EVALUATION OF TAGLN AS A DIAGNOSTIC MARKER IN BREAST CANCER

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE

OF BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MOLECULAR BIOLOGY AND GENETICS

By

Ayşe Sedef Köseer

August 2018

EVALUATION OF TAGLN AS A DIAGNOSTIC MARKER IN BREAST CANCER

By Ayşe Sedef Köseer

August 2018

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Işık Yuluğ (Advisor)

Özlen Konu Karakayalı

Bala Gür Dedeoğlu

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan

Director of the Graduate School of Engineering and Science

Abstract

EVALUATION OF TAGLN AS A DIAGNOSTIC MARKER IN BREAST CANCER

Ayşe Sedef Köseer

MSc in Molecular Biology and Genetics

Advisor: Işık G. Yuluğ

August 2018

Silencing of tumor suppressor genes via CpG hypermethylation in promoter regions is one of the frequent events occurring in different types of cancers. These genes have the potential as a diagnostic or a prognostic biomarker. Liquid biopsy is a relatively less invasive technique that is used for early diagnosis, therapy response prediction, minimal residual disease detection and real-time monitoring of tumor progression.

In this study, a 402 bp region (-286 bp to -80 bp for Section 1, -102 bp to +115 bp for Section 2) located in *TAGLN* promoter containing 22 CpGs was analyzed in breast cancer patients and healthy donors to evaluate the biomarker potential of *TAGLN* promoter methylation levels in breast cancer. *TAGLN* promoter region was significantly hypermethylated in breast cancer patients (77.3%) compared to healthy donors (68.2%). Among differentially methylated CpGs, 6 out of 22 were hypermethylated and one was hypomethylated in breast cancer patients. We also analyzed the relationship between *TAGLN* promoter methylation levels and the patient's clinicopathological parameters. Analyses revealed that *TAGLN* promoter is highly methylated in breast cancer patients over 50 years of age compared to the healthy donors in the same age group. *TAGLN* promoter methylation did not differ as related to various clinicopathological parameters of breast cancer patients. *TAGLN* promoter methylation levels diagnosed breast cancer patients with 74.45% specificity

and 57.58% sensitivity. Additionally, independent of the age group breast cancer patients (131.6 ng) exhibited higher levels of total cfDNA compared to healthy donors (56.4 ng). Pre- and postmenopausal breast cancer patients possessed higher total cfDNA levels compared to pre- and postmenopausal healthy donors. Total cfDNA levels did not differ in various clinicopathological parameters of breast cancer patients; however, total cfDNA levels diagnosed breast cancer patients with 73.33% specificity and 56.72% sensitivity.

In summary, breast cancer patient sera can be used to identify the tumor profile, and *TAGLN* promoter hypermethylation and total cfDNA levels could serve as a diagnostic biomarker in breast cancer.

Keywords: transgelin, TAGLN, SM22 alpha, Breast Cancer, DNA methylation, hypermethylation, biomarker, diagnosis, cell-free circulating DNA, cfDNA.

Özet

MEME KANSERİNDE TRANSGELIN GENİNİN TANIYICI BİR BELİRTEÇ OLARAK DEĞERLENDİRİLMESİ

Ayşe Sedef Köseer

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: Işık G. Yuluğ

Ağustos, 2018

Promotör bölgelerinde CpG hipermetilasyonu yoluyla tümör baskılayıcı genlerin susturulması, farklı kanser türlerinde sık karşılaşılan olaylardan biridir. Bu genler tanısal veya prognostik biyobelirteç olma potansiyeline sahiptir. Sıvı biyopsi, erken tanı, tedaviye yanıt tahmini, minimal kalıntı hastalık tespiti ve tümör ilerleyişinin gerçek zamanlı izlenmesi için kullanılan görece daha az invazif olan bir tekniktir.

