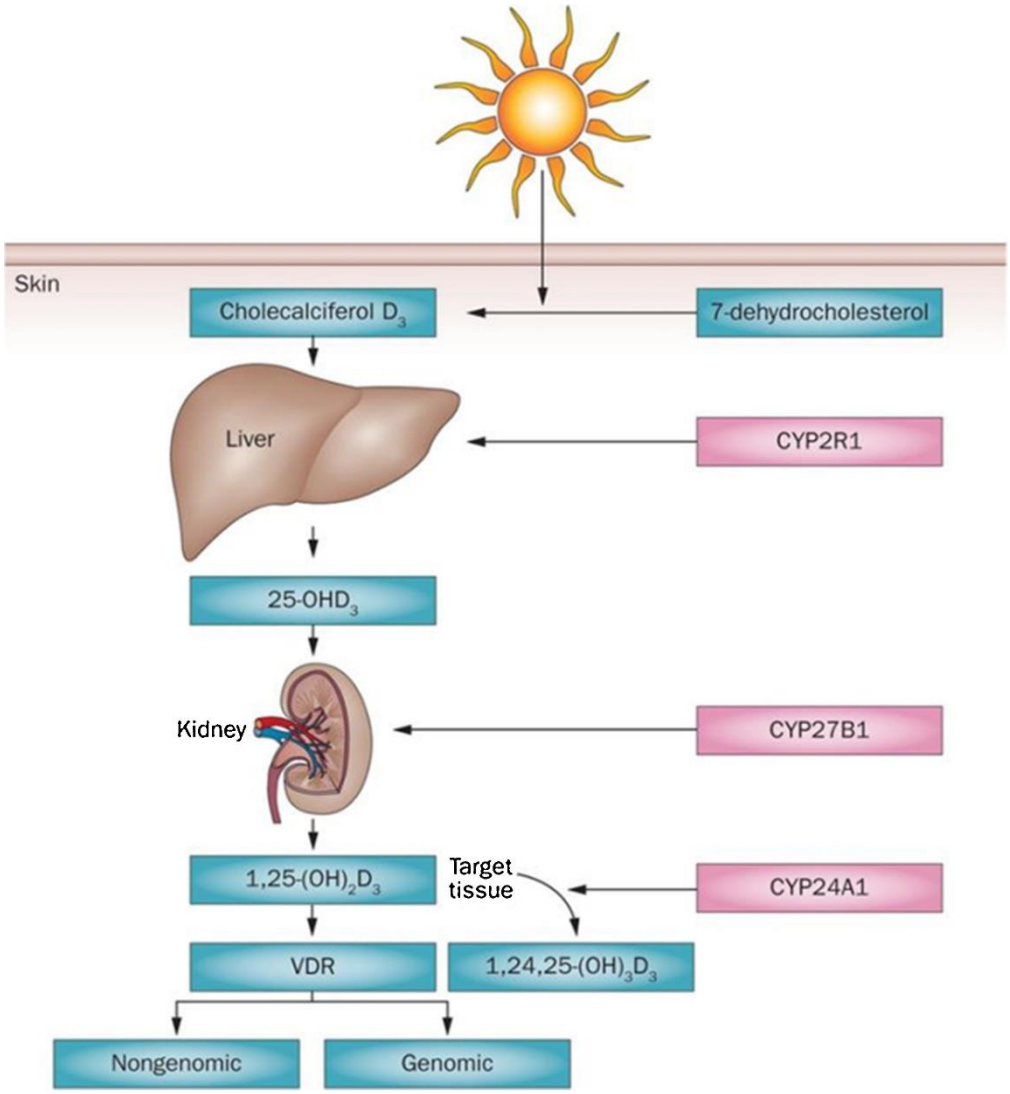

Figure 1. 7: Vitamin D metabolism. Vitamin D production starts in the skin tissue, when ultraviolet B photons convert the 7-dehydrocholesterol precursor of vitamin D to cholecalciferol. Cholecalciferol is inactive, and before it becomes 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), it undergoes two hydroxylation steps. Hepatic CYP2R1 mediated 25-hydroxylation and renal CYP27B1 mediated 1α-hydroxylation. Cholecalciferol is converted to the primarily circulating metabolite, 25-hydroxyvitamin D_3 ($25(OH)D_3$), in the liver. The biologically active metabolite, $1,25(OH)_2D_3$, is produced in the kidneys. $1,25(OH)_2D_3$ binds to the vitamin D receptor (VDR) and regulates genomic or non-genomic effects. Taken from Fathi et al. (2019).

1.3.2. VDR and vitamin D function

All genomic actions of $1,25(OH)_2D_3$ are mediated by the VDR. VDR is transcription factor and is part of the steroid hormone nuclear receptor family (Pike and Meyer, 2010; Haussler et al., 2010). It consists of 3 domains; the N-terminal DNA binding domain that has two zinc fingers that bind the grooves of the DNA at distinct sites (vitamin D responsive elements: VDREs), the C-terminal ligand binding domain and the hinge region that bind the two domains to each other. The ligand binding domain consists of 12 helices. The terminal helix works as a gate, closing around the bound ligand, forming an interface for co-activators and also facilitating interaction between VDR and its heterodimer partner, usually retinoid X receptor (RXR; Nishikawa et al., 1994). In the absence of RXR, VDR may form a heterodimer with a different partner or may indirectly bind to DNA through another transcription factor (Carlberg et al., 2013). VDR has been found to co-localise with pioneer factor PU.1 (Novershtern et al., 2011), GABPA, a DNA binding motif which overlaps with DR3 elements (Hong et al., 2016; Seuter et al., 2016), as well as other transcription factors (TF) like $RUNX_2$, TCF_4 and CEBPβ (Meyer et al., 2012). In the nucleus, VDR binds to the conserved genomic sequences, VDREs. There is a considerable amount of variability in VDRE sequences, however most that have the highest affinity for VDR are direct repeats of two hexameric binding sites including three spacing nucleotides called DR3 (Carlberg et al., 1993). VDR bound to its VDRE results recruits coregulatory complexes needed for its specific genomic activity. Depending on the target gene, co-activators or co-repressors are attracted to activate or repress gene expression (Fig. 1.8; Nagpal et al., 2005; Pike et al., 2012; Haussler et al., 2013).

ChIP-seq and ChIP-chip techniques have improved our understanding of the vitamin D mechanism at a genomic level. For instance, in mouse osteoblast, 1 200 VDR binding sites were identified at basal levels, upon $1,25(OH)_2D_3$ administration, the binding sites increased to 8 000 (Meyer et al., 2010b).

Notably, 1) the amount of VDR binding sites found on the genome is cell type-specific, 2) the transcription unit for activation is primarily, but not always the

VDR/RXR heterodimer, and 3) VDR binding sites are primarily, but not always, hexamer half-sites divided by 3 base pairs (Pike and Meyer, 2010).

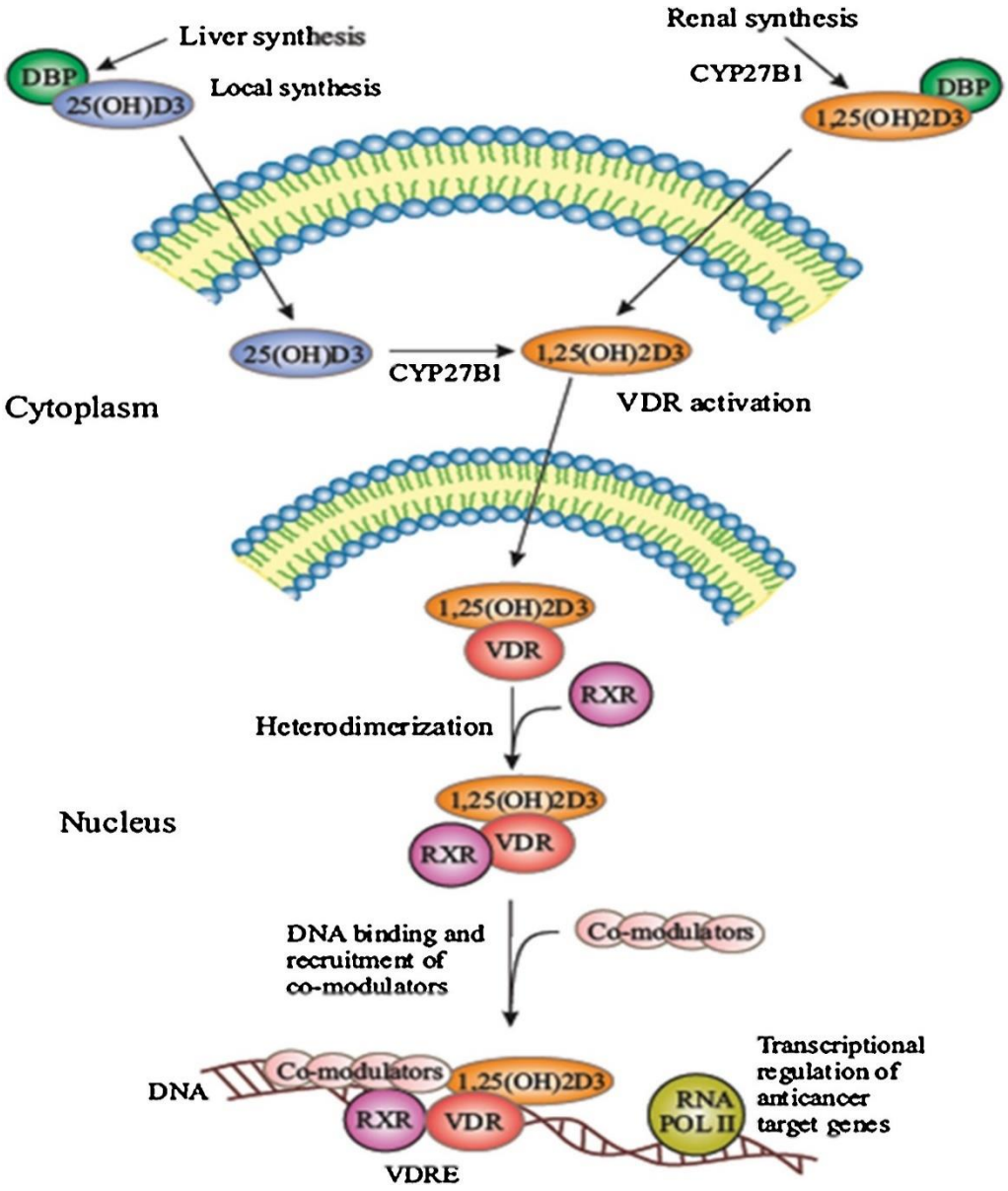

Figure 1. 8: The genomic mechanism of vitamin D and VDR. 25(OH)D_3 and 1,25(OH)$_2D_3$ circulate bound to a vitamin D binding protein (DBP). The biologically active metabolite 1,25(OH)$_2D_3$ binds to the vitamin D receptor (VDR) and liganded VDR forms a heterodimer with the nuclear retinoid X receptor (RXR) and is transported to the nucleus. The heterodimer complex binds to conserved genomic sequences, vitamin D response elements (VDREs) in a number of regulatory regions found in the promoter target genes, recruiting co-activators or co-repressors, causing positive or negative regulations in transcription of the genes. Taken from Fathi et al. (2019).

1.3.3. Vitamin D in cancer

Through these actions, vitamin D modulates calcium and bone metabolism (Fleet et al., 2012). Studies have gathered evidence that $1,25(OH)_2D_3$ does not only function in calcium and bone metabolism (Chiang and Chen, 2009). Wide sets of data have suggested that vitamin D can mediate the whole tumorigenesis process, from beginning to metastasis and microenvironment interactions in the cell (Giammanco et al., 2015). The mechanisms vitamin D affects cell behaviour regulation like proliferation, autophagy, angiogenesis, differentiation, apoptosis, inflammation and immune pathways (Ylikomi et al., 2002; Chiang and Chen, 2013). For example, vitamin D is commonly known to stop cell cycle progression by resulting in cell cycle arrest at the G_0-G_1 transition. In numerous cell lines, $1,25(OH)_2D_3$ supplementation has induced the expression of cyclin dependent kinases inhibitors (CDKIs) such as p21WAF1/CIP1 and p27kip1 to mediate cell cycle arrest (Liu et al., 1996; Wang et al., 1996; Jiang et al., 1994; Moffatt et al., 2001; Boyle et al., 2001).

In a study done on mouse models, $1,25(OH)_2D_3$ signalled through VDR has been shown to decrease prostate tumour growth and regulate cancer-related cellular events, but there is still lack of proper evidence. Calcium intake levels were also tested, because calcium has been reported to suppress renal production of vitamin D resulting in higher prostate cancer risk. It was found that increased vitamin D serum levels (higher than what is needed to protect bone; 1,000 IU/kg) can reduce the progression of prostate cancer. In addition, $1,25(OH)_2D_3$ levels in the prostate were inversely proportional to proliferation and increased dietary calcium levels caused increase in tumour growth because of the suppression of serum 25(OH)D levels. VDR ChIP-seq showed that vitamin D played a role on the inhibition of genes that control proliferation, promoting apoptosis and DNA protective pathways. Lastly, VDR binding peaks were found near genes that take place in immune response (Fathi et al., 2019). The study showed that vitamin D signalling regulates early stage prostate cancer progression and that manipulating vitamin D serum levels can change the route of early prostate cancer (Fathi et al., 2019).

Evidence has shown that adjustable variables such as vitamin D levels influence cancer risk and immune system function (Dou et al., 2016; Ogino et al., 2018). The important role that inflammation and the immune system plays in the etiology of cancer in undeniable as well as post-transplant malignancies caused by long-term immunosuppression (Fathi et al., 2019). In addition, supplemental or dietary intake of vitamin D and systemic vitamin D levels have been associated with reduced risks of cancer mortality and incidence (Dou et al., 2016). For example, gastrointestinal cancer occurs in an environment rich in microbiota and intestinal associated immune tissue, and has been a model disease for immunology-molecular pathological epidemiology (MPE). MPE is an emanate field that links environmental/lifestyle to immunity diseases or molecular pathologies as well as provide biomarkers, precision medicine, prevention strategies and treatment programs. A study was done on the correlation between vitamin D plasma levels and incidence of colorectal carcinoma subtypes and gastrointestinal cancer using immuno-MPE (Song et al., 2016). The extent of vitamin D for cancer prevention was found to be stronger in cancers having higher levels of lymphocytic infiltrates. This could be because certain immune cells have the ability to convert 25(OH)D to $1,25(OH)_2D_3$, which then creates an inhibitory effect against neoplastic growth in patients (Song et al., 2016; Ogino et al., 2018). Additionally, higher levels of 25(OH)D were negatively correlated to colorectal cancer risk, irrespective of VDR expression level in tumour cells (Jung et al., 2014). Moreover, there has been recent epidemiologic evidence supporting the importance of sufficient vitamin D administration and sunlight exposure, for the prevention of numerous types of cancer (Shui and Giovannucci, 2015).

1.3.4. Vitamin D in breast cancer

It has been suggested that vitamin D may prevent and ameliorate several cancer prognoses, including BCa. A study by Huss et al. (2014) reported a U-shaped correlation between pre-diagnostic vitamin D serum levels and BCa-related death risk. Poor survival was seen in patients with the lowest and highest vitamin D levels, compared to patients with intermediate levels of vitamin D. Numerous

research studies have supported the association with low vitamin D levels and poor BCa prognosis (Yao et al., 2017; Jacobs et al., 2016; Freedman et al., 2007; Rose et al., 2013), as well as patients with high vitamin D levels that were at high risk of BCa death (Huss et al., 2014).

Saracligil et al. (2017) showed that vitamin D reduces the growth of MCF-7 BCa cells in a dose dependent manner. Notably, vitamin D deficiency is common among BCa cases (reviewed by Atoum and Alzoughool, 2017) and consequently, it has been considered as a therapeutic agent in BCa therapy (Pilon et al., 2014; Chang et al., 2015). However the efficacy of vitamin D supplementation as adjunct therapy for BCa is not conclusive, with clinical trials reporting inconsistent results (Table 1). For example, Madden et al. (2018) showed positive results with vitamin D effects on BCa while Ismail et al. (2018) showed a negative association.

Vitamin D employs its function through the VDR, situated in ductal epithelial cells and lobule of normal mammary glands (Berger et al., 1988; Narbaitz et al., 1981). BCa lesions have been reported to express more VDR than normal breast tissue (Friedrich et al., 2002). Since female breast cancer patients with intermediate compared to low levels of vitamin D may have greater survival, it could be assumed that expression of VDR in BCa is also correlated with better prognosis (Huss et al., 2019). A few studies have shown an association between VDR expression and BCa survival, as well as tumour prognostic features. However, these studies have inconsistent results and most did their assessment with a small number of breast tumours (Al-Azhri et al., 2017; Berger et al., 1991; Ditsch et al., 2012; Eisman et al., 1986; Freake et al., 1984; Friedrich et al., 2002). In a recent study however, VDR immunohistochemical staining was done on more than 700 primary and invasive breast tissue from the Malmö diet and cancer study (MDCS; Elebro et al., 2017). It was found that increased VDR expression in BCa cell nuclei was associated with better prognosis and lower BCa death risk. Moreover, female patients with VDR-positive tumours had a higher rate of survival than those with VDR-negative tumours (Huss et al., 2019). Therefore, vitamin D status is a

modifiable BCa risk factor. Sufficient vitamin D concentrations have the potential of preventing BCa and may reduce its aggressiveness. Vitamin D deficiency is correlated with higher risk of BCa. Also, elevated VDR levels in BCa tissue may be a lead in targeting this receptor for treatment (Hemida et al., 2019) or making it more effective in chemotherapeutic treatments of BCa, thus possibly improving the genomic stability of BCa cells.

Table 1: Efficacy of vitamin D supplementation as adjunct therapy in female breast cancer patients.

Sample (Country)	Intervention	Effects on 25(OH)D status	Effect on prognosis	Reference
n = 60 **(USA)**	VD[a]-HD[b]: oral 50 000 IU/week for 12 weeks (<40 ng/ml). VD-ss[c]: 1200 mg/day calcium + 600 IU/day VD (Viactiv chews) (>40 ng/ml).	Baseline: 63% VD deficient (<20 ng/ml) or insufficient (20-31 ng/ml). At 12 weeks: 25(OH)D >40 ng/ml achieved for 42 subjects, with no opposing effects. At 16 weeks: Treatment with letrozole, more women with 25(OH)D levels >66 ng/ml (median level) reporting no disability from joint pain than women with levels <66 ng/ml (p = 0.026).	Vitamin D3 supplementation with 50,000 IU p/week is safe, significantly increases 25(OH)D levels, and may reduce disability from AI[d]-induced arthralgias	Khan et al. (2010)
n = 260 **(Spain)**	<30 ng/ml: oral calcium 1000 mg + D3 800 IU/day, + oral D3 16 000 IU every 2 weeks. ≥30 ng/ml: calcium 1000 mg + D3 800 IU/day.	At baseline: 25(OH)D <30 ng/ml (90% of women). After supplementation every 2 weeks: 50% failed to reach sufficient concentrations at 3 months.	40 ng/ml 25(OH)D may prevent AI arthralgia development but higher doses are needed to achieve this level in women who are deficient at baseline.	Prieto-Alhambra (2011)
n = 246 **(USA)**	Dosage was stratified by low dose (<10 000 IU/week) or high dose (>10 000 IU/week)	At baseline: 33.3% of patients were VD deficient at the start of therapy.During neoadjuvant chemotherapy: 60% of the subjects received a VD dose < 10,000 units/week. VD use was associated with improved disease-free survival (DFS; p = 0.026).	VD supplementation in patients with non-metastatic HER2 (+) breast cancer is associated with improved DFS.	Zeichner et al. (2015)

Table 1: Continued…

n = 5 417 **(Ireland)**	Initiation of *de novo* VD post-diagnosis of invasive BCa	20% reduction in breast cancer-specific mortality in *de novo* VD users compared to non-users (p = 0.048) Reduction increased to 49% if VD was introduced within 6 months of BCa diagnosis (p < 0.001) *De novo* VD use, post-diagnosis: associated with a reduction in breast cancer-specific mortality.	VD has the potential as a non-toxic and inexpensive agent to improve survival in breast cancer patients.	Madden et al. (2018)
n = 50 **(Egypt)**	Serum level 25(OH)D was measured	VD deficiency: 25(OH)D <20 ng/ml. 15 patients had VD deficiency: Positively associated with larger tumour size (p < 0.001), advanced stage (p = 0.001), lymph node positivity (p = 0.012) and HER2/neureceptor expression (p = 0.002).	VD deficiency had a negative effect on overall and disease-free survival in the breast cancer cases.	Ismail et al. (2018)

[a] Vitamin D

[b] 25(OH)D level > 40 ng/ml at baseline continued on standard VD supplementation and calcium only

[c] 25(OH)D level ≤ 40 ng/ml at baseline began 50 000 IU of oral vitamin D_3 every week

[d] Aromatase inhibitors

1.3.5. Vitamin D and the epigenome

Vitamin D interacts with the epigenome on many levels. The genes that play important roles in vitamin D signalling have promoters with large CpG islands and thus can be silenced by DNA methylation. The genes include those coding for VDR, CYP2R1, CYP27B1 and CYP24A1. Also, VDR directly interacts with coactivators and corepressors which then come into contact with chromatin modifiers like, histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and chromatin remodelers (Fig. 1.9; Pan et al., 2010).