Bu çalışmada, *TAGLN* promotör metilasyon düzeylerinin meme kanserinde biyobelirteç potansiyelini değerlendirmek için, 402 bç'lik bir bölge (Bölüm 1 için -286 bç ile -80 bç, Bölüm 2 için -102 bç ile +115 bç) ve 22 CpG içeren *TAGLN* promotör bölgesi meme kanseri hastalarında ve sağlıklı bağışçılarda analiz edildi. *TAGLN* promotör bölgesi meme kanseri hastalarında (%77.3) sağlıklı bağışçılara (%68.2) kıyasla isttistiksel olarak anlamlı orande hipermetile olduğu tespit edilmiştir. Meme kanseri hastalarında bakılan 22 farklı CpG arasında, 6 tanesi hipermetile ve 1 tanesi hipometile olarak bulunmuştur. Ayrıca *TAGLN* promotör metilasyon düzeyleri ile hastanın klinikopatolojik parametreleri arasındaki ilişki analiz edilmiştir. Analizler, *TAGLN* promotör bölgesinin 50 yaş üzeri meme kanseri hastalarında, aynı yaş grubundaki sağlıklı bağışçılara göre yüksek oranda metillendiğini ortaya çıkarmıştır. *TAGLN* promotör metilasyonu, meme kanseri hastalarının çeşitli klinikopatolojik parametrelerinde farklılık göstermediği bulunmuştur. *TAGLN* promotör metilasyon seviyeleri, meme kanseri hastalarını %74.45 özgüllük ve

%57.58 hassaslık değerleri ile tespit edebilmiştir. Ek olarak, yaş grubundan bağımsız olarak meme kanseri hastaları (131.6 ng) sağlıklı bağışçılara (56.4 ng) kıyasla daha yüksek toplam cfDNA seviyelerine sahipti. Toplam cfDNA seviyeleri, meme kanseri hastalarının çeşitli klinikopatolojik parametrelerinde farklılık göstermemiş, ancak toplam cfDNA seviyesinin %73.33 özgüllük ve %56.72 hassaslık ile meme kanseri hastalarını teşhis edebildiği gösterilmiştir. Özetle, tümör profilini tanımlamak için meme kanseri hasta serumu kaynak olarak kullanılabilir. *TAGLN* metilasyonu ve toplam cfDNA seviyesi, meme kanserinde tanısal amaçla belirteç olarak kullanılabilir.

Anahtar kelimeler: transgelin, TAGLN, SM22 alfa, Meme Kanseri, DNA metilasyonu, hipermetilasyon, biyobelirteç, tanı, hücreden bağımsız dolaşan serbest DNA, cfDNA.

To my beloved family,

Acknowledgement

I would like to express my gratitude to my supervisor Assoc. Prof. Dr. Işık Yuluğ for her endless support; her encouragement and care were invaluable starting from the first day. During my tough times she made sure that I was on the right track. I have gained many beautiful memories during my Master's studies. Her knowledge and guidance inspired me to become an independent researcher in the science field.

I was very lucky to be one of the members of the Yuluğ Group and I met beautiful people here. Eda Süer was a very unique person whose perspective of life has helped me during my tough times. I am very thankful for her wonderful insights on both life and science, and for her friendship. I am also thankful for Nazlı Değer, Gizem Sunar and Ilgım Özerk for their positive energy and fun conversations. I would also like to thank Ilayda Kaya with whom I was lucky to meet, for her endless support and companionship.

I am grateful to Ayşe Gökçe Keşküş, Seniye Targen and Nevin Belder for their endless support and friendship that helped get me through many many tough times. I also would like to thank Bilkent MBG family past and present members including Pelin Makas, Seda Birkan, Yıldız Kahyaoğlu, Füsun Elvan, Yavuz Ceylan and Abdullah Ünnü. I would like to express my gratitude to Claudia Cerella for her mentorship and her invaluable advice throughout my years during my studies.

I am especially grateful to Uğur Kahya for his positive attitude that helped me to get through all my challenges, frustrations and exhaustions. I do not know what would I have done without his support.

Lastly, my utmost thanks go to my family for their unconditional patience, support and love. Their efforts made this thesis possible.

This project was supported by TUBITAK 1001 grant, KBAG 215Z149 and Bilkent University.

Table of Contents

List of Figures

List of Tables

Abbreviations

BS	Bisulfite sequencing	MeDIP	Methylated DNA immunoprecipitation
BSP	Bisulfite sequencing PCR	MSP	Methylation specific PCR
cfDI	Cell-free DNA Integrity	NGS	Next generation sequencing
cfDNA	Cell-free DNA	NST	No special type
CGI	CpG Island	PR	Progesterone receptor
CNA	Circulating nucleic acid	QMSP	Quantitative methylation specific PCR
CpG	Cytosine-phosphate-Guanine	ROC	Receiver-operator characteristics
CTC	Circulating tumor cell	RT	Room temperature
DCIS	Ductal carcinoma *in situ*	TAGLN	Transgelin
DCT	Disseminated tumor cell	TN	Triple negative
EMT	Epithelial to mesenchymal transition	TNBC	Triple-negative breast cancer
ER	Estrogen receptor	TNM	Tumor-node-metastasis
Her2	Human epidermal growth factor	TSG	Tumor suppressor gene
HR	Hormone receptor	TSS	Transcription start site
IDC	Infiltrative ductal carcinoma		
ILC	Infiltrative lobular carcinoma		
LCIS	Lobular carcinoma *in situ*		
Lum A	Luminal A		
Lum B	Luminal B		

Chapter 1. Introduction

1.1 Breast Cancer

Cancer is a multistep process in which normal cells evolve progressively by acquiring genetic alterations and mutations results in aberrant cellular growth[1]. Among the cancers observed worldwide, breast cancer (BC) is the most common cancer and the leading cause of mortality in women[2]. BC is a clinically and genetically a heterogeneous disease, which complicates the treatment of BC patients and increases the mortality rate[3].

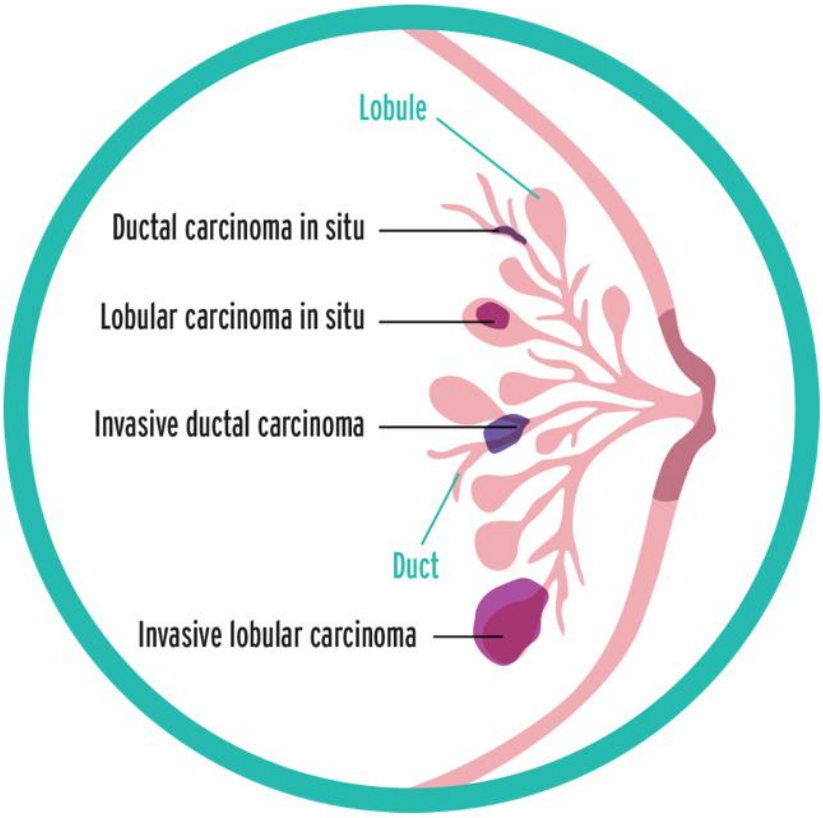

Figure 1.1: Structure of breast tissue, and pathological breast carcinomas.

Normal breast tissue structure and development of different pathological breast carcinomas, ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), respectively. Taken from AstraZeneca website[4]

Healthy human mammary gland is composed of secretory and adipose tissues with the support of connective fibrous tissue[5]. Secretory tissue consists of a lobular/ductal system that transports milk to the nipple[6]. The breast ductal system consists of several smaller ductules that originate from the nipple and reach the lobules[7]. The lining of the ductal walls is composed of two layers of epithelial cells that are at the root of most breast carcinomas[8]; however, any cell along the lobular/ductal system may result in malignancies. The various origins result in the pathological classification of BC[9].

1.1.1. Pathological classification of breast cancer

Pathological classifications are mostly based on observations of biopsy samples under microscopy. World Health Organization (WHO) classification of breast tumors includes benign and malignant tumors but most of them are observed only rarely. The most frequent types of BC are listed as follows[10]:

(1) Ductal Carcinoma *in situ* (DCIS) is the most frequently observed class of noninvasive/pre-invasive (*in situ*) BC. Abnormal cell proliferation starts in the milk ducts and has not invaded beyond the epithelial layer of ductal walls[11]. DCIS can elevate the risk of invasive BC development further in life.