The chromatin environment controls genomic activity in the whole genome. Post-translational modifications at the histone N-terminal tails, causing a shift at the nucleosomes, the chromatin relaxes and there's gene activation (Meyer et al., 2013). Histones undergo modifications such as lysine acetylation, lysine and arginine methylation as well as serine and threonine phosphorylation (Esteller, 2008). When genes are silenced epigenetically, CpG island hypermethylation occur through the lack of acetylation on histone 3 (H3) and histone 4 (H4), no methylation on lysine 4 (K4) on H3 (H3K4), and addition of methylation at K9 and K27 on H3 (H3K29 & H3K27; Esteller, 2008). Coactivators recruited by VDR, such as p160 steroid receptor coactivator proteins (SRC1, 2 & 3) have lysine acetyltransferase activity. In a study by Seuter et al. (2013), THP-1 cells treated with $1,25(OH)_2D_3$ induced H3K27 acetylation at promoter regions of many VDR target genes. In MDA-MB453 BCa cells, $1,25(OH)_2D_3$ supplementation regulated expression of the potent cyclin-dependent kinase inhibitor, p21 through histone methylation and acetylation (Saramäki et al., 2009). Therefore, histone methylation can cause gene activation or silencing, depending on what histone site is methylated as well as the methylation degree (i.e. mono-, di-, or tri-methylation), the amino acids affected and where they are situated at the histone tail (Esteller, 2008).

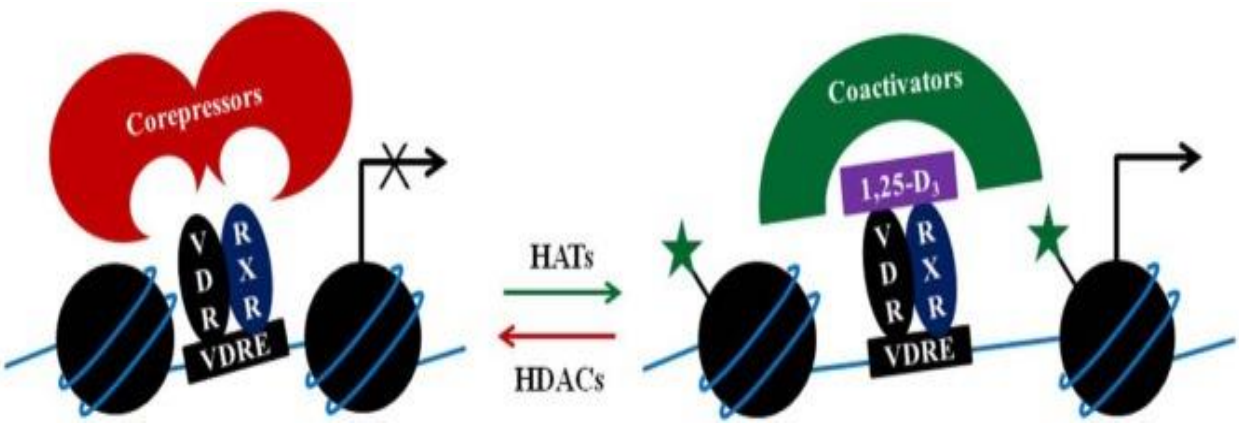

Figure 1. 9: Illustration of a two-step co-regulator model. In the absence of $1,25(OH)_2D_3$, the VDR/RXR complex may bind corepressors resulting in gene repression, by recruiting histone deacetylases (HDACs). Upon ligand binding, corepressors are replaced by coactivators, such as histone acetyltransferases (HATs). Acetylation of histones (green stars) allows for chromatin relaxation and gene transcription. Taken from Fetahu et al. (2014).

On one hand, vitamin D is regulated by epigenetic mechanisms while on the other hand, it plays specific roles in regulating epigenetic reactions. Regulation of gene expression is a refined mechanism and if deregulated, can result in pathological conditions. The role of vitamin D in maintaining normal epigenetic function emphasises the importance of this hormone in physiology (review by Fetahu et al., 2014). However, the detailed mechanism of its actions is still not fully understood.

1.4. Hypothesis

Given the genomic effects of vitamin D, it was hypothesized that vitamin D directly interacts with the epigenome to maintain chromosomal stability and regulate gene expression. Here, we hypothesise that vitamin D will increase global DNA methylation in hypomethylated breast cancer cells by increasing *DNMT* expression or inhibiting *TET* expression.

1.5. Aim

Since breast cancer has been associated with vitamin D deficiency and a loss of global DNA methylation, the aim of the study was to assess the molecular mechanism governing vitamin D-induced changes in global DNA methylation in MCF-7 BCa cells.

1.6. Objectives:

1) To establish control and experimental MCF-7 BCa cells to be treated with and without $1,25(OH)_2D_3$.
2) To assess the effect of $1,25(OH)_2D_3$ on cell viability and proliferation with a trypan blue and MTT assay, respectively.
3) To extract high quality, intact genomic DNA and quantify changes in global methylation and hydroxymethylation in MCF-7 BCa cells supplemented with or without $1,25(OH)_2D_3$.
4) To extract high quality, intact RNA from MCF-7 BCa and quantify the effect of $1,25(OH)_2D_3$ on the expression of genes encoding proteins involved in cytosine modification (*DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3*) as well as *VDR*.
5) To perform a statistical analysis to evaluate the effect of $1,25(OH)_2D_3$ on DNA methylation and the expression on genes regulating DNA methylation in MCF-7 BCa cells.
6) To validate the observed effect of $1,25(OH)_2D_3$ in non-malignant cells by repeating objectives 1-6 in the non-cancerous, control cell line, HEK293.

7) To perform a bioinformatics analysis on *DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3* to identify putative transcription factor binding sites for VDR, RXR, PU.1, $RUNX_2$, TCF_4, CEBPβ and DNA binding motif, GABPA.

2.1. Cell culture and treatment

Michigan Cancer Foundation-7 (MCF-7; passage 31) breast cancer cells and Human embryonic kidney 293 cells (HEK293; passage 12) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Biowest, Riverside, MO) supplemented with 5% (v/v) FBS (Pan Biotech, Aidenbach, Germany) and 1% (v/v) Penicillin-Streptomycin mix (Sigma Aldrich, St. Louis, MO) to about 80-90% confluency. Once confluent, cells were treated with 10 nM $1,25(OH)_2D_3$ (Sigma Aldrich, St. Louis, MO), 100 nM $1,25(OH)_2D_3$, or vehicle control (10 nM or 100 nM ethanol; Sigma Aldrich, St. Louis, MO) for 18 h. Cultures were kept at 37 °C in 5% CO_2. Following treatment, cells were detached by incubation in 2 ml trypsin (Sigma, St. Louis, MO) at 37 °C for 5 min. The cell suspension was centrifuged at 1 000 × *g* and the supernatant discarded. Cell pellets were re-suspended in 1 ml phosphate buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4; Sigma, St. Louis, MO). Cells were counted and viability estimated with the trypan blue (Sigma, St. Louis, MO) assay. Cell were either harvested and stored at -80 °C for RNA (5×10^5) and DNA (1×10^6) extraction, or used directly for an MTT assay (1×10^4). All experiments were done at least three times, with at least two technical replicates included in each independent experiment. HEK293 cells were included in the study as a control cell line representative of cells where global hypomethylation is normal.

2.1.1. Trypan blue assay

To determine cell viability, the trypan blue (Sigma, St. Louis, MO) exclusion assay was used. This assay is based on the principle that viable cells have intact cell membranes that exclude dyes like trypan blue, whereas non-viable cells do not (Strober, 2001). Thus, the cells that took up trypan blue were considered non-viable, while viable cells remained clear. A 10X dilution of cell aliquots in trypan blue solution were prepared (5 µl of cells and 45 µl of trypan blue). The solution

(10 µl) was loaded onto a haemocytometer and all cells present in each square of the haemocytometer was counted. The number of viable cells were counted by taking the average live cells per square multiplied by the dilution factor (10), multiplied by the chamber conversion factor (10 000). The percentage viability was calculated by taking the number of viable cells and dividing it by the total cell count (viable and non-viable) within the squares on the haemocytometer, and multiplying it by 100.

2.1.2. MTT assay

To asses cell proliferation the MTT assay was used. This colorimetric assay uses the reduction of yellow tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to detect cell proliferation as it measures the growth rate of cells by means of the linear relationship between cell activity and absorbance (Mahajan et al., 2012). Viable cells have NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT reagent to an insoluble crystalline product with a deep purple colour called formazan (Bahuguna et al. 2017) that can be quantified at 570 nm. For the assay, cells were seeded at a density of 1 x 10^5 cells / 45 µl culture media. The cells were left to attach for 24 h. Cells were treated with $1,25(OH)_2D_3$, vehicle control, or tissue culture media (serving as a blank) and incubated for 18 h. Following incubation, 5 mg/ml MTT (Sigma Aldrich, St. Louis, MO) was added to the cells. After a 4 h incubation, 55 µl of 10% (w/v) SDS (Sigma Aldrich, St. Louis, MO) in 0.01 M HCl (solubilizing agent; ACE Chemicals, Theta, Johannesburg) was added and the cells were further incubated for 16 h. Absorbance was measured at 570 nm on the Multiskan® GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The darker the solution, the greater the metabolic cell activity and number of viable cells (Barnabé, 2017). The MTT assay was performed on 3 independent biological replicates, with each experiment containing 3 technical replicates.

2.2. Genomic DNA (gDNA) extraction

To quantify the change in DNA methylation induced by $1,25(OH)_2D_3$, gDNA was extracted using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, Irvine, CA). All experiments were performed at 4 ºC unless stated otherwise. Centrifugation steps were done at 16 000 *x g* for 1 min. The frozen cell pellets (1 $x10^6$ cells) were re-suspended in a mixture of 200 µl DNA elution buffer and 3µl ß-mercaptoethanol; a protein reductant that breaks disulphide bonds between and within the proteins (Wingfield, 2016). 200 µl BioFluid & Cell Buffer and 20 µl Proteinase K was added. The solution was mixed thoroughly and incubated at 55 °C for 15 min. Thereafter the same volume of Genomic Binding Buffer (1:1) was added to the cell lysate. The mixture was transferred to a Zymo-Spin™ IIC-XL column in a collection tube and centrifuged. The collection tube was discarded with the flow through and 400 µl DNA Pre-Wash Buffer was added to the column in a new collection tube and centrifuged. The collection tube was emptied and 700 µl g-DNA Wash Buffer was added and centrifuged followed by another 200 µl g-DNA Wash Buffer and centrifugation step. The collection tube and its flow through was discarded. To elute the DNA, the column was inserted into a clean micro centrifuge tube and 30 µl DNA Elution Buffer was added to the column. The sample was incubated at 55 °C for 7 min and centrifuged. Eluted DNA was stored at – 80 °C.

2.3. RNA extraction

RNA was extracted using the Direct-zol™ RNA MiniPrep Plus Kit (Zymo Research, Irvine, CA). All experiments were performed at 4 ºC unless stated otherwise. Centrifugation steps were done at 16 000 *x g* for 30 sec unless stated otherwise. A mixture of 1 µl ß-mercaptoethanol and 600 µl of Tri-Reagent™ (Sigma, St Louis, MO) was added directly to the frozen cell pellets (5 x 10^5 cells) and the sample was aspirated thoroughly. Tri-reagent is a ready-to-use phenol-based reagent which functions to maintain the integrity of RNA during homogenisation while simultaneously disrupting and breaking down cell components (Rio et al., 2010). To recover the RNA through precipitation, 400 µl ethanol (Sigma, St Louis, MO) was added to the cell lysate and aspirated. The mixture was transferred onto a Zymo-Spin™ IIICG column in a collection tube and centrifuged. To remove gDNA contamination, a DNase I treatment was done where a mixture of 15 µl DNase I and 75 µl DNA Digestion Buffer was added directly onto the column matrix. This reaction mixture was incubated at room temperature for 20 min. After incubation, 400 µl Direct-zol™ RNA Pre-Wash was added to the column and centrifuged for 30 sec. The flow through was discarded and the step repeated. 700 µl RNA Wash Buffer was added to the column and centrifuged for 2 min to ensure complete removal of the wash buffer. To elute RNA, the column was transferred to an RNase-free tube and 20 µl DNase/RNase-Free Water was directly added to the column matrix and centrifuged. Eluted RNA was stored at – 80 °C.

2.4. Quality control

2.4.1. DNA and RNA purity

To assess the purity of the extracted DNA and RNA, the absorbance of the samples was quantified at 230, 260 and 280 nm on a NanoDrop® Spectrophotometer. DNA and RNA samples were measured at 260 nm because the heterocyclic rings of their nucleotides absorb ultraviolet (UV) light at this wavelength while the sugar-phosphate backbone does not contribute to absorption. At 280 nm, protein contamination was measured because proteins, particularly with aromatic amino acids like tyrosine, tryptophan, phenylalanine and tryptophan absorb UV light at 280 nm. A 260/280 ratio of 1.8 is generally accepted as pure for DNA and for pure RNA, a ratio of 2.0 is accepted (Wilfinger et al., 1997). The ratio for RNA is higher than DNA because RNA absorbs more UV light than DNA. This is because DNA is double stranded and thus absorbs UV light less strongly due to the stacking interactions between the bases (Alexander and Griffiths, 1993).

Another absorbance ratio used to test DNA and RNA purity was the A260/230 ratio. Phenolate ions, thiocynates and other organic compounds absorb UV light at 230 nm, therefore the A260/230 ratio measures the level of these contaminants. The expected A260/230 ratio ranges between 2.0-2.2. If the ratio is lower than expected, it may indicate that contaminants are present. The advantages of using UV light to measure RNA and DNA absorbance lies in the instrument used for quantification; the NanoDrop® Spectrophotometer that can detect as little as 2 ng/µl nucleic acid. Furthermore, only 1 µl of your sample is lost during quantification and a measurement can be obtained in less than 30 seconds (Wieczorek et al., 2012).

2.4.2. DNA and RNA integrity

To assess the integrity of the extracted DNA and RNA, the samples were electrophoresed through an agarose gel. Agarose gel electrophoresis separates molecules based on their size and charge. The molecules are migrated under the influence of an electric field, where negatively charged molecules such as DNA and RNA move towards the positive pole (anode). The smaller molecules migrate faster through gel, while the larger ones migrate slower (Sambrook and Russel, 2001). Under UV light, high-quality and high-molecular-weight DNA (> 50 kb) should be seen on the gel for the DNA to be deemed fully intact (Mayjonade et al., 2016). With RNA, two distinct bands of 18S and 28S with high molecular weight must be prominently seen on the gel for intact RNA. If there is gDNA contamination in the RNA samples, it will be visualised on the gel as gDNA runs slower through the gel matrix than RNA (Wieczorek et al., 2012).

For gel electrophoresis, a 10X TBE buffer stock solution was made, consisting of 0.89 M Trisaminomethane (Tris; Merck, Modderfontein, South Africa), 0.89 M Boric acid (Merck, Modderfontein, South Africa) and 0.5 M Ethylenediaminotetra-acetic acid (EDTA; Sigma Aldrich, St. Louis, MO) at a pH of 8.0 adjusted with 0.1 M NaOH (Sigma Aldrich, St. Louis, MO). This buffer was then diluted to a 1X TBE buffer solution which was used to run the agarose gels. To make a 1% (w/v) agarose gel for the DNA/RNA samples, 1.5 g of agarose (Lonza, Basel, Switzerland) was dissolved in 150 ml of 1X TBE buffer by heating the solution. After cooling the solution, 3 µl of Ethidium bromide (Sigma, St. Louis, MO) was added. Ethidium bromide is used because it binds to DNA/RNA and after illuminating the gel with a UV light source, the DNA/RNA bands can be visualised (Sigmon and Larcom, 1996). Using a 6X DNA/RNA loading dye (Sigma, St. Louis, MO), 1 µl of the loading dye was mixed with 5 µl of DNA/RNA sample and the solution was added to the wells. The gel was subjected to an electromotive force of 90 mV for 30 min.

2.5. Global DNA methylation analysis

To quantify changes in global DNA methylation level in response to $1,25(OH)_2D_3$ supplementation, an enzyme-linked immunosorbent assay (5-mC DNA ELISA Kit; Zymo Research, Irvine, CA) was used. A standard curve was prepared from methylated (positive) and unmethylated (negative) DNA standards (Table 2.1). The extracted DNA samples (100 ng) together with the standard curve samples were brought up to a 100 µl of 5-mC coating buffer and denatured for 5 min at 98 °C. After denaturation, the tubes were immediately incubated for 10 min on ice. The remaining steps were done under low light due to the light sensitive antibodies, as it affects the activity of the conjugates used for detection (Vancells, 2017). The samples were added to the wells, covered in foil and incubated at 37 °C for 1 h. The buffer in the wells was discarded and the wells were washed 3 times with 200 µl 5-mC ELISA buffer. After washing, 200 µl of the same buffer was added to the wells, covered in foil and incubated for 30 min at 37 °C. During this incubation time, the antibody mix was prepared (Table 2.2). After incubation, the buffer in the wells were discarded and 100 µl of the antibody mix were added to the wells. Covered in foil again, the wells were incubated for 1 h at 37°C. The antibody mix was discarded and the wells were washed 3 times with 200 µl 5-mC ELISA buffer. 100 µl HRP developer solution was added to the wells after the wash and the colour was left to develop for 1 h. The absorbance was measured at a wavelength of 415 nm on a Multiskan GO® Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). To quantify the methylation level, a standard curve was constructed, with the absorbance on the y-axis and % 5-mC on the x-axis. The standard curve samples were performed on 2 technical replicates while sample DNA was assessed in at least 3 independent biological replicates; each containing 3 technical replicates.

Table 2. 1: Reaction components for the methylated and unmethylated DNA standards.

% 5-mC	0% methylated DNA (µl)	100% methylated DNA (µl)
0	10.0	0
5	9.5	0.5
10	9.0	1.0
25	7.5	2.5
50	5.0	5.0
100	0	10

Table 2. 2: Reaction components for the antibody mix used to quantify global DNA methylation level.

	Dilution	Volume (µl)	Example for 18 wells (µl)
5-mC ELISA Buffer	N/A	(number of wells + 2) × 100	2 000
Anti-5-Methylcytosine	1: 2 000	Buffer Volume ÷ 2 000	1
Secondary Antibody	1: 1 000	Buffer Volume ÷ 1 000	2
Total volume		**x**[a]	

[a] The total volume was dependent on the number of wells used in the procedure.

2.6. DNA hydroxymethylation analysis

To quantify changes in DNA hydroxymethylation level in response to $1,25(OH)_2D_3$ supplementation, an enzyme-linked immunosorbent assay (Quest 5-hmC DNA ELISA Kit; Zymo Research, Irvine, CA) was used. A standard curve was prepared from hydroxymethylated (positive) and unmethylated (negative) DNA standards (Control A-E) provided with the kit (Table 2.3). A 100 µl/well of 1ng/µl anti-5-hydroxymethylcytosine polyclonal antibody was added to the wells, covered in foil and incubated at 37 °C for 1 h. The buffer was discarded and the wells were washed 3 times with 200 µl 1X ELISA buffer. After washing, 200 µl of the same buffer was added to the wells, covered in foil and incubated at 37 °C for 30 min. The extracted DNA samples (100 ng) together with the standard curve samples were brought up to a 100 µl of 1X ELISA buffer and denatured for 5 min at 98 °C. After denaturation, the samples were immediately transferred to ice for 10 min. The buffer was discarded and 100 µl of the denatured sample and control DNA were added to the each well. The wells were covered in foil and incubated at 37 °C for 1 h. After incubation, the buffer was discarded and the wells washed 3 times with 200 µl 1X ELISA buffer. The 100X anti-DNA HRP antibody solution was diluted in 1X ELISA buffer to final 1X concentration. 100 µl of the antibody mix was added to each well, covered in foil and incubated at 37 °C for 30 min. The buffer in the wells were discarded and washed 3 times with 200 µl 1X ELISA Buffer. 100 µl HRP developer solution was added to the wells after the wash and the colour was left to develop for 1 h. The absorbance was measured at a wavelength of 405 nm on a Multiskan GO® Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). To quantify the hydroxymethylation level, a standard curve was constructed, with the absorbance on the y-axis and % 5-mC on the x-axis. This data is then used to measure the concentration of the unknown samples in comparison to the linear portion of the standard curve. The standard curve samples were performed in 3 technical replicates, while sample DNA was assessed in at least 3 independent biological replicates; each containing 3 technical replicates.

Table 2. 3: Concentration 5-hydroxymethylation of the standards included in the analysis.

Control DNA set (100 ng/µl)	% 5-hmC
Control A	0
Control B	0.03
Control C	0.12
Control D	0.23
Control E	0.55

2.7. Quantitative reverse transcriptase PCR (RT-qPCR)

To assess the effect of $1,25(OH)_2D_3$ on *DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3*, and *VDR* expression, RT-qPCR was done using the SensiFast™ cDNA synthesis & SYBR No-Rox Kits (Bioline, London, UK). A master mix was prepared on ice for cDNA synthesis (Table 2.4). The reaction mixture was transferred to the SimpliAmp thermal cycler (Thermo Fisher Scientific, Waltham, MA) to allow primer annealing (25 °C for 10 min), reverse transcription (42 °C for 15 min) and inactivation (85 °C for 5 min). After synthesis the sample was cooled on ice. The cDNA amplification for specific genes was performed on the CFX-96 Thermal Cycler (Bio-Rad, Hercules, CA; Table 2.5 – 2.6). The primers for *DNMT3A, DNMT3B, TET1, TET2 and TET3* were designed using the PRIMER3 software (version 4.1.0; http://primer3.ut.ee/), while suitable primer pairs for *DMNT1*, *VDR, ACTB, GAPDH* and *B2M* were obtained from RTPrimerDB (Table 2.7). This experiment was done on at least three independent biological replicates, with two technical replicates for each gene.

Table 2. 4: Reaction components of the cDNA synthesis master mix.

Reagent	Volume (µl)
5x TransAmp Buffer	4
Total RNA (500 ng)	x[a]
RNase free-water	Up to 20
Reverse Transcriptase	1
Total volume	**20**

[a] The volume of RNA was dependent on the RNA concentration

Table 2. 5: Reaction components of the qPCR reaction.

Reagent	Volume (µl)
2x SensiFAST SYBR No-ROX Mix	5
10 µM Forward Primer	x[a]
10 µM Reverse Primer	x[b]
Template	1.5
RNase free-water	x[c]
Total volume	**10**

[a] The forward primer concentration varied for the different genes (100 nM: DNMT1, TET1, TET2, TET3; 200 nM: DNMT3A, DNMT3B, TET3, ACTB, GAPDH, B2M; 250 nM: VDR).

[b] The reverse primer concentration varied for the different genes (100 nM: DNMT1, TET1, TET2, TET3; 200 nM: DNMT3A, DNMT3B, TET3, ACTB, GAPDH, B2M; 250 nM: VDR).

[c] RNase free-water volume was dependent on the forward and the reverse primer volumes and RNA concentration.

Table 2. 6: The PCR reaction done in a 3-step cycle.

Cycles	Temperature (°C)	Time	Reaction
1	95.0	2 min	Polymerase activation
	95.0	5 sec	Denaturation
40	61.9	10 sec	Annealing
	72.0	5 sec	Extension

Table 2. 7: Primer sequences used to amplify the target and reference genes.

	Gene	Primer Sequences[a]	Product Size (bp)	RTprimerDB number	Reference
Target genes	*DNMT1*	FP: GTGGGGGACTGTGTCTCTGT RP: TGAAAGCTGCATGTCCTCAC	204	3628	Bestor et al., 1988
	DNMT3A	FP: CTGTGGGAGCCTCAATGTT RP: GCAGTTGTTGTTTCCGCAC	175	N/A[b]	
	DNMT3B	FP: CTCGTGTGGGGAAAGATCAA RP: CCAAATTAAAGTGCTGGCTGA	187	N/A	
	TET1	FP:AGATAAGGGCAGTGGAAAAGAA RP: TGGGGTTCGGTTTCACTTTT	151	N/A	
	TET2	FP: GTCCTATTGCTAAGTGGGTGG RP: CGTGCCGTATTTCCTCAGC	194	N/A	
	TET3	FP: GACACACCTGCCAAGAGAG RP: TATCACTCACCGCTCCTCC	152	N/A	
	VDR	FP: CTGACCCTGGAGACTTTGAC RP: TTCCTCTGCACTTCCTCATC	277	2787	Vienonen et al., 2003
Reference	*ACTB*	FP: CTGGAACGGTGAAGGTGACA RP:AAGGGACTTCCTGTAACAATGCA	140	1	Vandesompele et al., 2002
	GAPDH	FP: GTCAGTGGTGGACCTGACCT RP: AGGGGAGATTCAGTGTGGTG	395	2093	Wichmann et al., 2002
	B2M	FP: TATCCAGCGTACTCCAAAGA RP: GACAAGTCTGAATGCTCCAC	165	1528	Max et al., 2001

[a] The Forward (FP) and reverse (RP) primer sequences are given in the 5' - 3' direction.

[b] Not applicable – These primers were designed using Primer3.

2.8. Bioinformatics

Bioinformatics was used to identify putative transcription factor binding sites for VDR, RXR, PU.1, $RUNX_2$, TCF_4, CEBPβ and DNA binding motif, GABPA (Umesono et al., 1991; Carlberg et al., 1993) in *DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3*. The Genomatix software (MatBase version 11.1, released on June 2019, and https://www.genomatix.de/) was used to search for GABPA motifs in or near the candidate genes. The JASPAR software (version 8.0, released on January 2020, http://jaspar.genereg.net/) was used to find the transcription factor binding sites (VDR, RXR, PU.1, $RUNX_2$, TCF_4, and CEBPβ).

2.9. Statistical analysis

Statistical analysis was performed using IMB® SPSS® Statistics (v. 26; SPSS Inc. Chicago, IL). Gene expression was normalised using the Biogazelle qBase software and expressed as a fold change (using ΔΔCq) relative to the control (Kannan, 2016). $1,25(OH)_2D_3$-induced changes in cellular proliferation, DNA methylation and hydroxymethylation level, as well as gene expression was assessed using a Mann-Whitney test. Variance was evaluated using the Levene's test for equality of variance prior to use of the *t*-test. Correlation between VDR and the other target genes was analysed by computing the Pearson's correlation coefficients. A two-tailed p-value < 0.05 was considered significant.

3. Results

3.1 Quality Control

3.1.1. Nucleic acids used in the study were intact and pure

To assess the integrity of the extracted DNA and RNA, gel electrophoresis was done. Extracted DNA was consistently intact as illustrated by the high molecular weight band at the top of the agarose gel (Fig. 3.1A). The extracted RNA was also intact as illustrated by the presence of two bands representing the 28S and 18S rRNA subunits (Fig. 3.1B). On average, DNA extracted was relatively pure and free of protein and RNA contamination with 260/280 ratios of 1.8-2.0 and 260/230 ratios ranging between 2.0-2.2. The extracted RNA was also pure and free of phenol and DNA contamination with 260/280 and 260/230 ratios ranging between 1.8-2.0 and 2.0-2.2.

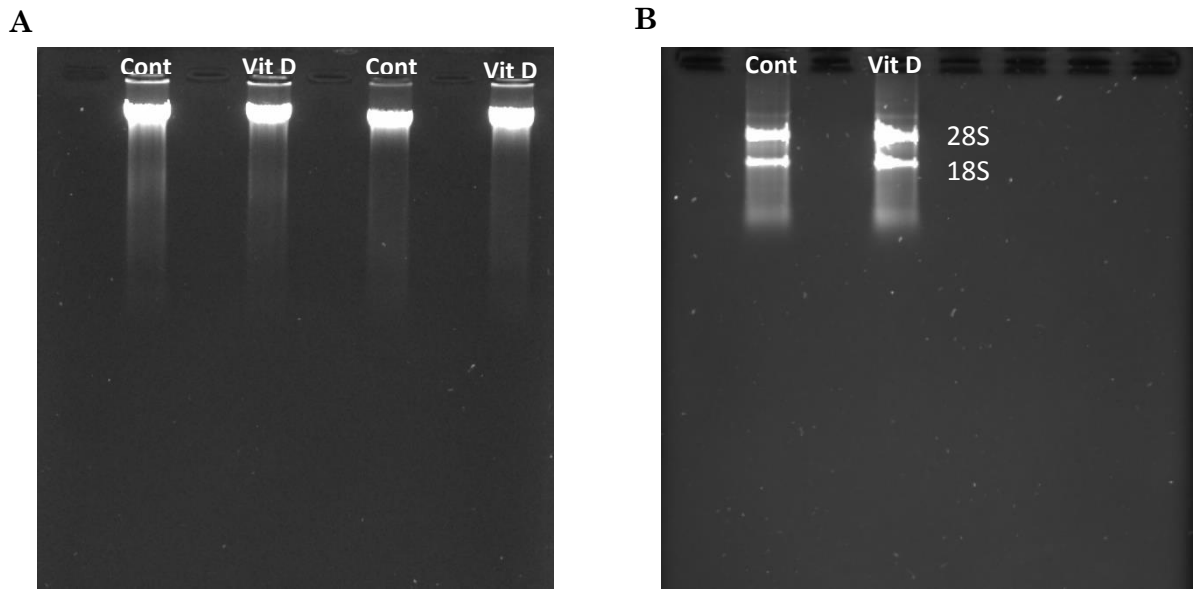

Figure 3. 1: The gels show intact DNA and RNA. The representative gels show extracted DNA (A) and RNA (B) from the cells supplemented with (Vit D) or without $1,25(OH)_2D_3$ (Cont).

3.1.2. Primers specifically amplified the gene of interest after cDNA synthesis

To assess whether the correct cDNA product was amplified during RT-qPCR of *DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, VDR, ACTB, GAPDH* and *B2M*, gel electrophoresis was conducted on the RT-qPCR products (Fig. 3.2). The observed product sizes matched the expected sizes, predicted by in silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr), with a 204 bp product for *DNMT1*, a 175 bp product for *DNMT3A*, a 187 bp product for *DNMT3B*, a 151 bp product for *TET1*, a 194 bp product for *TET2*, a 152 bp product for *TET3*, a 277 bp product for *VDR*, a 140 bp product for *ACTB*, a 395 bp product for *GAPDH*, and a 165 bp product for *B2M*. No products were amplified in the absence of a cDNA template (NTC). Additionally, the melt curve of each gene was analysed to verify if only one product was amplified using the Bio-Rad CFX Maestro software. With the exception of *TET3* and *VDR* showing smaller peaks below the threshold, PCR products for all the other genes showed one peak on the melt curve above the threshold line, which correlates with one product being amplified (Fig. 3.3).

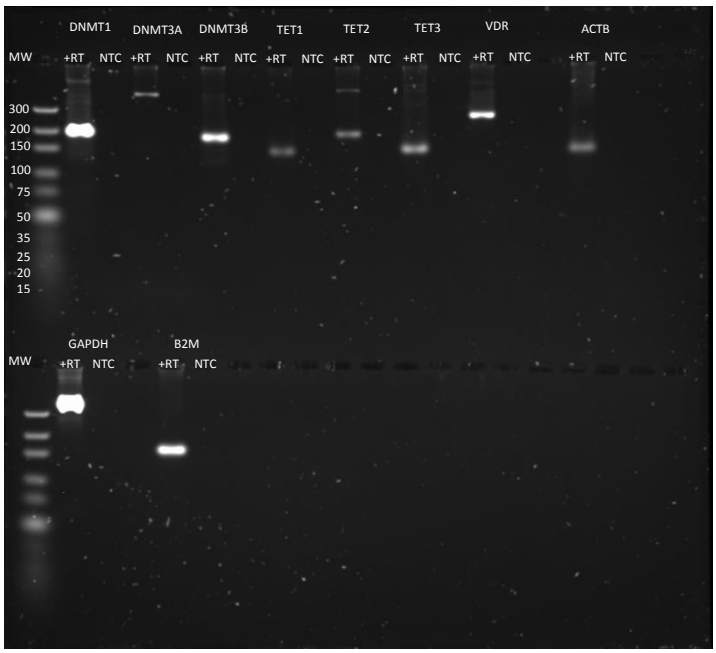

Figure 3. 2: Primers specifically amplified the gene of interest after cDNA synthesis. The agarose gel shows the RT-qPCR products of each primer pair (*DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, VDR, ACTB, GAPDH* and *B2M*). For each gene, a single band is observed at the expected base pair (bp) size (MW: molecular weight marker, +RT: reverse transcriptase, NTC: no template control).

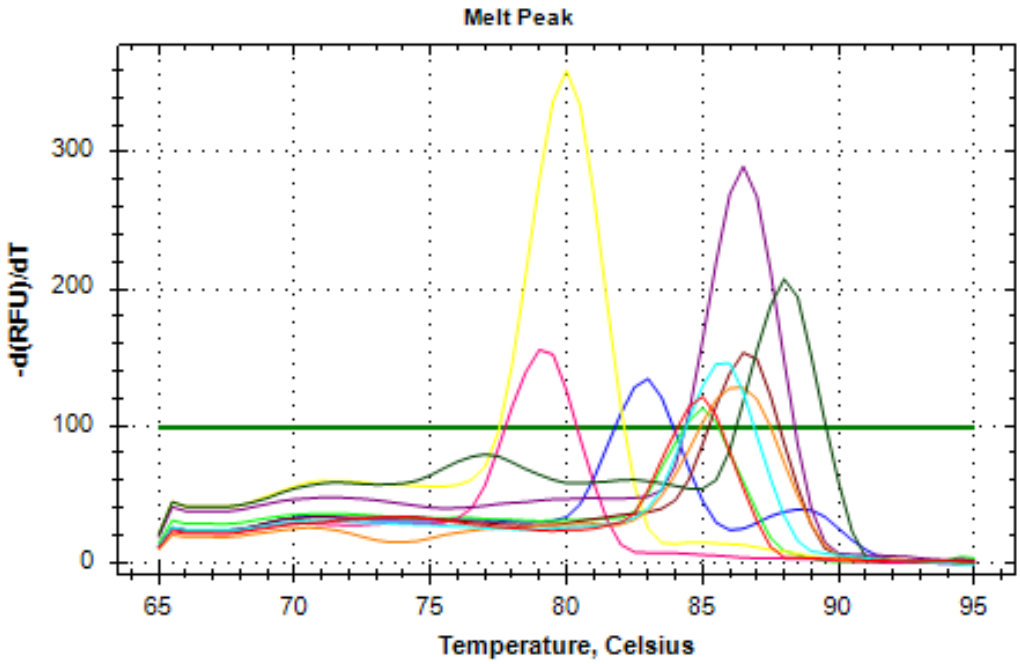

Figure 3. 3: Single melt peaks supports the specificity of primers used for RT-qPCR in the study. The melt curve shows one peak above the threshold for all the primer pairs (maroon = *DNMT1,* red = *DNMT3A,* light blue = *DNMT3B,* pink = *TET1,* light green = *TET2,* dark blue = *TET3,* dark green = *VDR,* orange = *ACTB,* purple = *GAPDH*, yellow = *B2M*). *TET3* and *VDR* primers displayed smaller peaks below the threshold.

3.1.3. Reference gene stability

The target genes were normalised against reference genes to obtain the normalised expression level. However, the house keeping genes used needed to be stably expressed. To analyse the stability of the reference genes (*ACTB*, *GAPDH* and *B2M*), the average expression stability value (M-value) of the reference genes was determined. The most stable reference genes have the lowest M-values, with the accepted limit for stability being an M-value of 0.50 (Yan et al., 2012). The M-values showed that *GAPDH* and *ACTB* were unstable (M-value > 0.50) in MCF-7 cells, and *B2M* could not be analysed due to inadequate Cq data values. In HEK293 cells, *B2M* and *ACTB* were stably expressed (M-value < 0.50) while *GAPDH* was acceptably stable (Table 3.1). Thus, for MCF-7 cells, gene expression data was normalised against the global mean scaled to the control values, while in HEK293 cells, gene expression was normalised against *B2M* and *ACTB*.

Table 3. 1: The M-values for the reference genes in MCF-7 and HEK293 cells.

Reference gene	M-value	
	MCF-7	**HEK293**
ACTB	1.928	0.183
GAPDH	1.928	0.183
B2M	N/A[a]	0.533

[a]Not available

3.2. 1,25(OH)$_2$D$_3$ induced no significant change in cell viability of MCF-7 or HEK293 cells

To determine whether 1,25(OH)$_2$D$_3$ influence cell viability, the trypan blue assay was performed. Neither 10 nM, nor 100 nM 1,25(OH)$_2$D$_3$ supplementation significantly altered cell viability in MCF-7 or HEK293 cells. There was also no difference in cell viability between MCF-7 and HEK293 cells with response to both concentration (Fig. 3.4).

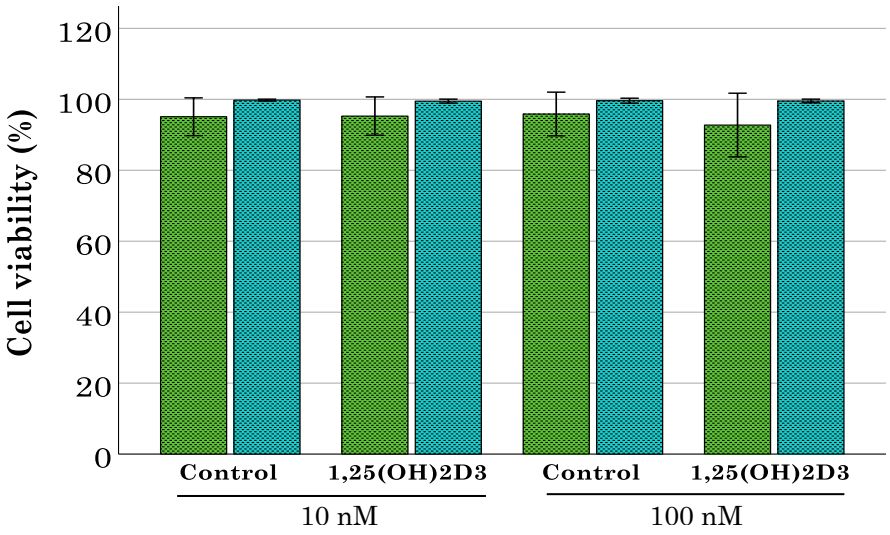

Figure 3. 4: 1,25(OH)$_2$D$_3$ supplementation did not significantly influence cell viability. The bar graph shows MCF-7 (green) and HEK293 (blue) cell viability in response to 10 nM or 100 nM 1,25(OH)$_2$D$_3$ supplementation. Error bars show ± 1SD (n = 6).

3.3. 1,25(OH)$_2$D$_3$ increased methylation in MCF-7 cells but had no effect on HEK293 cells

To assess the effect of vitamin D on DNA methylation, global methylation was quantified in response to 1,25(OH)$_2$D$_3$ supplementation in MCF-7 and HEK293 cells. Global methylation was significantly induced in MCF-7 cells after an 18 h in vitro supplementation with 10 nM, but not 100 nM 1,25(OH)$_2$D$_3$ ($P < 0.050$, Fig 3.5A). In HEK293 cells, there was no significant change in global methylation with 10 nM or 100 nM 1,25(OH)$_2$D$_3$ supplementation (Fig. 3.5B). Without 1,25(OH)$_2$D$_3$ supplementation, global methylation was significantly higher in MCF-7 than in HEK293 cells ($P < 0.010$). Interestingly, 10 nM 1,25(OH)$_2$D$_3$ supplementation also showed significantly higher levels of global methylation in MCF-7 cells compared to HEK293 cells ($P < 0.001$, Fig. 3.6). A similar trend was observed in response to 100 nM 1,25(OH)$_2$D$_3$.

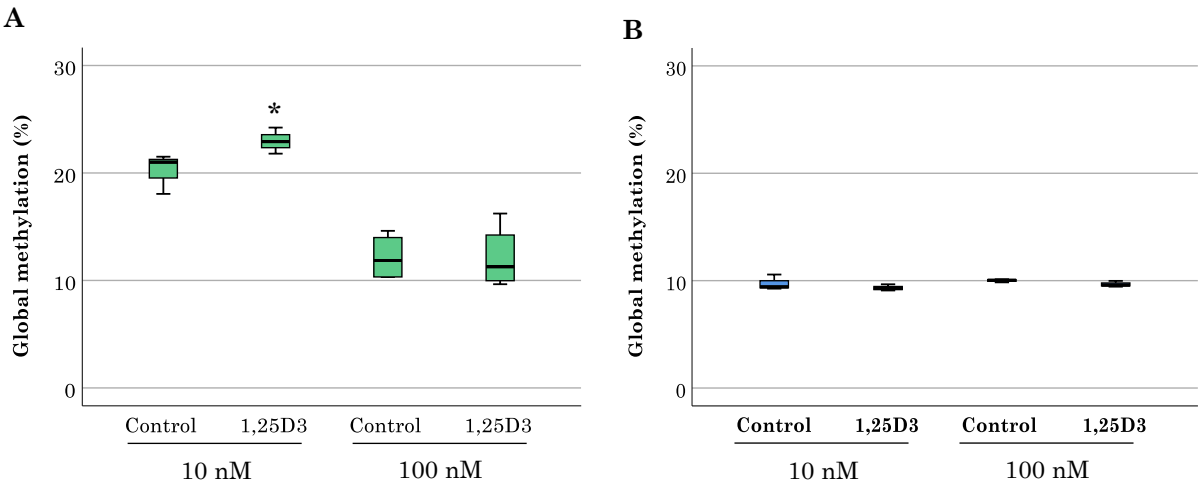

Figure 3. 5: 1,25(OH)$_2$D$_3$ significantly increased global methylation in MCF-7, but not HEK293 cells. The boxplots show global methylation level, quantified by ELISA, in MCF-7 (A) and HEK293 (B) cells. Pairwise comparisons showed that 10 nM 1,25(OH)$_2$D$_3$ significantly increased global methylation level in MCF-7 cells relative to the control (* $P < 0.050$). However, there was no change in global methylation with 100 nM 1,25(OH)$_2$D$_3$ supplementation in MCF-7 cells, nor did 1,25(OH)$_2$D$_3$ have any effect on HEK293 global methylation. Error bars show ± 1SD (n = 6).