(2) Lobular Carcinoma *in situ* (LCIS) is another non-invasive BC where abnormal cellular proliferation in lobules has not spread beyond the lobular walls[12].

(3) Invasive Ductal Carcinoma (IDC) is the most frequently observed class of invasive BC. Approximately 80% of all BC cases are IDC[13], mostly affecting older women. Abnormal cellular proliferation starts in the milk ducts and invades and infiltrate the fatty tissue of the breast[14]. IDC may invade lymph nodes in later stages by duct and tubule penetration[15]. IDC consists of numerous subtypes, including tubular, medullary, mucinous, papillary and cribriform. Uncategorized cases are called “no special type” or “NST”[16].

(4) Invasive Lobular Carcinoma (ILC) is the second commonly observed type of invasive BC. Approximately 10% of all invasive BC cases are ILC. The origin of the carcinoma is the lobules, and it invades the lobular walls and spreads to breast tissue[17]. In later stages, lymph nodes and other areas of the body may also be invaded.

1.1.2. Histological Grading and Molecular Classification of Breast Cancer

Invasive breast carcinomas can be divided into grades based on their differentiation degree and growth patterns including nuclear polymorphism, tubule formation and mitotic rate[18]. The tumor is examined to assess the existence of normal structures of milk ducts, shape and size of the tumor nucleus and cell division rate. Numerical scoring of 1-3 is used for each factor; the scores are summed together and the grade is assigned[19]. Based on this grading system, maximum scores for grades 1, 2 and 3 are 5, 6-7 and 8-9, respectively. High-grade tumors are less differentiated, associated with poorer prognosis, and may require immediate treatment.

The tumor-node-metastasis (TNM) notation system is used to describe the stage and the extent of a solid tumor for prognosis evaluation[20]. In the past, TNM was assessed by evaluating T, the size and the invasion status of the primary tumor; N, the involvement of nearby regional lymph nodes; and M, the status of distant metastasis[21]. In 2018, the American Joint Committee on Cancer (AJCC) updated the staging guidelines adding the tumor grade, hormone receptor status and HER2 status[22]. Stage 0 is used for non-invasive BC that has not spread outside of the lobules or ducts. Stage I is used for the invasive type of BC and early invading cells can be visualized. Stage II is subcategorized into IIA and IIB. Stage IIA is used if breast tissue does not possess a solid tumor but cancer cells can be visualized in nearby lymph nodes, tumor size is 2 centimeters or smaller and has invaded nearby lymph nodes, or tumor size is between 2 and 5 centimeters and has not invaded the lymph nodes. Stage IIB is used if tumor size is between 2 and 5 centimeters with lymph node invasion or tumor size is larger than 5 centimeters without any lymph node invasion. Stage III is subcategorized into IIIA, IIIB and IIIC. Stage IIIA is used if tumor size is smaller than 2 centimeters and cancer cells can be visualized in 4-9 lymph nodes, tumor size is larger than 5 centimeters with clusters of lymph node or breastbone invasion. Stage IIIB is used if cancer cells have spread to the skin of the breast or chest wall and spread to lymph nodes. Stage IIIC is used if cancer cells can be visualized in 10+ lymph nodes, lymph nodes close to the collar bone or lymph nodes close to the breastbone or underarm. Stage IV describes invasive BC that has invaded the other areas of the body including brain, bones, lungs and liver (Figure 1.2).

Stage	Definition
Stage 0 is carcinoma *in situ*	Tumors that have not grown beyond their site of origin and invaded the neighboring tissue. They include: - ductal carcinoma *in situ* - lobular carcinoma *in situ*
Stage 1	Tumor size <2 cm, metastases to other organs and tissues not available
Stage 2a	Tumor <2 cm in cross-section with involvement of the lymph node or tumor from 2 to 5 cm without involvement of the axillary lymph nodes
Stage 2b	Tumor more than 5 cm in cross-section (the result of axillary lymph node research is negative for cancer cells) or tumor from 2 to 5 cm in diameter with the involvement of axillary lymph nodes
Stage 3a	Also called local spread of *breast cancer*: tumor more than 5 cm with spread to axillary lymph nodes or tumor of any size with metastases in axillary lymph nodes, which are knitted to each other or with the surrounding tissues
Stage 3b	Tumor of any size with metastases into the skin, chest wall or internal lymph nodes of the mammary gland (located below the breast inside of the chest)
Stage 3c	Tumor of any size with a more widespread metastases and involvement of more lymph nodes
Stage 4	Defined as the presence of tumors (regardless of the sizes), spread to parts of the body that are located far removed from the chest (bones, lungs, liver, brain or distant lymph nodes)