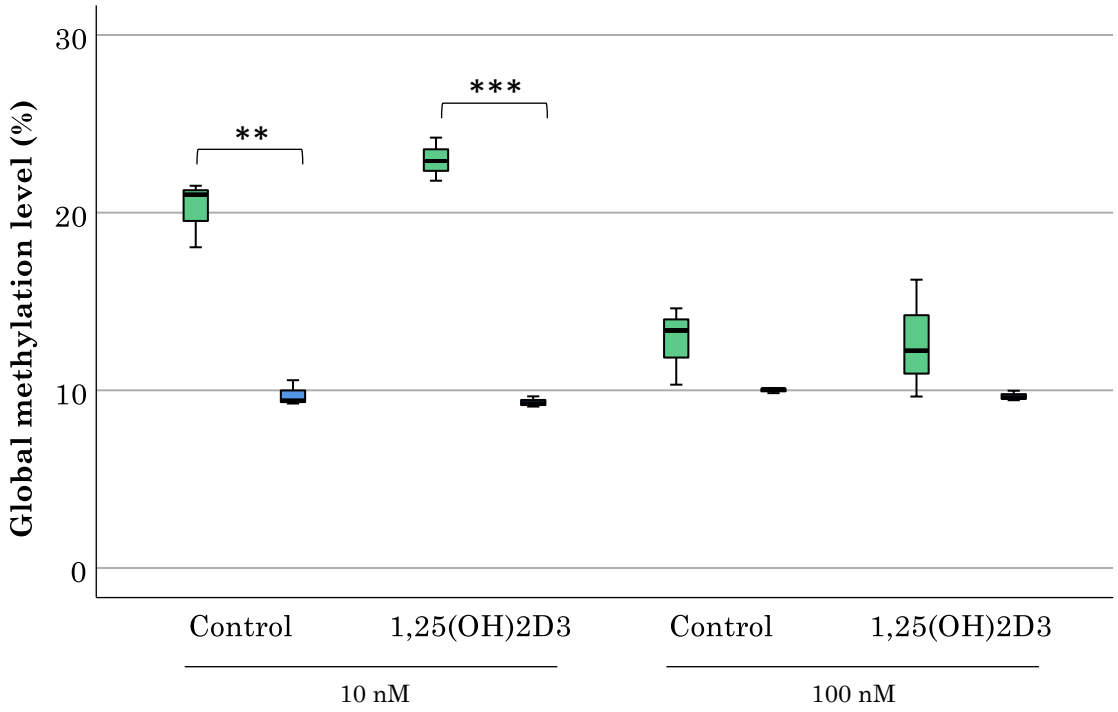

Figure 3. 6: MCF-7 cells have higher global methylation levels than HEK293 cells, irrespective of treatment. The boxplot shows global methylation level in MCF-7 (green) and HEK293 (blue) cells. Pairwise comparisons showed significantly higher global methylation in MCF-7 cells compared to HEK293 cells both in response to the 10 nM vehicle control (** $P < 0.010$) and 10 nM $1,25(OH)_2D_3$ supplementation (*** $P < 0.001$). A similar trend is present in response to 100 nM vehicle control and $1,25(OH)_2D_3$. Error bars show ± 1SD (n = 6).

3.4. $1,25(OH)_2D_3$ induce *de novo DNMT3B* expression in MCF-7 cells and maintenance *DNMT1* expression in HEK293 cells

To assess whether the changes in DNA methylation in MCF-7 cells, in response to 10 nM $1,25(OH)_2D_3$ supplementation, correspond to a change in the expression of genes encoding DNA methylating enzymes, *DNMT1*, *DNTM3A* and *DNMT3B* mRNA levels were quantified by RT-qPCR. In MCF-7 cells, 10 nM $1,25(OH)_2D_3$ significantly induced *de novo DNMT3B* expression ($P < 0.050$, Fig. 3.7B), but induced no change in either *DNMT1* or *DNMT3A* expression (Fig. 3.7A and C). In HEK293 cells, maintenance *DNMT1* was significantly induced ($P < 0.05$, Fig.

3.8A), yet no significant change occurred in *DNMT3A* or *DNMT3B* expression (Fig. 3.8B and C).

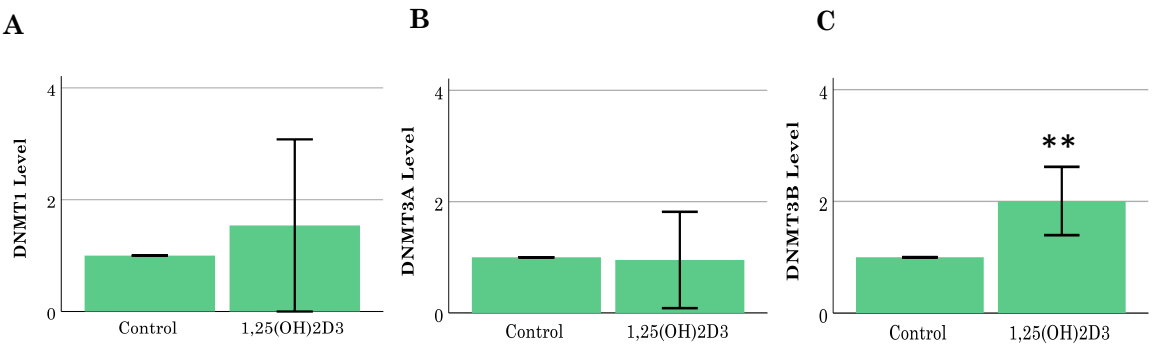

Figure 3. 7: 1,25(OH)$_2$D$_3$ induced *DNMT3B* expression, but had no effect on *DNMT1* and *DNMT3A* in MCF-7 cells. The bar graphs show *DNMT1* (A), *DNMT3A* (B) and *DNMT3B* (C) fold change in response to 10 nM 1,25(OH)$_2$D$_3$ supplementation relative to the control, as quantified by RT-qPCR, in MCF-7 cells. Pairwise comparisons showed a significant induction in *DNMT3B*, but not *DNMT3A* or *DNMT1* expression relative to the control (** P < 0.010). Error bars show ± 1SD (n = 6).

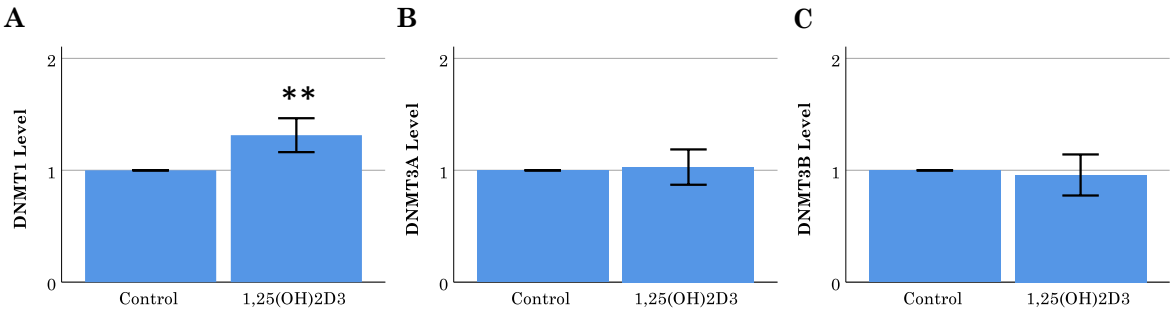

Figure 3. 8: 1,25(OH)$_2$D$_3$ supplementation induced *DNMT1* expression, but not *DNMT3A* or *DNMT3B* in HEK293 cells. The bar graphs show *DNMT1* (A), *DNMT3A* (B) and *DNMT3B* (C) fold change in response to 10 nM 1,25(OH)$_2$D$_3$ supplementation relative to the control, as quantified by RT-qPCR, in HEK293 cells. Pairwise comparisons showed a significant induction in *DNMT1*, but not *DNMT3A* or *DNMT3B* expression relative to the control (** P < 0.010). Error bars show ± 1SD (n = 6).

3.5. 1,25(OH)$_2$D$_3$ downregulates *TET3,* while inducing *TET2* expression in MCF-7 cells

To assess whether the changes in DNA methylation in MCF-7 cells, in response to 10 nM 1,25(OH)$_2$D$_3$ supplementation, correspond to a change in the expression of genes encoding DNA demethylating enzymes, *TET1*, *TET2* and *TET3* were quantified by RT-qPCR. 1,25(OH)$_2$D$_3$ exerted no significant change in *TET1* expression, but significantly increased *TET2* expression ($P < 0.01$) and decreased *TET3* expression ($P < 0.01$) in MCF-7 cells (Fig. 3.9A - C). In HEK293 cells, there was a significant increase in *TET1* expression ($P < 0.05$), but no significant change in *TET2* and *TET3* expression (Fig. 3.10A - C).

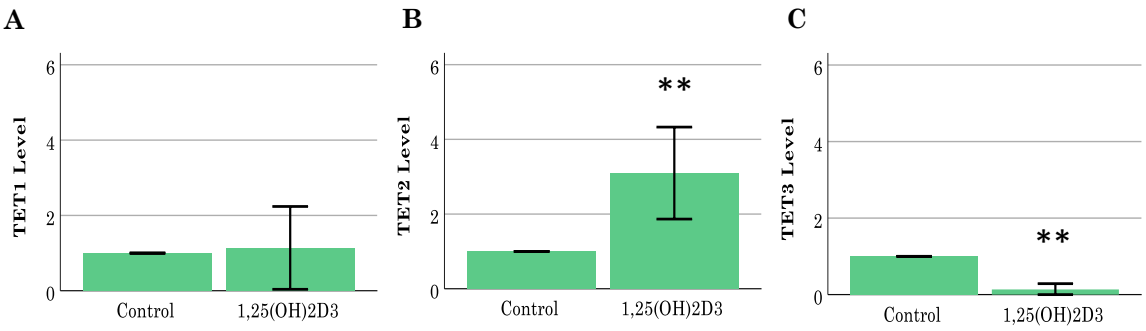

Figure 3. 9: 1,25(OH)$_2$D$_3$ significantly induced *TET2*, while downregulating *TET3* expression in MCF-7 cells. The bar graphs show *TET1* (A), *TET2* (B) and *TET3* (C) fold change in response to 10 nM 1,25(OH)$_2$D$_3$ supplementation relative to the control, as quantified by RT-qPCR, in MCF-7 cells. Pairwise comparisons showed a significant increase in *TET2* (** $P < 0.010$), and significant decrease in *TET3* (** $P < 0.010$) expression relative to the control. Error bars show ± 1SD (n = 6).

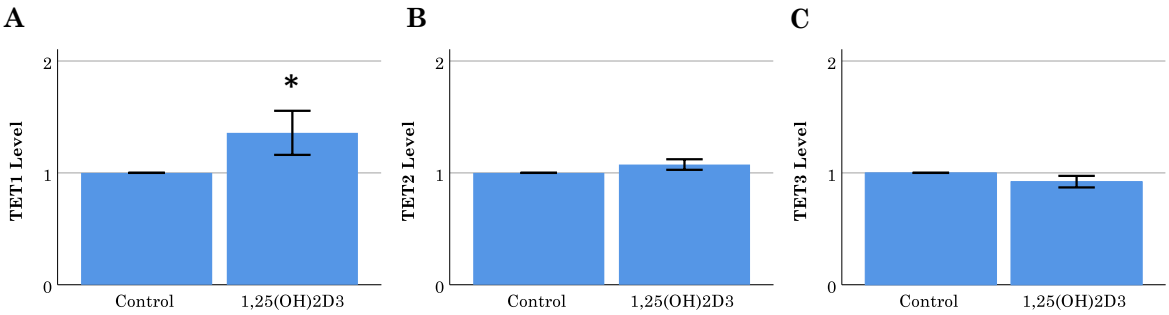

Figure 3. 10: $1,25(OH)_2D_3$ significantly induced *TET1* expression, but not *TET2* and *TET3* expression in HEK293 cells. The bar graphs show *TET1* (A), *TET2* (B), and *TET3* (C) fold change in response to 10 nM $1,25(OH)_2D_3$ supplementation relative to the control, as quantified by RT-qPCR, in HEK293 cells. Pairwise comparisons showed a significant increase in *TET1* but not *TET2* or *TET3* expression relative to the control (* $P < 0.050$). Error bars show ± 1SD (n =6).

3.6. $1,25(OH)_2D_3$ increased hydroxymethylation in MCF-7 cells, while decreasing hydroxymethylation in HEK293 cells

To confirm whether the $1,25(OH)_2D_3$-induced changes in *TET* expression in MCF-7 cells and HEK293 cells, corresponded to marked changes in demethylation events in the cells, global hydroxymethylation level was quantified in response to 10 nM $1,25(OH)_2D_3$ supplementation. Global hydroxymethylation was significantly increased in MCF-7 cells ($P < 0.050$, Fig. 3.11A), while being decreased in HEK293 cells ($P < 0.050$, Fig. 3.11B) after an 18 h *in vitro* supplementation with 10 nM $1,25(OH)_2D_3$. To assess whether a higher dose of $1,25(OH)_2D_3$ would enhance the effect, experiments were repeated in the presence of 100 nM $1,25(OH)_2D_3$. No significant change in global hydroxymethylation relative to the respective controls occurred in either cell line at the higher dose (Fig. 3.11A and Fig. 3.11B). Comparing hydroxymethylation profiles between the cells, showed no significant difference under control conditions. However, in response to 10 nM $1,25(OH)_2D_3$ supplementation, MCF-7 cells showed significantly higher levels of hydroxymethylation compared to HEK293 cells ($P < 0.010$). But, in response to 100 nM $1,25(OH)_2D_3$ supplementation, MCF-7 cells showed significantly lower levels of hydroxymethylation compared to HEK293 cells ($P < 0.050$, Fig. 3.12).

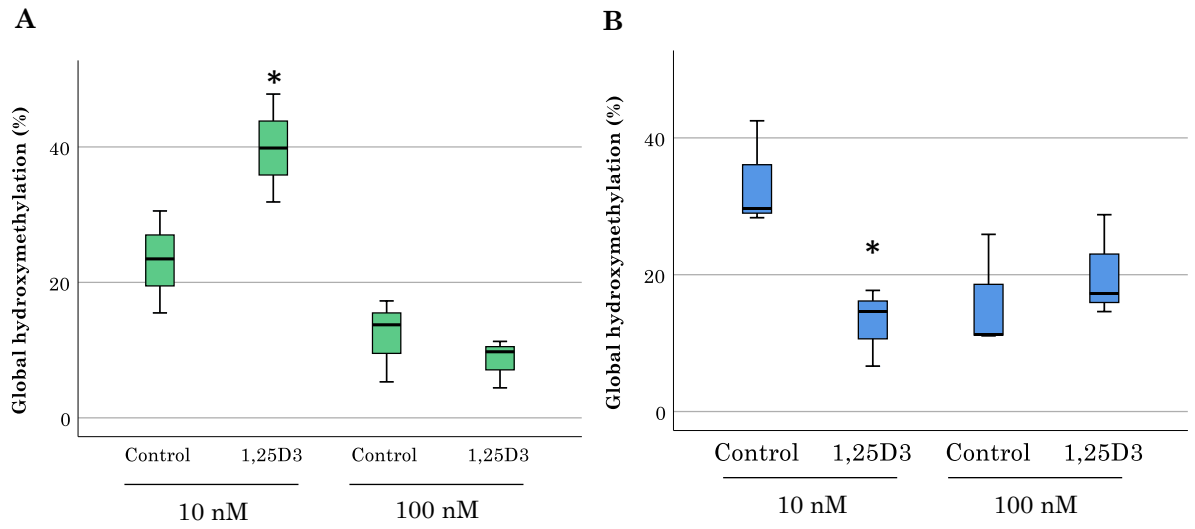

Figure 3. 11: 1,25(OH)$_2$D$_3$ significantly increased hydroxymethylation level in MCF-7 cells, while decreasing hydroxymethylation in HEK293 cells. The boxplots show global hydroxymethylation level, quantified by ELISA, in MCF-7 (A) and HEK293 (B) cells. Pairwise comparisons showed that 10 nM 1,25(OH)$_2$D$_3$ significantly increased global hydroxymethylation in MCF-7 cells (* P < 0.050), while decreasing hydroxymethylation level in HEK293 cells (* P < 0.050) relative to the control. Error bars show ± 1SD (n = 3).

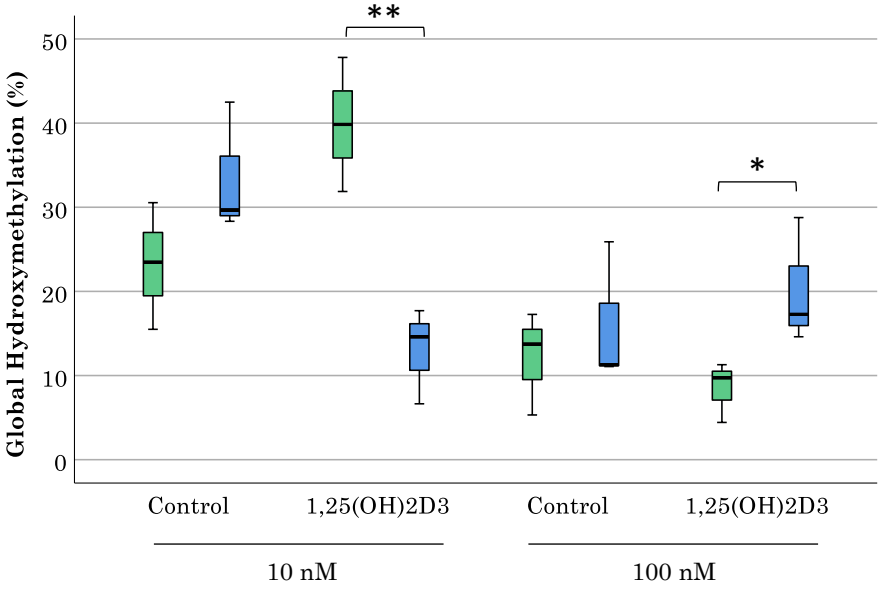

Figure 3. 12: The level of demethylation events in response to 1,25(OH)$_2$D$_3$ supplementation is cell-type specific and concentration dependent. The boxplot shows global hydroxymethylation level in MCF-7 (green) and HEK293 (blue) cells. Pairwise comparisons showed that MCF-7 cells had significantly higher levels of hydroxymethylation in response to 10 nM 1,25(OH)$_2$D$_3$ compared to HEK293 cells (** P < 0.010), while HEK293 cells had significantly higher hydroxymethylation levels in response to 100 nM 1,25(OH)$_2$D$_3$ compared to MCF-7 cells (* P < 0.050). Error bars show ± 1SD (n = 3).

3.7. 1,25(OH)$_2$D$_3$ supplementation induced *VDR* expression in MCF-7, cells while decreasing *VDR* in HEK293 cells

To assess whether 1,25(OH)$_2$D$_3$-induced changes in the DNA methylation profile, as well as *DNMT* and *TET* expression levels, corresponded to a change in *VDR* expression, *VDR* mRNA level was quantified by RT-qPCR. 10 nM 1,25(OH)$_2$D$_3$ significantly induced *VDR* expression in MCF-7 cells ($P < 0.050$, Fig. 3.13A), while downregulating *VDR* expression relative to the control in HEK293 cells ($P < 0.050$, Fig. 3.13B).

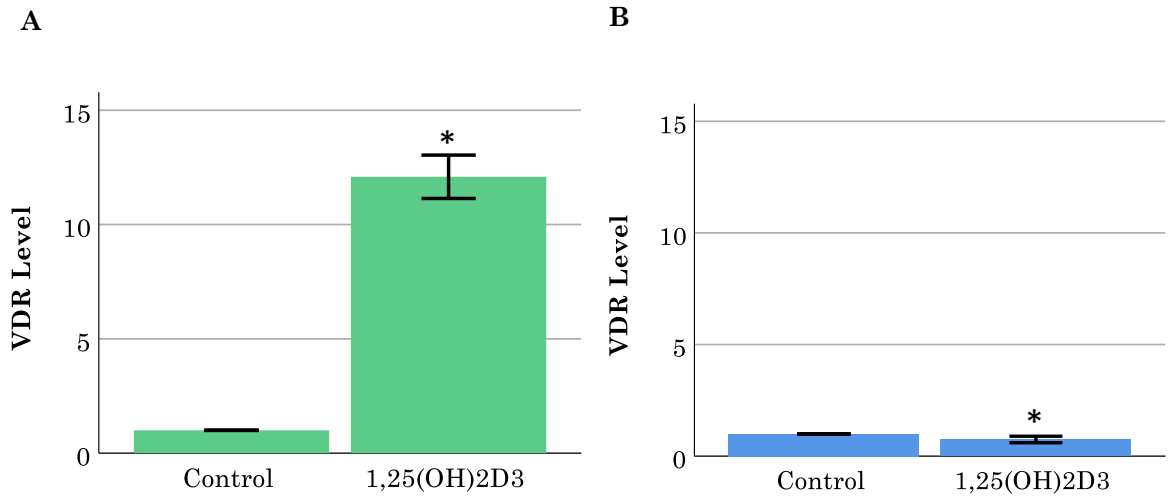

Figure 3. 13: 1,25(OH)$_2$D$_3$-mediated *VDR* autoregulation appears to be cell-type specific. The bar graphs show *VDR* fold change in response to 10 nM 1,25(OH)$_2$D$_3$ supplementation relative to the control, as quantified by RT-qPCR, in MCF-7 (A) and HEK293 (B) cells. Pairwise comparisons showed a significant induction in *VDR* expression in MCF-7 cells in response to 1,25(OH)$_2$D$_3$ relative to the control (* $P < 0.050$), while 1,25(OH)$_2$D$_3$ significantly reduced the expression of *VDR* in HEK293 cells (* $P < 0.050$). Error bars show ± 1SD (n = 3).