Figure 1.2: Breast cancer staging

Staging of BC according to the size, lymph node and distant metastasis status. Taken from Atoum Manar, TNM staging and classification (familial and nonfamilial) of BC in Jordanian females, Indian J Cancer, 2010, 47, R194-198, by permission of Wolters Kluwer Medknow Publications[23].

It is presumed that there is an association between diversity of gene expression and diversity of corresponding phenotypes in breast tumors[9]. mRNA expression profiling has been used to subcategorize breast tumors into six intrinsic subtypes originating from two distinct cell types (luminal epithelial and basal-like cells)[24–27]: luminal A (Lum A), luminal B (Lum B), HER2/ERBB2-enriched, normal-like, basal and claudin-low. Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) expression levels are fundamental for the prognosis and treatment decision. Approximately 65% of observed BC cases are hormone receptor (HR) positive with better prognosis as opposed to HR-negative BC. Lum A and Lum B BC subtypes are HR-positive or HER2-negative, where proliferative genes (i.e. Ki-67) are shown to be highly expressed in the Lum B subtype[28] with a poorer prognosis compared to the Lum A subtype[29]. Lum B tumors are generally higher-grade tumors. HER2/ERBB2-enriched BC tumors constitute 15% to 25% of invasive BC. The HER2-enriched subtype displays a poor prognosis and this may derive from the incomplete eradication of tumor cells after therapy resulting in a higher risk of early relapse[30]. Although HER2-enriched breast tumors show a poor prognosis, this subtype is sensitive to HER2-targeted treatments[31]. Basal tumors and claudin-low tumors, triple negative (TN), are constituted of HR-negative

and HER2-negative. Basal tumors are observed in 60% to 90% triple-negative BC (TNBC) patients with expression profiles that are similar with epithelial cells that lack hormone receptors and HER2 expressions while inducing proliferative gene expressions[32]. Claudin-low tumors present a TN phenotype with high expression of epithelial-to-mesenchymal transition (EMT), cancer stem cell-related and immune response-related genes[33]. Claudin-low tumors are also associated with poor survival rates compared to luminal type BC. Normal-like BC resembles Lum A type with HR-positive, HER2-negative and low levels of Ki-67 features. Even though normal-like BC has a good prognosis, it is slightly worse than Lum A type[24]. ER-positive Lum A types of breast tumors are associated with the best prognosis while claudin-low and basal tumors are recognized as more aggressive and with poorer prognosis[28]. Table 1.1 demonstrates common features of intrinsic subtypes of BC, and their prognostic status respectively.

Table 1.1: Molecular classification of breast cancer

Molecular Subtype	ER/PR/Her2 status	Grade	Prognosis
Normal-like	ER+/-, PR-, Her2-	1/2/3	Intermediate
Lum A	ER+, PR+, Her2-	1/2	Good
Lum B	ER+/-, PR+/-, Her2+/-	2/3	Intermediate
Her2-enriched	ER-, PR-, Her2+	2/3	Poor
Basal	ER-, PR-, Her2-	3	Poor
Claudin-low	ER-, PR-, Her2-	1/2/3	Poor

Adapted from Dai et al.[24]

1.1.3. Inherited breast cancer

It is estimated that 5% to 10% of all BC cases have a hereditary background, which is due to autosomal dominant mutations[34]. Among these hereditary cases, approximately 30% are associated with germline mutations occurring in DNA repair

genes, *BRCA1* and *BRCA2*[35]. Recently, a set of susceptible genes was discovered with the help of a next generation sequencing technique. These genes are subcategorized as high penetrant genes with low frequency and moderate penetrant genes. Penetrance is defined as the risk of causing any type of cancer[36]. *TP53*, a tumor suppressor gene that is vital for the regulation of cell growth, is a high penetrant gene, and mutated in 1% of BC cases[37]. *TP53* gene mutation increases the risk for BC development 60-fold[38]. *ATM*, vital for DNA double strand breaks and cell cycle progression, is a moderate penetrant gene. *ATM* mutation increases the risk for BC 2-fold[39].