3.8. *DNMT3B* genes showed a positive correlation with *VDR* in response to 1,25(OH)$_2$D$_3$ in MCF-7 cells, but not in HEK293 cells

To observe whether a change in *VDR* expression relates directly to a change in the expression of *DNMTs* and *TETs*, the correlation coefficient between *VDR* and the target genes were calculated in response to 1,25(OH)$_2$D$_3$. *VDR* showed a strong positive correlation with *DNMT3B* ($r = 1$; $P < 0.001$) and *TET2* ($r = 1$; $P < 0.0001$)

in MCF-7 cells. *VDR* showed a negative correlation with *TET3* (r = -1; P < 0.0001). There was no significant correlation between *VDR* and *DNMT1*, *DNMT3A* or *TET1* (Fig. 3.14), nor did *VDR* expression correlate with the expression level of any of the genes in HEK293 cells (Fig. 3.15).

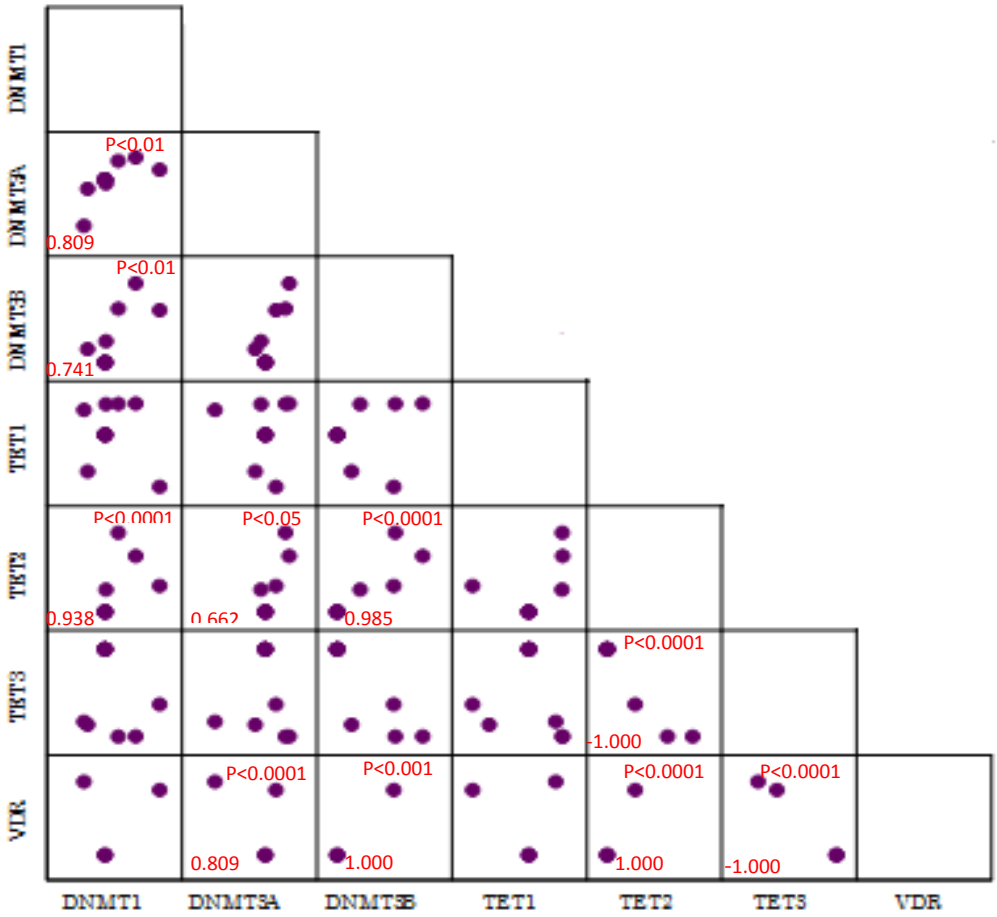

Figure 3. 14: The cross-correlation between *VDR* and the target genes in MCF-7 cells. The scatterplots show the correlation between genes in MCF-7 cells in response to 10 nM $1,25(OH)_2D_3$. The Pearson's correlation coefficients (r) for significant correlations are in the bottom left corner illustrating correlation strength (0.10-0.29 weak correlation, 0.30-0.49 moderate correlation, > 0.50 strong correlation).

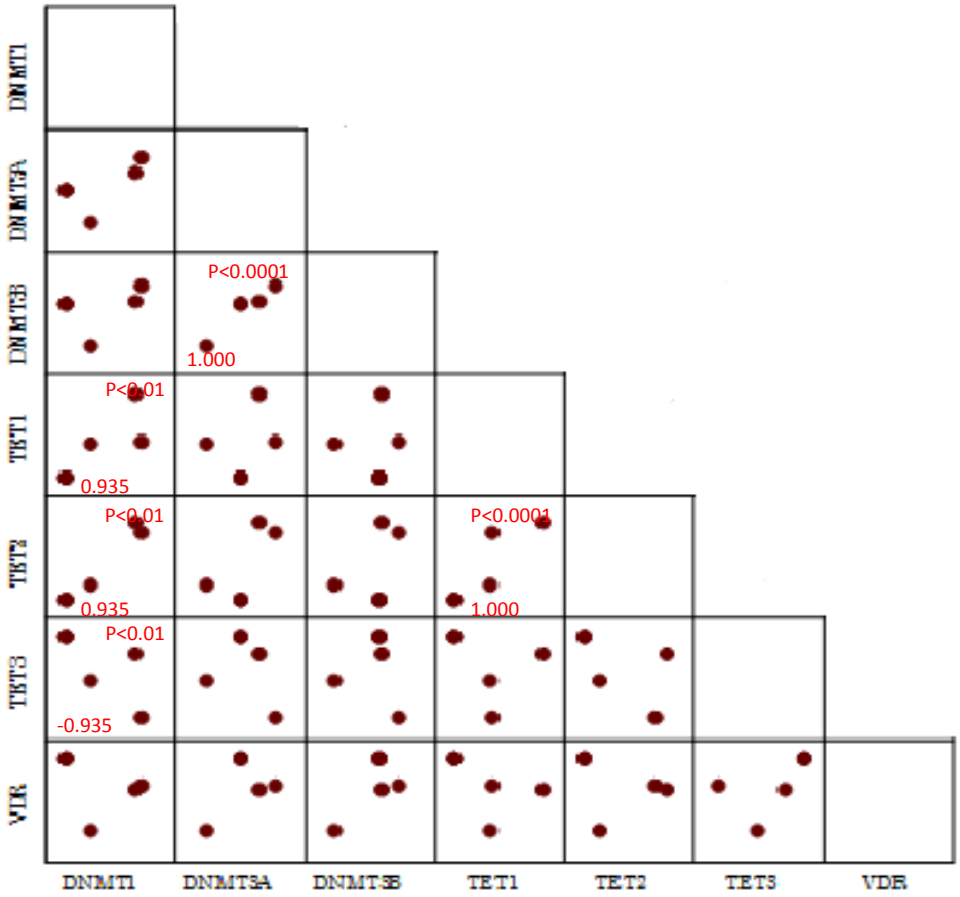

Figure 3. 15: The cross-correlation between *VDR* and the target genes in HEK293 cells. There was no significant relationship between *VDR* and *DNMT1*, *DNMT3A*, *DNMT3B*, *TET1*, *TET2* and *TET3*. Pearson's correlation coefficients (r) for significant correlations are in the bottom left corner illustrating correlation strength (0.10-0.29 weak correlation, 0.30-0.49 moderate correlation, > 0.50 strong correlation).

3.9. $1,25(OH)_2D_3$ induced no significant change in cell proliferation of MCF-7 and HEK293 cells

To determine if the changes in the epigenome influenced cell proliferation, an MTT assay was performed. $1,25(OH)_2D_3$ did not significantly change cell proliferation in MCF-7 or HEK293 cells in response to 10 nM or 100 nM $1,25(OH)_2D_3$ supplementation. There was also no difference in cell proliferation between MCF-7 and HEK293 cells with response to treatment (Fig. 3. 16).

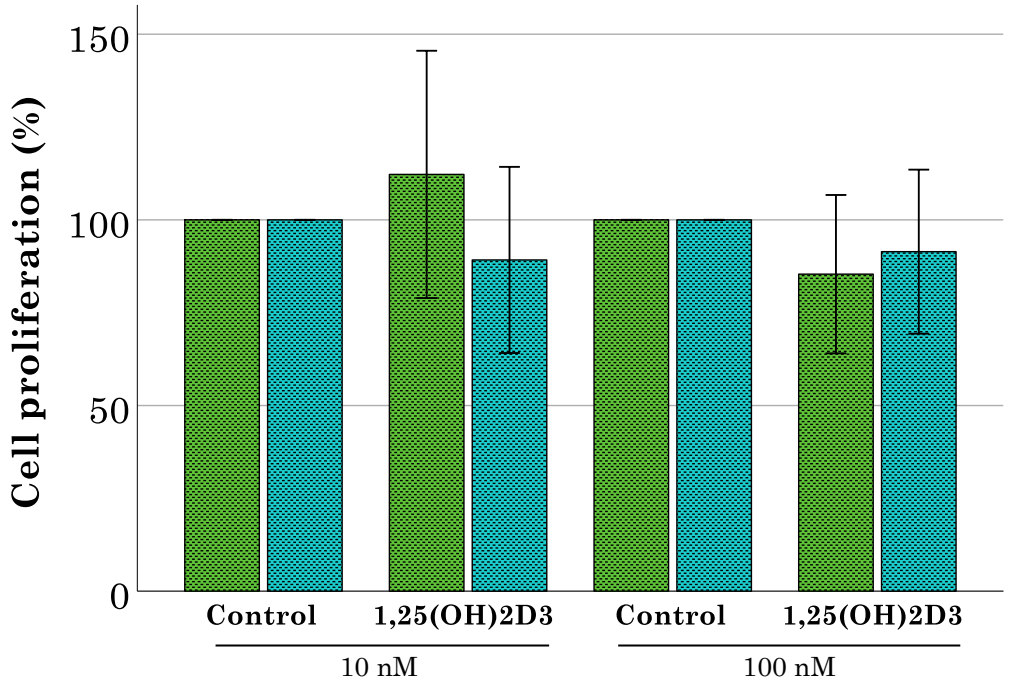

Figure 3. 16: $1,25(OH)_2D_3$ had no significant effect on cell proliferation in MCF7- or HEK293 cells. The bar graph shows cell proliferation, quantified by an MTT assay, in response to 10 nM and 100 nM $1,25(OH)_2D_3$ supplementation in MCF-7 (green) and HEK293 (blue) cells. Cell proliferation was not significantly influenced by $1,25(OH)_2D_3$ supplementation. Error bars show ± 1SD (n = 3).

3.10. All six genes contained VDR and RXR transcription factor binding sites but also had additional TF binding sites

JASPAR was used to identify putative transcription factor binding sites (TFBSs) for VDR, RXR, PU.1, $RUNX_2$, TCF_4, CEBPβ in *DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3*. All six genes had VDR and RXR TFBSs. GABPA motifs in or near the candidate genes were identified using Genomatix. GABPA core DNA binding motifs were identified in *DNMT1*, *DNMT3A*, *DNMT3B*, *TET1* and *TET2*. Interestingly no GABPA DNA binding motifs were found for *TET3*. A GABPA binding site was found in the 3' untranslated region (UTR) of *DNMT1*. There was a matrix similarity of 89% with the core GABPA DNA binding site housed in the 20 nucleotide element. Two GABPA binding sites were found in 3' UTR of *DNMT3A* and three more were found in the 5' flanking sequence. No GABPA binding motifs were identified in the 5' UTR and 3'UTR of *TET1*, however five sites

were found in the 5' flanking sequence. One GABPA binding site was identified in the 5' UTR of *TET2* (Fig. 3.17A-F). All matrix similarities were above 80%.

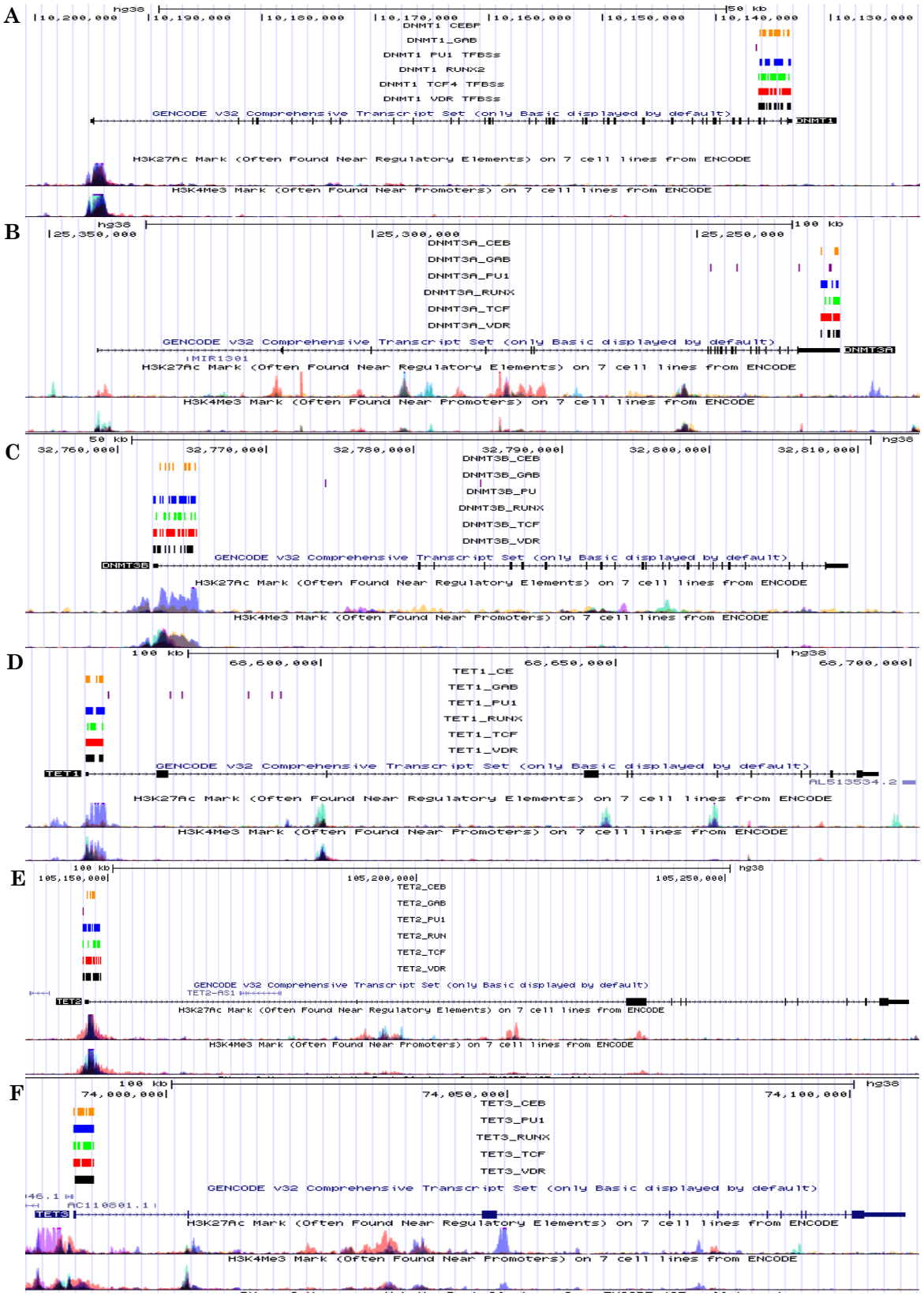

Figure 3. 17: Putative VDR binding sites located in *DNMTs* and *TETs*. The UCSC genome browser was used to annotate CEBPβ (orange), GABPA (purple), PU.1 (blue), $RUNX_2$ (green), TCF_4 (red), VDR (black) and RXR (black) binding sites in *DNMT1* (A). *DNMT3A* (B), *DNMT3B* (C), *TET1* (D), *TET2* (E) and *TET3* (F). Gene diagrams are drawn to scale.

This study aimed to assess the molecular mechanism governing vitamin D-induced changes in global DNA methylation in MCF-7 BCa cells, displaying aberrant global hypomethylation, and to compare the observed effects on a naturally hypomethylated embryonic kidney cell line, HEK293. The results showed that $1,25(OH)_2D_3$ increase global methylation levels in MCF-7 cells by upregulating the expression of *DNMT3B,* while decreasing the expression of *TET3*. This suggested the possible role of $1,25(OH)_2D_3$ in increasing genome stability in hypomethylated breast cancer cells.

4.1. Quality Control

4.1.1. Nucleic acids used in the study was intact and pure

Extracted DNA and RNA from the cells was intact. Obtaining intact DNA and RNA of a relatively good purity was essential for this project, since degraded or partially degraded RNA can have an impact on gene expression (Vermeulen et al., 2011). This proves to be particularly essential when therapeutic or prognostic conclusions are dependent on analyses such as these. Moreover, degraded DNA might have an impact on quantifying the methylation status, because it might lead to a false reading (Rhein et al., 2015). Thus, good nucleic acid integrity and purity is required for reliable results.

4.1.2. Primers specifically amplified the gene of interest after cDNA synthesis

The correct cDNA product was amplified on the agarose gel for all the genes used in RT-qPCR. A single product, represented by a single band on an agarose gel and a single melt peak above the threshold on the qPCR melt curve, was observed for every primer pair used in the study. For VDR and TET3, a smaller shoulder peak was observed, melting at a lower temperature to the main product. According to Poritz and Ririe (2014), smaller peaks below the threshold can be caused by

primer-dimers, while specific primers should only generate one peak. The smaller peaks observed for VDR and TET3 were possibly primer-dimers, which may have in qPCR, interfered with accurate quantification.

4.1.3. Reference gene stability

While *ACTB*, *GAPDH* and *B2M* were the selected reference genes for this study, only *ACTB* and *GAPDH* were stably expressed in HEK293 cells, while all three were unstable in MCF-7 cells. This suggests that these housekeeping genes are not suitable for comparative analysis of gene expression in MCF-7 cells. In agreement Tilli et al. (2016), showed that *RPL13A*, *PGK1*, *ACTB*, *DIMT1*, *GAPDH* and *B2M* are not stably expressed across breast cancer cell lines and identified a set of *novel* housekeeping genes (*CCSER2*, *SYMPK*, *ANKRD17*) and traditional housekeeping gene, *PUM1*, as persistently stable for clinical and research analyses. Moreover, a study by Révillion et al. (2000) showed that *GAPDH* expression was associated with BCa cell proliferation and *B2M* expression has been shown to be significantly different in BCa molecular subtypes, as well as being correlated to the regulation of apoptosis in BCa (Li et al., 2014). This could explain the unstable nature of the reference genes in the MCF-7 cells. Thus, choosing the correct housekeeping genes for specific cell lines is important as this has an immense effect on their expression.

4.2. 1,25(OH)$_2$D$_3$ induced no significant change in cell viability of MCF-7 or HEK293 cells

Cell viability was not significantly changed in MCF-7 and HEK293 cells in response to 10 nM or 100 nM 1,25(OH)$_2$D$_3$ supplementation. This may possibly mean that a higher dose of 1,25(OH)$_2$D$_3$ does not exert toxic effects to the cells. Although there have been studies reporting vitamin D to decrease apoptosis in peripheral blood mononuclear cells (Tabasi et al., 2015), there have been studies reporting vitamin D having no effect on cell viability as well as no toxic effect with a higher dose in microglial cells (Djukic et al., 2014). However, contradictory to these studies, Baek et al. (2011) reported that vitamin D$_3$ significantly reduced cell

viability in gastric cancer and cholangiocarcinoma cells. Additionally, vitamin D_3, synergistically worked with other anti-cancer drugs like adriamycin and paclitaxel to suppress cell viability. Also, a study by Costa et al. (2009) showed that 100 nM $1,25(OH)_2D_3$ caused a 50% reduction in viability of MCF-7 cell lines as well as another study that showed an 18% apoptosis level in MCF-7 cells after 24 h treatment with $1,25(OH)_2D_3$, 28% after 48 h and 38.5% after 72 h (Saracligil et al., 2017). Although there are studies showing pro-apoptotic effects induced by vitamin D in certain non-cancerous and cancerous cell lines, our study has shown that vitamin D has no effect on the cell viability of MCF-7 breast cancer cells, and this may be due to dose- and time-dependent factors.

4.3. $1,25(OH)_2D_3$ increased methylation in MCF-7 cells but had no effect on HEK293 cells

10 nM $1,25(OH)_2D_3$ increased global methylation in MCF-7 BCa cells but not in HEK293 cells. This increase in global methylation in the BCa cells, suggests that vitamin D may enhance chromosomal stability in breast cancer cells; enhancing genomic control over gene expression. This global methylation increase may also suggest an anti-cancerous effect of vitamin D treatment on breast cancer through methylation changes. This is in agreement with O'Brien et al. (2018) who reported that 25(OH)D concentrations are affiliated with CpG DNA methylation in various vitamin D-related genes and that CpG methylation in those genes may work alongside 25(OH)D to impact breast cancer risk. A study by Zhu et al. (2018) also showed that vitamin D supplementation increased global methylation in a dose-dependent manner in obese and overweight African Americans who were vitamin D deficient (25(OH)D < 20 ng/ml). The lack of effect in the embryonic kidney cells may relate to the developmental stage of the cells as it represents a period in development characterised by hypomethylation (Zhou et al., 2018). DNA methylation patterns are removed and thereafter re-established between different periods of development in mammals. Almost all methylation from the parents are first deleted during gametogenesis, then in early embryogenesis again, with demethylation and re-methylation happening every time (Zeng and Chen, 2019).

In early embryogenesis, demethylation occurs in the pre-implantation period; first in the zygote and then during the replication cycles of the morula and blastula (Fig. 4.1, Cedar and Bergman, 2012). The lack of change induced by vitamin D therefore, suggests that vitamin D has a cell-type specific effect on the epigenome and will not increase methylation in cells where hypomethylation is normal. Global methylation was also higher in MCF-7 cells than in HEK293 cells without $1,25(OH)_2D_3$ supplementation. Nevertheless, both cell lines were in a hypomethylation state, since hypermethylation is defined as methylation between ~70-100% (Komori et al., 2015). This result was in line with literature that has stated the genome of MCF-7 as well as MDAMB231 cell lines to be extensively hypomethylated in intergenic and intragenic regions (Wilson et al., 2007). There was higher global methylation in MCF-7 cells than in HEK293 cells in the presence of 10 nM $1,25(OH)_2D_3$, which was expected given the methylation effect of vitamin D on MCF-7 and the lack of change in methylation observed in HEK293 cells.

4.4. $1,25(OH)_2D_3$ induce *de novo DNMT3B* expression in MCF-7 cells and maintenance *DNMT1* expression in HEK293 cells

$1,25(OH)_2D_3$ increased *DNMT3B* expression, but exerted no effect on *DNMT1* and *DNMT3A* expression in MCF-7 cells. DNMT3B functions to specifically methylate gene bodies (introns and exons; Neri et al., 2017). An increase in gene body methylation is very likely to increase global levels – as observed in this study. It is thus possible that *DNMT3B* is an unidentified vitamin D target gene. Although a few studies show that DNA methyltransferases inhibitors like azacytidine and decitabine increase methylation in multiple cancer cell lines (Giri et al., 2019), our study showed that $1,25(OH)_2D_3$ treatment induced global methylation in MCF-7 breast cancer cells through *de novo* methylation by *DNMT3B* activation. In HEK293 cells, vitamin D had no effect on *DNMT3A* or *DNMT3B* expression but *DNMT1* expression was significantly increased. This was expected, based on the global methylation results, therefore showing that vitamin D primarily functions to maintain methylation patterns in HEK293 cells, instead of affecting *de novo* methylation.

4.5. 1,25(OH)$_2$D$_3$ downregulates *TET3,* while inducing *TET2* expression in MCF-7 cells

In line with the increase in global methylation we observed in the MCF-7 breast cancer cells, we saw a decrease in *TET3* expression. *TET3* is known to demethylate gene bodies and intergenic regions (Huang et al., 2014; Zhang et al., 2016). Therefore, if vitamin D decrease *TET3* expression, resulting in less demethylation events, while increasing *DNMT3B* expression, this could explain the increase in global methylation observed in the breast cancer cells. Vitamin D had the opposite effect on *TET2* expression, increasing its expression in the MCF-7 breast cancer cells. *TET2* is known to have tumour suppressor function acting on promoters or enhancer regions rather than gene bodies and intergenic regions (Huang et al., 2014). Since it is known that there are high levels of promoter methylation at tumour suppressor genes in cancer cells (Lao and Grady, 2011), it is possible that the anticancer effect of vitamin D may be related to demethylation of tumour suppressor genes as a result of vitamin D induced *TET* expression.

In HEK293 cells, there was no significant change in *TET2* and *TET3* expression but only an increase in *TET1* expression. *TET1* is known to act on promoters/transcription start site (TSS) regions (Hon et al., 2014; Huang et al., 2014). Although *TET1*, was significantly induced, its demethylation effect was not observed as it does not act on the larger part of the genome, such as the gene body. The promoter and regulatory regions in the genome of non-cancerous cells are known to be hypomethylated, thus *TET1* may have possibly had only minimal effects on the embryonic kidney cells. Overall, *TET* expression was minimal in HEK293 cells, supporting the idea that vitamin D acts specifically to correct aberrant methylation.

4.6. 1,25(OH)$_2$D$_3$ increased hydroxymethylation in MCF-7 cells, while decreasing hydroxymethylation in HEK293 cells

Global hydroxymethylation was measured to confirm whether enhanced *TET2* expression actually increased demethylation events in the genome of the MCF-7

breast cancer cells. With 10 nM $1,25(OH)_2D_3$ supplementation, MCF-7 cells showed an significant induction in global hydroxymethylation, supporting the proposed demethylation events of tumour suppressor genes by vitamin D. There was a decrease in global hydroxymethylation in HEK293 cells, also supporting the minimal effect of *TET* expression in the embryonic kidney cells, seeing as there was no significant change in *TET2* and *TET3* expression in response to $1,25(OH)_2D_3$. A higher dose of 100 nM $1,25(OH)_2D_3$ caused no change in global hydroxymethylation in the two cell lines relative to the controls. This suggests that more vitamin D does not exert an effect on the expression of the cells and that a higher dose causes no toxic effects.

Comparing the two cell lines, it was seen that HEK293 cells have higher levels of hydroxymethylation compared to MCF-7 cells in the absence of 10 nM $1,25(OH)_2D_3$. According to Munari et al. (2016), normal kidney cells have high levels of 5hmC while solid tumours exhibit reduced levels of 5hmC, possibly due to perturbations in the TET enzyme functions (Yang et al., 2013). For instance, oncometabolites like 2-hydroxyglutarate, have been shown to inhibit TET function in some cancers (Figueroa et al., 2010), resulting in decreased enzymatic activity levels (Müller et al., 2012). Additionally, Munari et al. (2016) also suggested that an imbalance in *DNMT1* expression can cause a loss in 5hmC or other types of enzymes facilitating 5hmC metabolism may be altered. Such as Thymine DNA Glycosylase (TDG) which is dysregulated in tumour cells, can cause the deamination of 5hmC contributing to the reduction of 5hmC levels. In the presence of 10 nM $1,25(OH)_2D_3$, MCF-7 cells had higher global hydroxymethylation compared to HEK293 cells, emphasising the higher levels of *TET* expression seen in MCF-7 cells compared to HEK293 cells that we observed in this study.

4.7. 1,25(OH)$_2$D$_3$ supplementation induced *VDR* expression in MCF-7, cells while decreasing *VDR* in HEK293 cells

To assess whether 1,25(OH)$_2$D$_3$-induced changes in the DNA methylation profile, *DNMT* and/or *TET* expression corresponded with a change in *VDR* expression. There was a significant increase in *VDR* expression in MCF-7 cells. A number of studies have reported 1,25(OH)$_2$D$_3$ can cause upregulation of *VDR* in vitro and in vivo. A study that administered 1,25(OH)$_2$D$_3$ in vitro and in vivo to rats showed an increased in VDR concentration (Cusano et al., 2018). Another in vitro study on human skin osteosarcoma and fibroblast cell's exposure to 1,25(OH)$_2$D$_3$ resulted in 3 to 5 fold increase in VDR number (Reinhardt and Horst, 1998). The increase in VDR expression seen in this study, may support the effect on the increase in demethylation observed in MCF-7 cells, as it has been shown that VDR is methylated and under-expressed in MCF-7 cells and vitamin D demethylates VDR, enhancing its expression (Marik et al., 2010).

1,25(OH)$_2$D$_3$ however, significantly decreased *VDR* expression relative to the control in HEK293 cells. Regulating *VDR* is thought to be an essential mechanism through which cellular responsiveness to vitamin D is regulated, because vitamin D activity correlates to VDR and vitamin D concentration (Cusano et al., 2018). However, in embryonic cells, VDR expression and it metabolising enzymes has been shown to be low (Blomberg Jensen et al., 2012). This suggests that the VDR pathway is downregulated during embryonic development. In line with our findings, Bais et al. (2011) reported a decrease in *VDR* mRNA level in HEK293 cells. This supports our point, that VDR expression may be reduced in HEK293 cells, so that it does not affect the methylation events during embryonic development.

4.8. *DNMT3B* genes showed a positive correlation with *VDR* in response to 1,25(OH)$_2$D$_3$ in MCF-7 cells, but not in HEK293 cells

In MCF-7 cells we observed a strong positive correlation between *VDR* and *DNMT3B*, as well as between *VDR* and *TET2*. This then shows a synergistic increase in both *VDR* and *DNMT3B* as well as *TET2* with response to vitamin D treatment. *VDR* and *TET3* exerted a negative relationship, meaning that an increase in *VDR* expression may lead to a decrease in *TET3* expression, causing less demethylation. Lastly, there was no relationship between *VDR* and *DNMT1*, *DNMT3A* and *TET1*. This then suggests, that a change in *VDR* expression, does not affect the expression of these genes in MCF-7 breast cancer cells in response to vitamin D treatment. The correlation between *VDR* and the target genes in response to 1,25(OH)$_2$D$_3$ were evaluated to observe whether a change in *VDR* expression affects the expression of the different targets. In HEK293 cells, there was surprisingly no correlation between VDR and all target genes. This suggests that the change in *VDR* expression does not influence a change in *DNMT* or *TET* expression in HEK293 cells.

4.9. 1,25(OH)$_2$D$_3$ induced no significant change in cell proliferation of MCF-7 and HEK293 cells

Cell proliferation was not significantly changed in MCF-7 and HEK293 cells in response to 10 nM or 100 nM 1,25(OH)$_2$D$_3$ supplementation. This suggests that vitamin D does not exert an effect on the proliferation of either cancerous or non-cancerous cells. This is in disagreement with studies that have shown vitamin D to be influential in cell proliferation and differentiation (Srikuea et al., 2012; Bikle, 2010). Most studies have described 1,25(OH)$_2$D$_3$ as having an inhibitory effect on proliferation in numerous cell types, usually associated with stimulating cell differentiation. Such as a study done on breast cancer cell lines including MCF-7 cells, showing that 1,25(OH)$_2$D$_3$ had pro-apoptotic and anti-proliferative effects at supra-physiological concentrations of 10-100 nM, usually used to study hormone effects in cell culture research (van den Bemd et al., 2000; Feldman et al., 2014).

Another study reported a negative correlation between vitamin D and cell proliferation, showing a decrease in cell proliferation as well as angiogenesis with vitamin D treatment (Nakagawa et al., 2005; Mantell et al., 2000). A paper published by Lu et al. (2017) however, showed that 10 nM $1,25(OH)_2D_3$ exerted no effect on corneal epithelial cell proliferation which is in agreement with our findings. Morales et al. (2004) also showed that 10 nM $1,25(OH)_2D_3$ had no effect on the cell count of osteoblast-like cells. The contradictory results on the effect of vitamin D and cell proliferation may be dose- and time-dependent.

4.10. All six genes contained VDR and RXR transcription factor binding sites but also had additional TF binding sites

Putative VDR and RXR TF binding sites were identified in all the *DNMT* and *TET* coding genes. Notably, the DNMTs and TETs also contained TF binding sites for other VDR binding partners including GABPA, PU.1, CEBPβ, TCF4, and RUNX2. This suggests that vitamin D and VDR may not only bind to RXR to mediate vitamin D-related functions on the epigenome, but may also recruit other transcription factor such as GABPA, PU.1, CEBPβ, TCF4, and RUNX2 to regulate gene expression.

GABPA DNA binding motifs were identified in *DNMT1*, *DNMT3A*, *DNMT3B*, *TET1* and *TET2*, but interestingly no GABPA DNA binding motifs were found for *TET3*. VDR can form different complexes and modify its DNA location through interaction with other TFs. In line with our findings, VDR is known to interact with GABPA in THP-1 cells, and motifs have been identified within VDR/RXR binding peaks (Neme et al., 2017). Through the ChIP-seq data of GABPA cistrome, the co-localisation of GABPA and VDR was confirmed, and furthermore VDR interaction with PU.1 was confirmed as well for modulating genes in immune and cellular signalling mechanism (Seuter et al., 2018). Through the analysis of ChIP-seq data for both VDR and PU.1, VDR-PU.1 interaction has been identified in open chromatin in $1,25(OH)_2D_3$-sensitive PU.1 loci, found closer to $1,25(OH)_2D_3$ target genes (Seuter et al., 2017).

Coregulatory factors like CEBPβ have been shown to be recruited to mediate *VDR* expression after ligand-dependent epigenetic function have occurred, like histone (H4) acetylation (Zella et al., 2010). This suggests that VDR-specific enhancers are able to combine signals from several environmental factors (i.e. vitamin D and retinoid acid) by interrelating with their associated TFs (Zella et al., 2006) in many different vitamin D-related genes, possibly including the target genes investigated in this study.

TCF_4 is known to play a role in chromatin remodelling and transcription by recruiting histone acetyltransferases like p300 (Bayly et al., 2004; Massari et al., 1999; Zhang et al., 2004). Kennedy et al. (2016) showed that TCF_4 caused a decrease in CpG methylation at genes with activated transcription, which in the case of this study were memory-related genes such as *Fos* and *IEG* (for forming memory at the hippocampus; Countryman et al., 2005). TCF_4 was observed to modulate methylation of these through negatively regulating *TET2* expression that is over-expressed in TCF_4 (+/−) mice. This was thought to be associated with gene body methylation (Huang et al., 2014) or for demethylating memory-related genes as a compensatory mechanism. Through the findings of this study, it supports our suggestion that TCF_4 plays a role in regulating methylation in not only *TET2* but the rest of the methylation and demethylation genes in response to $1,25(OH)_2D_3$.

$RUNX_2$ TF binding sites were also identified in the target genes, suggesting that it regulates the transcription of *DNMTs* and *TETs*. $RUNX_2$ is known to regulate vascular calcification (VC) of VC-related genes (Ducy et al., 1997). Its expression is upregulated in calcified vessels (Sierra and Towler, 2010), and it was found that treating vascular smooth muscle cells with vitamin D_3 increased *VDR* and *RUNX₂* expression. Though immunoprecipitation experiments, it was suggested that there is a correlation between VDR and $RUNX_2$. This functional relationship between VDR and $RUNX_2$ is essential for vascular calcification with response to vitamin D_3.

Based on the results observed this study supported by literature, it shows that DNMTs and TETs could be regulated through several other transcription factors and not only through VDR:RXR. Therefore, showing that vitamin D has the ability to take different transcriptional routes in order to mediate its epigenetic functions.

5.1. Rationale of the study and summary of the findings

This study investigated the molecular mechanism governing vitamin D-induced changes in global DNA methylation in MCF-7 BCa cells, and to observe its effects on a non-cancerous cell line HEK293 for comparison. $1,25(OH)_2D_3$ increased global methylation in MCF-7 cells by upregulating the expression of *DMT3B* while decreasing the expression of *TET3*, suggesting that vitamin D may enhance chromosol stability in the breast cancer cells. Vitamin D also increased *TET2* expression, which is known to demethylate promoter and enhancers, thus suggesting the possibility that the anticancer effect of vitamin D may be related to demethylation of tumour suppressor genes through induction of *TET*. This was confirmed by the increase of global hydroxymethylation seen in the breast cancer cells. No change in global methylation, *DNMT3* or *TET* expression was observed in HEK293 cells with $1,25(OH)_2D_3$ treatment, suggesting that vitamin D has a cell-type specific effect on the epigenome and will not increase methylation in cells where hypomethylation is normal; as seen in embryonic cells. Thus, vitamin D may act specifically to correct aberrant methylation and maintaining a sufficient vitamin D status could be key in promoting good health and well-being.

5.2. Implications of the study

This study aimed in expanding the knowledge on the molecular mechanism for vitamin D-mediated changes in global DNA methylation. We concluded that vitamin D may improve genomic stability and correct aberrant methylation through mediating *DNMT* and *TET* expression in breast cancer cells. This implies a necessity for a lifestyle that includes maintaining a sufficient vitamin D status in breast cancer patients as well as healthy individuals, to promote well-being and maintain a healthy epigenome.

5.3. Challenges and limitations

A limitation in this study was that we did not analyse protein level or enzymatic activity of the target genes. Nonetheless, the changes in methylation status, suggest increased enzyme activity. We did not measure changes in chromosomal stability and it remains to be seen whether the induced changes will be heritable from one cell to the next. Moreover, while we identified putative transcription factor binding sites for VDR in the *DNMTs* and *TETs*, suggesting that the effects we observed were mediated by VDR, we did not measure VDR binding to these target genes.

5.4. Future direction

Future work could include, measuring enzymatic activity of DNMTs and TETs in response to $1,25(OH)_2D_3$ treatment, as well as luciferase assays for VDR-mediated transactivation of target gene expression. Moreover, the effects can be validated by repeating some of the experiments in cells that do not express VDR (i.e. with siRNA mediated VDR knockdown), to confirm that what we are seeing is a result of VDR function. Seeing as vitamin D plays a role in several cancer types, in future studies, the effect of vitamin D on global methylation can also be tested in other cancer types such as colorectal, gastrointestinal and pancreatic cancer.

References

Abreu Velez AM, Howard MS. Tumor-suppressor Genes, Cell Cycle Regulatory Checkpoints, and the Skin. *N Am J Med Sci*. 2015:7(5):176-88.

Alexander RR, Griffiths JM. (1993). Basic Biochemical Methods, 2nd Ed. J.M. Wiley and Sons: New York.

Al-Azhri J, Zhang Y, Bshara W, Zirpoli GR, McCann SE, Khoury T, Morrison CD, Edge SB, Ambrosone CB, Yao S. Tumor expression of vitamin D receptor and breast cancer histopathological characteristics and prognosis. *Clin Cancer Res*. 2017:23(1):97–103.

Aslibekyan S, Dashti HS, Tanaka T, Sha J, Ferrucci L, Zhi D, Bandinelli S, Borecki IB, Absher DM, Arnett DK, Ordovas JM. PRKCZ methylation is associated with sunlight exposure in a North American but not a Mediterranean population. *Chronobiol Int*. 2014:31(9):1034-40.

Atoum M, Alzoughool F. Vitamin D and Breast Cancer: Latest Evidence and Future Steps. *Breast Cancer (Auckl)*. 2017.

Baek S, Lee YS, Shim HE, Yoon S, Baek SY, Kim BS, Oh SO. Vitamin D3 regulates cell viability in gastric cancer and cholangiocarcinoma. *Anat Cell Biol*. 2011:44(3):204-9.

Bahuguna A, Khan I, Bajpa, VK, Kang SC. MTT assay to evaluate the cytotoxic potential of a drug. *Bangladesh J Pharmacol*. 2017:12:115-118.

Bai S, Wang H, Shen J, Zhou R, Bushinsky DA, Favus MJ. Elevated vitamin D receptor levels in genetic hypercalciuric stone-forming rats are associated with downregulation of Snail. *J Bone Miner Res*. 2010:25(4):830-840.

Bayly R, Chuen L, Currie RA, Hyndman BD, Casselman R, Blobel GA, LeBrun DP. E2A-PBX1 interacts directly with the KIX domain of CBP/p300 in the induction of proliferation in primary hematopoietic cells. J Biol Chem. 2004:279:55362-55371.

Berger J1, Hauber J, Hauber R, Geiger R, Cullen BR. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. Gene. 1988:66(1):1-10.

Berger U, McClelland RA, Wilson P, Greene GL, Haussler MR, Pike JW, Colston K, Easton D, Coombes RC. Immunocytochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis. Cancer Res. 1991:51(1):239–44.

Bikle D. (2010). Extrarenal synthesis of 1,25-dihydroxyvitamin D and its health implications. In: Holick MF, editor. Nutrition and Health: Vitamin D. Humana Press; New York. 277–295.