1.2 Epigenetics and breast cancer

Cancer is a complex and a multistep process of normal cells that evolve progressively by acquiring genetic changes and gene expression alterations resulting in aberrant function in oncogenes and tumor suppressor genes. DNA methylation and histone modifications take part in fundamental roles in various cellular signaling networks, such as cell cycle, DNA repair, apoptosis, cell adhesion, inflammation and invasion. Epigenetic alterations can occur at tumor-related gene regions and can alter the function of these genes inducing malignant transformation including induction, development and progression without changing the original sequence[40]. Epigenetic changes are different from genetic changes since epigenetic changes can be found more frequently and are reversible following treatment with therapeutic agents[41].

1.2.1. DNA methylation alterations in breast cancer

At the molecular level, the 5' carbon of cytosine bases in CpG dinucleotides can be methylated via addition of a methyl group by DNA methyltransferases[40]. Although CG dinucleotides can be found genome-wide, they are mostly grouped in CpG islands. CpG islands are 200 to 500 bp length regions where the GC content is higher than 50-55% and the observed-to-expected CpG percentage is higher than 0.6[42,43]. CpG islands, for the most part, remain unmethylated in normal cells, ensuring gene expression regulations[44]. In cancer, gene expression regulation mechanisms are manipulated either by global hypomethylation resulting in the instability of the genome or by hypermethylation of promoter regions of tumor suppressor genes

resulting in aberrant growth[45,46]. In cancer, the frequency of hypomethylation of transcription regulatory genes is less than hypermethylation of CpG islands that reside in the gene promoter[47,48]. Nonetheless, some of the cancer-related DNA hypomethylation targets transcription control sequences[49–51]. The *PLAU*, protease urokinease, gene is found to be hypomethylated and overexpressed in breast tumor associated with tumor progression[52]. Hypomethylation of *PLAU* gene was found to be associated with tumor invasion[53]. Inactivation of tumor-suppressor genes by promoter hypermethylation is a frequent event observed in various cancers[54]. Hypermethylation of tumor suppressor genes were found to be involved in cancer initiation and progression[55]. Retinoblastoma, *RB*, is the first gene that was found to be hypermethylated, followed by *APC* and *RASSF1A*[56–58]. In BC, different genes have been found to be hypermethylated compared to normal tissues. These genes play a role in various mechanisms such as aberrant growth and proliferation (*p16*, *ERα*, *RARβ*), regulator of apoptosis (*RASSF1A*), DNA repair (*BRCA1*), and invasion and metastasis (*CDH1*)[59]. Among these genes, *RASSF1A*, hypermethylated in 60% of tumors but significantly lower in normal tissue, is the most promising biomarker in BC. *RASSF1A* methylation was also found in sera samples of BC patients[60].

1.2.2. DNA Methylation as a breast cancer biomarker

BC detection in early stages of development is crucial for therapeutic success. Despite the ubiquitous occurrence of unmethylated DNA, methylated DNA can be specifically identified even at lower levels. It was shown that aberrant promoter methylation can be detected in Fine Needle Aspirates and primary tumors[61]. DNA methylation was shown to be a potential biomarker for early detection and prognosis of BC[62]. *RASSF1A*, *CCND2*, *APC* and *HIN1* gene promoter methylations were able to classify invasive carcinomas, fibroadenomas and normal tissues[63]. Specifically *RASSF1A* and *CCDND2* gene promoter methylation levels are significantly higher in ER-positive than ER-negative BC[64]. Hypermethylation of *PGR*, *CDH13*, *TIMP3*, *TFF1*, *ESR1*, *HSD17B4* and *BCL2* gene promoters were found to be negatively correlated with ER status, while *ESR1*, *PTGS2*, *TGFBR2* and *CDH12* gene promoter hypermethylations were correlated with PR status[65]. A study performed in untreated lymph-node negative HR-positive BC patients showed that *PITX2* gene promoter hypermethylation leads to poorer prognosis[66]. Hypomethylation of gene promoters

were also found to be associated with BC subtypes. *NAV1* and *PER1* gene promoters were hypomethylated significantly in ER-positive/PR-positive BC[67].