Bikle DD. Vitamin D: newly discovered actions require reconsideration of physiologic requirements. *Trends Endocrinol Metab*. 2010:21:375–384.

Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol*. 2014:21(3):319–329.

Bikle DD, Murphy EW, Rasmussen H. The ionic control of 1,25-dihydroxyvitamin D3 synthesis in isolated chick renal mitochondria. The role of calcium as influenced by inorganic phosphate and hydrogen-ion. J. *Clin. Invest*. 1975:55: 299–304.

Bikle DD, Rasmussen H. The ionic control of 1,25-dihydroxyvitamin D3 production in isolated chick renal tubules. J. *Clin. Invest*. 1975:55: 292–298.

Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002:16: 6–21.

Boyle BJ, Zhao XY, Cohen P, Feldman D. Insulin-like growth factor binding protein-3 mediates 1-α-25-dihydroxyvitamin D3 growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. *J. Urol*. 2001:165:1319–1324.

Blomberg Jensen M, Jørgensen A, Nielsen JE, Steinmeyer A, Leffers H, Juul A, Rajpert-De Meyts E. Vitamin D metabolism and effects on pluripotency genes and cell differentiation in testicular germ cell tumors in vitro and in vivo. *Neoplasia*. 2012:14(10):952-63.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin, in press. The online GLOBOCAN 2018 database is accessible at as part of IARC's Global Cancer Observatory.

Brekelmans C, Seynaeve C, Bartels C, Tilanus-Linthorst M, Meijers-Heijboer E, Crepin C, van Geel A, Menke M, Verhoog L, van den Ouweland A, Obdeijn I, Klijn J. Effectiveness of Breast Cancer Surveillance in BRCA1/2 Gene Mutation Carriers and Women With High Familial Risk. *JCO*. 2001:19:924–930.

Calderon-Garciduenas AL, Ruiz-Flores P, Cerda-Flores RM, Barrera-Saldana HA. Clinical follow up of Mexican women with early onset of breast cancer and mutations in the BRCA1 and BRCA2 genes. *Salud publica de Mexico*. 2005:47(2):110–115.

Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF, Hunziker W. Two nuclear signalling pathways for vitamin D. *Nature*. 1993:361:657–660.

Carlberg C, Campbell MJ. Vitamin D receptor signaling mechanisms: Integrated actions of a well-defined transcription factor. *Steroids*. 2013:78:127–136.

Casiano CA, Mediavilla-Varela M, Tan EM. Tumor-associated antigen arrays for the serological diagnosis of cancer. *Mol. Cell*. Proteomics. 2006:5:1745–1759.

Castellano-Castillo D, Morcillo S, Clemente-Postigo M, Crujeiras AB, Fernandez-García JC, Torres E, Tinahones FJ, Macias-Gonzalez M. Adipose tissue inflammation and *VDR* expression and methylation in colorectal cancer. *Clin Epigenet*. 2018:10**:**60.

Cavalieri E, Chakravarti D, Guttenplan J, Jankowiak R, Muti P, Rogan E, Russo J, Santen RJ, Sutter T. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. *Biochim Biophys Acta*. 2006:1766:63–78.

Cedar H, Bergman Y. Programming of DNA methylation patterns. *Annual Review of Biochemistry*. 2012:81:97–117.

Chang S, Gao L, Yang Y, Tong D, Guo B, Liu L, Li Z, Song T, Huang C. miR-145 mediates the antiproliferative and gene regulatory effects of vitamin D3 by directly targeting E2F3 in gastric cancer cells. *Oncotarget*. 2015:6(10):7675-7685.

Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW. De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase. *J. Biol. Chem*. 2003:278:38084–38093.

Chen X, Bonnefoi H, Diebold-Berger S, Lyautey J, Lederrey C, Faltin-Traub E, Stroun M, Anker P. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. *Clin. Cancer Res*. 1999:5:2297–2303.

Cheung HH, Lee TL, Rennert OM, Chan WY. DNA Methylation of Cancer Genome. *Birth Defects Res C Embryo Today*. 2009:87(4):335–350.

Chiang KC, Chen TC. The anti-cancer actions of vitamin D. *Anticancer Agents Med Chem*. 2013:13(1):126–139.

Chiang KC, Chen TC. Vitamin D for the prevention and treatment of pancreatic cancer. *World J Gastroenterol*. 2009:15(27):3349–3354.

Cho YS, Park YG, Lee YN, Kim MK, Bates S, Tan L, Cho-Chung YS. Extracellular protein kinase A as a cancer biomarker: its expression by tumor cells and reversal by a myristate-

lacking Calpha and RIIbeta subunit overexpression. *Proc. Natl. Acad. Sci, U. S. A.* 2000:97:835.

Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiological Reviews*. 2016:96(1):365–408.

Church TR, Wandell M, Lofton-Day C, Mongin S.J, Burger M, Payne SR, Castaños-Vélez E, Blumenstein B.A, Rösch T, Osborn N, Snover D, Day RW, Ransohoff DF, and Ransohoff DF. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut*. 2014:63:317–325.

Countryman RA, Kaban NL, Colombo PJ. Hippocampus c-fos is necessary for long-term memory of a socially transmitted food preference. *Neurobiol Learn Mem*. 2005:84:175-183.

Costa JL, Eijk PP, van de Wiel MA, ten Berge D, Schmitt F, Narvaez CJ, Welsh J, Ylstra B. Anti-proliferative action of vitamin D in MCF7 is still active after siRNA-VDR knock-down. *BMC Genomics*. 2009:10:499.

Cvijic ME, Kita T, Shih W., DiPaola, R. S., and Chin, K. V. (2000) 'Extracellular catalytic subunit activity of the cAMP-dependent protein kinase in prostate cancer', Clin. Cancer Res, 6, pp. 2309.

Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *Lancet*. 2005:366(9499):1784-1793.

Ditsch N, Toth B, Mayr D, Lenhard M, Gallwas J, Weissenbacher T, Dannecker C, Friese K, Jeschke U. The association between vitamin D receptor expression and prolonged overall survival in breast cancer. *J Histochem Cytochem*. 2012;60(2):121–129.

Djukic M, Onken ML, Schütze S, Redlich S, Götz A, Hanisch UK, Bertsch T, Ribes S, Hanenberg A, Schneider S, Bollheimer C, Sieber C, Nau R. Vitamin d deficiency reduces the immune response, phagocytosis rate, and intracellular killing rate of microglial cells. *Infect Immun*. 2014:82(6):2585-94.

Dor Y and Cedar H. Principles of DNA methylation and their implications for biology and medicine. *The Lancet*. 2018:392(10149): 777-786.

Dou R, Ng K, Giovannucci EL., Manson JE, Qian ZR, Ogino S. Vitamin D and colorectal cancer: molecular, epidemiological and clinical evidence. *Br. J. Nutr*. 2016:115(9):1643-1660.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*. 1997:89: 747-754.

Duffy MJ. Serum tumor markers in breast cancer: are they of clinical value? *Clin. Chem*. 2006:52:345 - 351.

Eaton L. Early periods and late childbearing increase risk of breast cancer, study confirms. *BMJ*. 2002:324(7334):386.

Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA, Gehrke C. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res*. 1982:10(8):2709-2721.

Eisman JA, Suva LJ, Martin TJ. Significance of 1,25-dihydroxyvitamin D3 receptor in primary breast cancers. *Cancer Res*. 1986:46(10):5406–5408.

Elebro K, Bendahl PO, Jernström H, Borgquist S. Androgen receptor expression and breast cancer mortality in a population-based prospective cohort. *Breast Cancer Research and Treatment*. 2017:22:1-13.

Eskelinen M, Hippelainen M, Kettunen J, Salmela E, Penttila I, Alhava E. Clinical value of serum tumour markers TPA, TPS, TAG 12, CA 15-3 and MCA in breast cancer diagnosis; results from a prospective study. *Anticancer Res*. 1994:14:699–703.

Esteller M. Epigenetics in cancer. *N. Engl. J. Med*. 2008:358:1148–1159.

Fathi N, Ahmadian E, Shahi S, Roshangar L, Khan H, Kouhsoltani M, Dizaj SM, Sharifi S. Role of vitamin D and vitamin D receptor (VDR) in oral cancer. *Biomedicine & Pharmacotherapy*. 2019:109:391-401.

Feldman D, Pike JW, Adams JS. (2011). Vitamin D: Two-Volume Set. Academic Press; Cambridge. 230.

Fleet JC, DeSmet M, Johnson R, Li Y. Vitamin D and cancer: a review of molecular mechanisms. *Biochem J*. 2012:441(1):61-76.

Freake HC, Abeyasekera G, Iwasaki J, Marcocci C, MacIntyre I, McClelland RA, Skilton RA, Easton DF, Coombes RC. Measurement of 1,25-dihydroxyvitamin D3 receptors in breast cancer and their relationship to biochemical and clinical indices. *Cancer Res*. 1984:44(4):1677–81.

Freedman DM, Looker AC, Chang SC, Graubard BI. Prospective study of serum vitamin D and cancer mortality in the United States. *J Natl Cancer Inst*. 2007:99(21):1594–602.

Friedrich M, Villena-Heinsen C, Tilgen W, Schmidt W, Reichrat J, Axt-Fliedner R. Vitamin D receptor (VDR) expression is not a prognostic factor in breast cancer. *Anticancer Res*. 2002:22(3):1919–24.

Giammanco M, Di Majo D, La Guardia M, Aiello S, Crescimannno M, Flandina C, Tumminello FM, Leto G. Vitamin D in cancer chemoprevention. *Pharm. Biol*. 2015:53:1399–1434.

Giovanella L, Ceriani L, Giardina G, Bardelli D, Tanzi F, Garancini S. Serum cytokeratin fragment 21.1 (CYFRA 21.1) as tumour marker for breast cancer: comparison with carbohydrate antigen 15.3 (CA 15.3) and carcinoembryonic antigen (CEA). *Clin. Chem. Lab. Med*. 2002:40:298–303.

Giri AK, Aittokallio T. DNMT Inhibitors Increase Methylation in the Cancer Genome. *Front. Pharmacol*. 2019:10:385.

Gold P, Freedman SO. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J. Exp. Med*. 1965:121:439–462.

Goll MG, Kirpekar F, Maggert KA, Yoder JA, Hsieh CL, Zhang X, Golic KG, Jacobsen SE, Bestor TH. Methylation of tRNAAsp by the DNA methyltransferase homolog DNMT2. *Science*. 2006:311:395–398.

Gönenç A, Erten D, Aslan S, Akinci M, Simşek B, Torun M. Lipid peroxidation and antioxidant status in blood and tissue of malignant breast tumor and benign breast disease. *Cell Biol. Int*. 2006:30:376–380.

Guerrero-Preston R, Hadar T, Ostrow KL, Soudry E, Echenique M, Ili-Gangas C, Pérez G, Perez J, Brebi-Mieville P, Deschamps J, Morales L, Bayona M, Sidransky D, Matta J. Differential promoter methylation of kinesin family member 1a in plasma is associated with breast cancer and DNA repair capacity. *Oncology Reports*. 2014:32:505.

Gupta RP, Hollis BW, Patel SB, Patrick KS, Bell NH. CYP3A4 is a human microsomal vitamin D 25-hydroxylase. *J. Bone Miner. Res*. 2004:19:680–688.

Hartmann LC, Sellers TA, Frost MH, Lingle WL, Degnim AC, Ghosh K, Vierkant RA, Maloney SD, Pankratz VS, Hillman DW, Suman VJ, Johnson J, Blake C, Tlsty T, Vachon CM, Melton LJ, Visscher DW. Benign breast disease and the risk of breast cancer. *N Engl J Med*. 2005:353:229-237.

Haussler MR, Haussler CA, Whitfield GK, Hsieh JC, Thompson PD, Barthel TK, Bartik L, Egan JB, Wu Y, Kubicek JL, Lowmiller CL, Moffet EW, Forster RE, Jurutka PW. The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate healthful aging. *J Steroid Biochem Mol Biol*. 2010:121(1-2):88-97.

Haussler MR, Whitfield GK, Kaneko I, Haussler CA, Hsieh D, Hsieh JC, Hsieh JC, Jurutka PW. Molecular mechanisms of vitamin D action. *Calcif. Tissue Int*. 2013:92:77–98.

Hemida MA, AbdElmoneim NA, Hewala TI, Rashad MM, Abdaallah S. Vitamin D Receptor in Breast Cancer Tissues and Its Relation to Estrogen Receptor Alpha (ER-α) Gene Expression and Serum 25-hydroxyvitamin D Levels in Egyptian Breast Cancer Patients: A Case-control Study. *Clin Breast Cancer*. 2019:19(3):e407-e414.

Hervouet E, Peixoto P, Delage-Mourroux R, Boyer-Guittaut M, Cartron PF. Specific or not specific recruitment of DNMTs for DNA methylation, an epigenetic dilemma. *Clin Epigenetics*. 2018:10:17.

Heyn H, Carmona FJ, Gomez A, Ferreira HJ, Bell JT, Sayols S, Ward K, Stefansson OA, Moran S, Sandoval J, Eyfjord JE, Spector TD, Esteller M. DNA methylation profiling in breast cancer discordant identical twins identifies DOK7 as novel epigenetic biomarker. *Carcinogenesis*. 2013:34:102–108.

Hiramatsu K, Takahashi K, Yamaguchi T, Matsumoto H, Miyamoto H, Tanaka S, Tanaka C, Tamamori Y, Imajo M, Kawaguchi M, Toi M, Mori T, Kawakita M. N(1),N(12)-Diacetylspermine as a sensitive and specific novel marker for early- and late-stage colorectal and breast cancers. *Clin. Cancer Res*. 2005:11:2986–2990.

Hong AR, Kim YA, Bae JH, Min HS, Kim JH, Shin CS, Kim SY, Kim SW. A Possible Link Between Parathyroid Hormone Secretion and Local Regulation of GABPA in Human Parathyroid Adenomas. *J Clin Endocrinol Metab*. 2016:101(6):2594-601.

Hosseinpour F, Wikvall K. Porcine microsomal vitamin D(3) 25-hydroxylase (CYP2D25). Catalytic properties, tissue distribution, and comparison with human CYP2D6. *J. Biol. Chem*. 2000:275:34650–34655.

Houghton LA, Vieth R. The case against ergocalciferol (vitamin D2) as a vitamin supplement. *Am. J. Clin. Nutr*. 2006:84:694–697.

Huang Y, Chavez L, Chang X, Wang X, Pastor WA, Kang J, Zepeda-Martinez JA, Pape UJ, Jacobsen SE, Peters B, Rao A. Distinct roles of the methylcytosine oxidases Tet1 and Tet2 in mouse embryonic stem cells. Proc Natl Acad Sci USA. 2014:111:1361-1366.

Huss L, Butt S, Borgquist S, Almquist M, Malm J, Manjer J. Serum levels of vitamin D, parathyroid hormone and calcium in relation to survival following breast cancer. *Cancer Causes Control*. 2014:25(9):1131–1140.

Huss L, Butt ST, Borgquist S, Elebro K, Sandsveden M, Rosendahl A, Manjer J. Vitamin D receptor expression in invasive breast tumors and breast cancer survival. *Breast Cancer Res*. 2019:21:84.

Hyun K, Jeon J, Park K, Kim J. Writing, erasing and reading histone lysine methylations. *Exp Mol Med*. 2017:49(4):e324.

Ismail A, El-Awady R, Mohamed G, Hussein M, Ramadan SS. Prognostic Significance of Serum Vitamin D Levels in Egyptian Females with Breast Cancer. *Asian Pac J Cancer Prev*. 2018:19(2):571-576.

Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science. 2011:333(6047):1300-1303.

Jacobs ET, Kohler LN, Kunihiro AG, Jurutka PW. Vitamin D and colorectal, breast, and prostate cancers: a review of the epidemiological evidence. *J Cancer*. 2016:7(3):232–40.

Jardé T, Perrier S, Vasson MP, Caldefie-Chézet F. Molecular mechanisms of leptin and adiponectin in breast cancer. *Eur J Cancer*. 2011:47(1):33-43.

Jeon SM, Shin EA () Exploring vitamin D metabolism and function in cancer. *Exp Mol Med*. 2018:50:20.

Jeschke U, Mylonas I, Shabani N, Kunert-Keil C, Schindlbeck C, Gerber B, Friese K. Expression of sialyl lewis X, sialyl Lewis A, E-cadherin and cathepsin-D in human breast cancer: immunohistochemical analysis in mammary carcinoma in situ, invasive carcinomas and their lymph node metastasis. *Anticancer Res*. 2005:25:1615–1622.

Jiang H, Lin J, Su ZZ, Collart FR, Huberman E, Fisher PB. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. *Oncogene*. 1994:9:3397–3406.

Jones P.A, Baylin SB. The epigenomics of cancer. *Cell*. 2007:128:683–692.

Jones G, Prosser DE, Kaufmann M. 25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): its important role in the degradation of vitamin D. *Arch. Biochem. Biophys*. 2012:523:9–18.

Jung S, Qian ZR, Yamauchi M, Bertrand KA, Fitzgerald KC, Inamura K, Kim SA, Mima K, Sukawa Y, Zhang X. Predicted 25 (OH) D score and colorectal cancer risk according to vitamin D receptor expression. *Cancer Epidemiol. Prev. Biomark*. 2014:23(8):1628-1637.

Juwle A, Saranath D. BRCA1/BRCA2 gene mutations/SNPs and BRCA1 haplotypes in early-onset breast cancer patients of Indian ethnicity. *Medical oncology*. 2012:29(5):3272–81.

Khuwaja GA, Abu-Rezq AN. Bimodal breast cancer classification system. *Pattern Analysis and Applications*. 2004:7:235–242.

Kennedy AJ, Rahn EJ, Paulukaitis BS, Savell KE, Kordasiewicz HB, Wang J, Lewis JW, Posey J, Strange SK, Guzman-Karlsson MC, Phillips SE, Decker K, Motley ST, Swayze EE, Ecker DJ, Michael TP, Day JJ, Sweatt JD. Tcf4 Regulates Synaptic Plasticity, DNA Methylation, and Memory Function. *Cell Rep*. 2016:16(10):2666-2685.

Kettunen E, Hernandez-Vargas H, Cros MP, Durand G, Le Calvez-Kelm F, Stuopelyte K, Jarmalaite S, Salmenkivi K, Anttila S, Wolff H, Herceg Z, Husgafvel-Pursiainen K. Asbestos-associated genome-wide DNA methylation changes in lung cancer. *Int J Cancer*. 2017:141(10):2014-2029.

Kim MS, Fujiki R, Kitagawa H, Kato S. 1alpha,25(OH)2D3-induced DNA methylation suppresses the human CYP27B1 gene. *Mol. Cell. Endocrinol*. 2007:265(266):168–173.

Kim GD, Ni J, Kelesoglu N, Roberts RJ, Pradhan S. Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. *EMBO J*. 2002:21(15):4183-95.

Koh KP, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, Lahesmaa R, Orkin SH, Rodig SJ, Daley GQ, Rao A. Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell*. 2011:8(2):200-13.

Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*. 2013:502(7472):472-9.

Komori HK, Hart T, LaMere SA, Chew PV, Salomon DR. Defining CD4 T Cell Memory by the Epigenetic Landscape of CpG DNA Methylation. *J Immunol*. 2015.

Kufe DW, Pollock RE, Weichselbaum RR, Bast RC Jr, Gansler TS, Holland JF, Frei III E. 2003. Holland-Frei Cancer Medicine, 6th edition. Hamilton (ON): BC Decker.

Kuhajda FP, Offutt LE, Mendelsohn G. The distribution of carcinoembryonic antigen in breast carcinoma. Diagnostic and prognostic implications. *Cancer*. 1983:52:1257–1264.

Kurebayashi J, Nomura T, Hirono M, Okubo S, Udagawa K, Shiiki S, Ikeda M, Nakashima K, Tanaka K, Sonoo H. Combined measurement of serum sialyl Lewis X with serum CA15-3 in breast cancer patients. *Jpn. J. Clin. Oncol*. 2006:36:150–153.

Lao VV, Grady WM. Epigenetics and colorectal cancer. *Nat Rev Gastroenterol Hepatol*. 2011:8(12):686-700.

Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med*. 2000:343(2):78-85.

Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev*. 1996:10:142–153.

Lopes N, Carvalho J, Duraes C, Sousa B, Gomes M, Costa JL, et al. 1Alpha,25-dihydroxyvitamin D3 Induces de novo E-cadherin Expression in Triple-negative Breast Cancer Cells by CDH1-promoter Demethylation. *Anticancer Res*. 2012:32:249–257.

Lu X, Chen Z, Mylarapu N, Watsky MA. Effects of 1,25 and 24,25 Vitamin D on Corneal Epithelial Proliferation, Migration and Vitamin D Metabolizing and Catabolizing Enzymes. *Sci Rep*. 2017:7**:**16951.

Madden JM, Murphy L, Zgaga L, Bennett K. De novo vitamin D supplement use post-diagnosis is associated with breast cancer survival. *Breast Cancer Res Treat*. 2018:172(1):179-190.

Mahajan SD, Law WC, Aalinkeel R, Reynolds J, Nair BB, Yong KT, Roy I, Prasad PN, Schwartz SA. Chapter three - Nanoparticle-Mediated Targeted Delivery of Antiretrovirals to the Brain. *Methods in Enzymology*. 2012:509:41-60.

Marik R, Fackler M, Gabrielson E, Zeiger MA, Sukumar S, Stearns V, Umbricht CB. DNA methylation-related vitamin D receptor insensitivity in breast cancer. *Cancer Biol Ther*. 2010:10(1):44-53.

Massari ME, Grant PA, Pray-Grant MG, Berger SL, Workman JL, Murre C. A conserved motif present in a class of helix-loop-helix proteins activates transcription by direct recruitment of the SAGA complex. Mol Cell. 1999:4:63-73.

Mayjonade B, Gouzy J, Donnadieu C, Pouilly N, Marande W., Callot C, Langlade N, Muños S. Extraction of high-molecular-weight genomic DNA for long-read sequencing of single molecules. *Bio Techniques*. 2016:61(4):203–205.

Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature*. 2008:454:766–770.

Meyer MB, Benkusky NA, Pike JW. 1,25-Dihydroxyvitamin D3 induced histone profiles guide discovery of VDR action sites. *J. Steroid Biochem. Mol. Biol*. 2013.

Meyer MB, Goetsch PD, Pike JW. Genome-wide analysis of the VDR/RXR cistrome in osteoblast cells provides new mechanistic insight into the actions of the vitamin D hormone. *J. Steroid Biochem. Mol. Biol*. 2010b:121:136–141.

Meyer MB, Goetsch PD, Pike JW. VDR/RXR and TCF4/beta-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. *Mol. Endocrinol*. 2012:26:37-51.

Moffatt KA, Johannes WU, Hedlund TE, Miller GJ. Growth inhibitory effects of 1-α-25-dihydroxyvitamin D3 are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. *Cancer Res*. 2001:61:7122–7129.

Mohamed IN, Fatema E, Nada A, Kamilia A, Satyanarayana G, Hussaini SSQ. Breast Cancer: Conventional Diagnosis and Treatment Modalities and Recent Patents and Technologies. *Breast Cancer (Auckl)*. 2015:9(s2):17–34.

Morales O, Samuelsson MKR, Lindgren U, Haldose´ n L. Effects of 1,25-Dihydroxyvitamin D3 and Growth Hormone on Apoptosis and Proliferation in UMR 106 Osteoblast-Like Cells. *Endocrinology*. 2004:145(1):87–94.

Moulton T, Crenshaw T, Hao Y, Moosikasuwan J, Lin N, Dembitzer F, Hensle T, Weiss L, McMorrow L, Loew T, Kraus W, Gerald W, Tycko B. Epigenetic lesions at the H19 locus in Wilms' tumour patients. *Nature Genetics*. 1994:7:440–447.

Myers DJ, and Walls AL. 2018. Breast, Atypical Hyperplasia. Treasure Island (FL): StatPearls Publishing.

Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. *Endocr. Rev*. 2005:26:662–687.

Narbaitz R, Sar M, Stumpf WE, Huang S, DeLuca HF. 1,25-Dihydroxyvitamin D3 target cells in rat mammary gland. *Horm Res*. 1981:15(4):263-269.

Narvaez CJ, Matthews D, LaPorta E, Simmons KM, Beaudin S, Welsh J. The impact of vitamin D in breast cancer: genomics, pathways, metabolism. *Front Physiol*. 2014:5:213.

Negishi M, Chiba T, Saraya A, Miyagi S, Iwama A. Dmap1 plays an essential role in the maintenance of genome integrity through the DNA repair process. *Genes Cells*. 2009:14:1347–1357.

Neme A, Seuter S, Carlberg C. Selective regulation of biological processes by vitamin D based on the spatio-temporal cistrome of its receptor. *Biochim Biophys Acta*. 2017. 1860:952–961.

Neri F, Rapelli S, Krepelova A, Incarnato D, Parlato C, Basile G, Maldotti M, Anselmi F, Oliviero S. Intragenic DNA methylation prevents spurious transcription initiation. *Nature*. 2017:543:72–77.

Nishikawa R, Ji XD, Harmon RC, Lazar CS, Gill GN, Cavenee WK, Huang HJ. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci U S A*. 1994:91(16):7727-31.

Novershtern N, Subramanian A, Lawton LN, Mak RH, Haining WN, McConkey ME, Habib N, Yosef N, Chang CY, Shay T, Frampton GM, Drake AC, Leskov I, Nilsson B, Preffe, F, Dombkowski D, Evans JW, Liefeld T, Smutko JS, Chen J, Friedman N, Young RA, Golub TR, Regev A, Ebert BL. Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell*. 2011:144: 296-309.

O'Brien KM, Sandler DP, Xu Z, Kinyamu HK, Taylor JA, Weinberg CR. Vitamin D, DNA methylation, and breast cancer. *Breast Cancer Res*. 2018:20(1):70.

Ogino S, Nowak JA, Hamada T, Milner Jr DA, Nishihara R. Insights into pathogenic interactions among environment, host, and tumor at the crossroads of molecular pathology and epidemiology. *Annu. Rev. Pathol. Mech. Dis*. 2018:14:83-103.

Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases DNMT3A and DNMT3B are essential for de novo methylation and mammalian development. *Cell*. 1999:99:247–257.

Pan L, Matloob AF, Du J, Pan H, Dong Z, Zhao J, Feng Y, Zhong Y, Huang B, Lu J. Vitamin D stimulates apoptosis in gastric cancer cells in synergy with trichostatin A /sodium butyrate-induced and 5-aza-2′-deoxycytidine-induced PTEN upregulation. *FEBS J*. 2010:277:989–999.

Petrucelli N, Daly MB, Pal T. BRCA1- and BRCA2-Associated Hereditary Breast and Ovarian Cancer. *GeneReviews*. 2016.

Pfeifer GP. Defining Driver DNA Methylation Changes in Human Cancer. Int J Mol. 2018:19(4):1166.

Pike JW, Meyer MB, Bishop KA. Regulation of target gene expression by the vitamin D receptor - an update on mechanisms. *Rev. Endocr. Metab. Disord*. 2012:13:45–55.

Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D3. *Endocrinol Metab Clin North Am*. 2010:39:255–269.

Pilon C, Urbanet R, Williams TA, Maekawa T, Vettore S, Sirianni R, Pezzi V, Mulatero P, Fassina A, Sasano H, Fallo F. 1alpha,25-Dihydroxyvitamin D(3) inhibits the human H295R cell proliferation by cell cycle arrest: a model for a protective role of vitamin D receptor against adrenocortical cancer. *J Steroid Biochem Mol Biol*. 2014:140: 26–33.

Pixberg CF, Schulz WA, Stoecklein NH, Neves RPL. Characterization of DNA methylation in circulating tumor cells. *Genes*. 2017:6:1053–1075.

Poritz MA, Ririe KM. Getting things backwards to prevent primer dimers. *The Journal of molecular diagnostics: JMD*. 2014:16(2):159–162.

Postlind H, Axén E, Bergman T, Wikvall K. Cloning, structure, and expression of a cDNA encoding vitamin D3 25-hydroxylase. *Biochem. Biophys. Res. Commun*. 1997:241:491–497.

Preston-Martin S, Pike MC, Ross RK, Henderson BE. Epidemiologic evidence for the increased cell proliferation model of carcinogenesis. *Environ Health Perspect Suppl*. 1993:101:137–138.

Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE. Increased cell division as a cause of human cancer. *Cancer Res*. 1990:50:7415–7421.

Rahmaniyan M, Patrick K, Bell NH. Characterization of recombinant CYP2C11: a vitamin D 25-hydroxylase and 24-hydroxylase. *Am. J. Physiol. Endocrinol. Metab*. 2005:288:E753–E760.

Rhee I, Bachman K, Park B, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, Baylin SB, Vogelstein B. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*. 2002:416:552–556.

Rhein M, Hagemeier L, Klintschar M, Muschler M, Bleich S, Frieling H. DNA methylation results depend on DNA integrity-role of post mortem interval. *Front Genet*. 2015:6:182.

Rio DC, Ares M Jr, Hannon GJ, Nilsen TW. Purification of RNA using TRIzol (TRI reagent). *Cold Spring Harb Protoc*. 2010(6):pdb.prot5439.

Rose AA, Elser C, Ennis M, Goodwin PJ. Blood levels of vitamin D and early stage breast cancer prognosis: a systematic review and meta-analysis. *Breast Cancer Res Treat*. 2013:141(3):331–9.

Sambrook J, Russel DW. (2001). Molecular Cloning: A Laboratory Manual, 3rd Ed. Cold Spring Harbor Laboratory Press: New York, NY.

Saracligil B, Ozturk B, Unlu A, Abusoglu S, Tekin G. The effect of vitamin D on MCF-7 breast cancer cell metabolism. *Bratisl Lek Listy*. 2017:118(2):101-106.

Saramäki A, Diermeier S, Kellner R, Laitinen H, Vaïsänen S, Carlberg C. Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (CDKN1A) gene in response to 1alpha, 25-dihydroxyvitamin D3. *J. Biol. Chem*. 2009:284:8073–8082.

Schon K, Tischkowitz M. Clinical implications of germline mutations in breast cancer: TP53. *Breast Cancer Res Treat*. 2017:167(2):417-423.

Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleicacids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011:11:426–437.

Seuter S, Neme A, Carlberg C. Epigenomic PU.1-VDR crosstalk modulates vitamin D signaling. *Biochim Biophys Acta*. 2017:1860:405–415.

Seuter S, Pehkonen P, Heikkinen S, Carlberg C. Dynamics of 1α, 25-dihydroxyvitamin D3-dependent chromatin accessibility of early vitamin D receptor target genes. *Biochim. Biophys. Acta*. 2013:1829:1266–1275.

Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA, Wong KK, Brandstetter K, Wittner B, Ramaswamy S, Classon M, Settleman J. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*. 2010:141(1):69-80

Shui I, Giovannucci E. Vitamin D Status and Cancer Incidence and Mortality, Sunlight, Vitamin D and Skin Cancer. 2015:33.

Sierra OL, Towler DA. Runx2 trans-activation mediated by the MSX2-interacting nuclear target requires homeodomain interacting protein kinase-3. *Mol Endocrinol*. 2010:24:1478-1497.

Sigmon J, Larcom LL. The effect of ethidium bromide on mobility of DNA fragments in agarose gel electrophoresis. *Electrophoresis*. 1996:17(10):1524-1527.

Song M, Nishihara R, Wang M, Chan AT, Qian ZR, Inamura K, Zhang X, Ng K, Kim SA, Mima K. Plasma 25-hydroxyvitamin D and colorectal cancer risk according to tumour immunity status. *Gut*. 2016:65(2):296-304.

Seuter S, Neme A, Carlberg C. ETS transcription factor family member GABPA contributes to vitamin D receptor target gene regulation. *J Steroid Biochem Mol Biol*. 2018:177:46–52.

Sever R, Brugge JS. Signal Transduction in Cancer. *Cold Spring Harb Perspect Med*, 2015:5:a006098.

Silva J, Silva JM, Garcia V, Garcia JM, Dominguez G, Bonilla F. RNA is more sensitive than DNA in identification of breast cancer patients bearing tumor nucleic acids in plasma. *Genes Chromosomes Cancer*. 2002:35:375–376.

Silva JM, Dominguez G, Villanueva MJ, Gonzalez R, Garcia JM, Corbacho C, Provencio M, Espana P, Bonilla F. Aberrant DNA methylation of the p16INK4a gene in plasma DNA of breast cancer patients. *Br. J. Cancer*. 1999:80:1262–1264.

Singewald N, Schmuckermair C, Whittle N, Holmes A and Ressler K. Pharmacology of cognitive enhancers for exposure-based therapy of fear, anxiety and trauma-related disorders. 2014.

Sharipov FK, Kireev GV, Koloiarova NE, Khodzhaev AV, Beknazarov Z. Peroxidation of serum lipids in patients with breast cancer. *Klin. Lab. Diagn*. 2003:13–15.

Shewach and Kuchta. Introduction to Cancer Chemotherapeutics. *Chem Rev*. 2009:109(7):2859-2861.

Steenland K, Stayner L, Deddens J. Mortality analyses in a cohort of 18 235 ethylene oxide exposed workers: Follow up extended from 1987 to 1998. *Occupational and Environmental Medicine*. 2004:61(1):2–7.

Steenland K, Whelan E, Deddens J, Stayner L, Ward E. Ethylene oxide and breast cancer incidence in a cohort study of 7576 women (United States). *Cancer Causes and Control*. 2003:14(6):531–539.

Strober W. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol*. 2001: A3:3B.

Srikuea R, Zhang X, Park-Sarge OK, Esser KA. VDR and CYP27B1 are expressed in C2C12 cells and regenerating skeletal muscle: potential role in suppression of myoblast proliferation. *Am J Physiol Cell Physiol*. 2012:303:C396–405.

Szyf M. Targeting DNA methylation in cancer. *JL Eurotext*. 2006:93(9):961-972.

Tabasi N, Rastin M, Mahmoudi M, Ghoryani M, Mirfeizi Z, Rabe SZ, Reihani H. Influence of vitamin D on cell cycle, apoptosis, and some apoptosis related molecules in systemic lupus erythematosus. *Iran J Basic Med Sci*. 2015;18(11):1107–1111.

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*. 2009:324(5929):930-5.

Thompson JA. Molecular cloning and expression of carcinoembryonic antigen gene family members. *Tumour Biol*. 1995:16:10–16.

Tsai HC, Baylin SB. Cancer epigenetics: linking basic biology to clinical medicine. *Cell Res*. 2011:21:502–517.

Tuoresmäki P, Väisänen S, Neme A, Heikkinen S, Carlberg C. Patterns of Genome-Wide VDR Locations. *PLoS ONE*. 2014:9(4):e96105.

Vancells JC. Alexa Fluor® conjugated secondary antibodies – FAQs. Available at:http://www.abcam.com/secondary-antibodies/alexa-fluor-conjugated-secondary-antibodies---faqs (Accessed: 26 October 2017).

Van den Bemd G-JCM, Pols HAP, Leeuwen JPTM. Anti-Tumor Effects of 1,25-Dihydroxyvitamin D3 and Vitamin D Analogs. *Curr Pharm Des*. 2000:6:717–732.

Veurink M, Koster M, Berg LT. The history of DES, lessons to be learned. *Pharm World Sci*. 2005:27(3):139–43.

Vorobiof D, Sitas F, Vorobiof G. Breast cancer incidence in South Africa. *Journal of Clinical Oncology*. 2001:19(18s):125s-127s.

Wang J, Han X, Sun Y. DNA methylation signatures in circulating cell-free DNA as biomarkers for the early detection of cancer. *Sci China Life Sci*. 2017:60(4):356-362.

Wang QM, Jones JB, Studzinski GP. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1–S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. *Cancer Res*. 1996:56:264–267.

Waterland RA. Epigenetic mechanisms and gastrointestinal development. *J Pediatr*. 2006:149: S137–S142.

Wilfinger WW, Mackey K, Chomczynski P. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques*. 1997:22(3):474-476, 478-481.

Wingfield PT. Use of Protein Folding Reagents. *Curr Protoc Protein Sci*. Author manuscript; available in PMC. 2016.

Wingren C, Borrebaeck CA. Antibody microarrays: current status and key technological advances. *Omics*. 2006:10:411–427.

Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *New Engl J Med*. 2006:354:270–282.

Yao S, Kwan ML, Ergas IJ, Roh JM, Cheng TD, Hong CC, McCann SE, Tang L, Davis W, Liu S, et al. Association of serum level of vitamin D at diagnosis with breast cancer survival: a case-cohort analysis in the pathways study. *JAMA Oncol*. 2017:3(3):351–357.

Yan J, Yuan F, Long G, Qin L, Deng Z. Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. *Mol Biol Rep*. 2012:39:1831–1838.

Ylikomi T, Laaksi I, Lou YR, Martikainen P, Miettinen S, Pennanen P, Purmonen S, Syvala H, Vienonen A, Tuohimaa P. Antiproliferative action of vitamin D. *Vit Horm*. 2002:64: 357–406.

Zaenker P, Ziman MR. Serologic Autoantibodies as Diagnostic Cancer Biomarkers—A Review. *Cancer Epidemiol Biomarkers Prev*. 2013:22(12):2161–81.

Zhang BN, Cao XC, Chen JY, Chen J, Fu L, Hu XC, Jiang ZF, Li HY, Liao N, Liu DG, Tao O, Sha, ZM, Sun Q, Wang S, Wang YS, Xu BH, Zhang J. Guidelines on the diagnosis and treatment of breast cancer (2011 edition). *Gland Surg*. 2012:39–61.

Zeichner SB, Koru-Sengul T, Shah N, Liu Q, Markward NJ, Montero AJ, Glück S, Silva O, Ahn ER. Improved clinical outcomes associated with vitamin D supplementation during adjuvant chemotherapy in patients with HER2+ nonmetastatic breast cancer. *Clin Breast Cancer*. 2015:15(1):e1-11.

Zella LA, Kim S, Shevde NK, Pike JW. Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D3. *Mol Endocrinol*. 2006:20:1231–1247.

Zella LA, Meyer MB, Nerenz RD, Lee SM, Martowicz ML, Pike JW. Multifunctional enhancers regulate mouse and human vitamin D receptor gene transcription. *Mol Endocrinol*. 2010:24:128–147.

Zeng Y, Chen T. DNA Methylation Reprogramming during Mammalian Development. *Genes (Basel)*. 2019:10(4):257.

Zhang J, Kalkum M, Yamamura S, Chait BT, Roeder RG. E protein silencing by the leukemogenic AML1-ETO fusion protein. *Science*. 2004:305:1286-11289.

Zhang HY, Liang F, Jia ZL, Song ST, Jiang ZF. PTEN mutation, methylation and expression in breast cancer patients. *Oncol Lett*. 2013:6:161–168.

Zhou W, Dinh HQ, Ramjan Z, Weisenberger DJ, Nicolet CM, Shen H, Laird PW, Berman BP. DNA methylation loss in late-replicating domains is linked to mitotic cell division. *Nat Genet*. 2018:50(4):591-602.

Zhu H, Huang Y, Bhagatwala J, Parikh S, Havens R, Kotak I, Dong Y. Vitamin D Supplementation Increases Global DNA Methylation in Overweight and Obese African Americans with Vitamin D Deficiency. *AHA Journals*. 2018:129: Issue suppl_1.

Source	**URL** **(Date Accessed)**
Barnabé M. Cell viability assays: MTT assay application and protocol, Quartzy. 2017.	https://blog.quartzy.com/2017/05/01/cell-viability-assays-mtt-protocol (9 April-2018).
Dana-Farber. The Connection between Pregnancy and Breast Cancer Risk. 2017.	http://blog.dana-farber.org/insight/2015/01/does-pregnancy-increase-risk-of-breast-cancer/ (9 July-2018).
Fact sheet No. 297: Cancer. World Health Organization. 2006.	http://www.who.int/mediacentre/factsheets/fs297/en/index.html (31 July-2018)
GLOBOCAN	https://gco.iarc.fr/today/home (11 December-2018)
GLOBOCAN	http://quadcitiesdaily.com/?p=262013 (16 January-2019)
Kannan S. 4 Easy Steps to Analyze Your qPCR Data Using Double Delta Ct Analysis. 2016.	http://bitesizebio.com/24894/4-easy-steps-to-analyze-your-qpcr-data-using-double-delta-ct-analysis/ (10 October-2017)
HHS (U.S. Department of Health and Human Services). 2011. Report on Carcinogens, 12th Edition. Washington, D.C.: U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program.	http://ntp.niehs.nih.gov/go/roc12 (8 July-2018)

Male Breast Cancer Treatment. National Cancer Institute. 2006.	http://www.cancer.gov/cancertopics/pdq/treatment/malebreast/healthprofessional. (31 July-2018)
Prakash S Bisen	https://www.researchgate.net/publication/279725968_Cancer_A_Worldwide_Menace_KEY_WORDS/figures?lo=1 (12 July-2019)
Vancells JC. Alexa Fluor® conjugated secondary antibodies – FAQs	http://www.abcam.com/secondary-antibodies/alexa-fluor-conjugated-secondary-antibodies---faqs (26 October-2017)
Wieczorek, D., Delauriere, L., and Schagat, T. Methods of RNA Quality Assessment. *Promega Corperation*. 2012.	https://worldwide.promega.com/resources/pubhub/methods-of-rna-quality (2 March-2018).
World Health Organisation Vol. 60. Some Industrial Chemicals. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. Lyon: International Agency for Research on Cancer. 1999.	https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Industrial-Chemicals-1994 (20 December 2018)
What Are the Key Statistics About Breast Cancer in Men? 2007. American Cancer Society	http://www.cancer.org/docroot/CRI/content/CRI_2_4_1X_What_are_the_key_statistics_for_male_breast_cancer_28.asp?sitearea (31 July 2018)
World Cancer Report	http://www.iarc.fr/en/Publications/PDFsonline/World-Cancer-Report/WorldCancer-Report (31 July-2018)