1.3 Liquid Biopsy and Circulating cell-free DNA

Carcinogenesis is a complex advancing process including multiple genetic and epigenetic modifications, and continuous monitoring of tumor-specific changes is therefore essential[68]. Biopsies aid researchers to predict progression and therapy response of a disease by revealing the genetic profile of the tumor[69]. However, biopsy cannot be performed routinely on patients since it is a highly invasive procedure. In particular, biopsies are not performable or repeatable in stage IV cancer patients, both practically and ethically[70]. Thus, biopsy can provide only temporary and limited data related with the tumor, and might not be able to represent tumor heterogeneity[71]. Biopsy is also a risky procedure that can increase the possibility of seeding and spreading of cancer cells to other sites[70]. Liquid biopsy, analysis of circulating cell-free nucleic acids (CNAs) in bloodstream, is a promising tool for early detection, minimal residual disease detection, prediction of therapy response and real time monitoring tumor progression[72]. Circulating cell-free DNA (cfDNA) was first mentioned in 1948 and defined as extracellular nucleic acids in human circulation by Mandel and Metais[73]. These extracellular molecules are present mostly in double-stranded form with a wide range of molecular weight[74,75] that are distinctly lower than genomic DNA. CfDNAs can be found as nucleoprotein complexes in circulation[76]. In healthy individuals, concentration of blood cfDNA can be measured as approximately 1ng/ml[77]. High levels of cfDNA were detected in systemic lupus erythematosus[78], rheumatoid arthritis[79] and other diseases. Most of the studies about cfDNA were performed using serum and plasma but other extracellular fluids have also been used (i.e., saliva[79], urine[80], pleural effusions[81], cerebrospinal fluid[82] etc.). In particular, serum-based studies have shown cfDNA's potential in diagnosis and prognosis[83–85] and in addition as a predictor of treatment-response in cancer[86]. Plasma-based studies have also shown cfDNA's utility in translational oncology[87,88].

1.3.1. Tumor origin of cfDNAs

The source of nucleic acids found in the circulation is thought to be cells that have undergone apoptosis[89] and necrosis[90]. Since a hallmark of apoptosis is DNA degradation, a ladder pattern of cfDNA in the plasma can be the evidence that apoptosis is one of the sources of the observed nucleic acids in extracellular fluids[91]. Additionally, in the tissue environment, digested DNA fragments can be detected due to the engulfment of necrotic cells by macrophages via phagocytosis[92]. However, the length of fragmented cfDNAs that are present in circulation due to necrosis was found to be longer than the apoptotic limit[93]. It was also reported that active nucleic acid secretion into the extracellular environment in the form of exosomes for intercellular communication may be one of the sources of cfDNA[94,95].

Blood cfDNA levels were shown to be elevated in patients with a malignant disease[96]. cfDNA concentrations can be affected by various cancer-dependent factors such as tumor size, location, and stage[97]. However, the effects of such factors differ between cancer types. In lung cancer, several studies have reported that cfDNA concentration is increased in advanced tumor stages[98], whereas another study reported that cfDNA concentration is independent of stage[99]. In BC, cfDNA concentrations were associated with tumor stage[83] and size[100,101], but not with hormone receptor status[100,101]. Other studies did not verify the association of cfDNA concentration with tumor stage, size or histological grades[102]. These contradictions can be also seen in studies where the correlation between cfDNA levels and metastasis status was investigated[103,104]. In cancer patients, the source of high levels of cfDNA levels has been found to be the primary tumor. Genomic content can be actively released from solid tumors as well as tumor cells (CTCs). CTCs, whose existence was first reported in 1869 by Thomas Ashworth, are small number of cells that are secreted to the bloodstream during EMT and establish micrometastasis in distal sites of the body[105]. Disseminated tumor cells (DCTs) are the subclass of CTCs that are capable of localizing in distant sites and progress towards metastasis[106]. In the bloodstream, CTCs release fragments of DNA due to their high turnover as a result of apoptosis or necrosis[107]. CTC and DCT levels have been found to be correlated with cfDNA levels in prostate cancer patients[108]. In gastrointestinal cancer patients, cfDNA levels were found be varied during and after therapy, which suggests a correlation between tumor load and cfDNA levels[109]. In BC patients, high

levels of methylated cfDNAs were found to be correlated with the presence of CTC[110]. Liver, kidney and spleen are the organs that are reported to be responsible for disappearance of CTCs and cfDNAs[111–114]. The cleaning process of cfDNAs from the circulation may be independent of their methylation status[115].

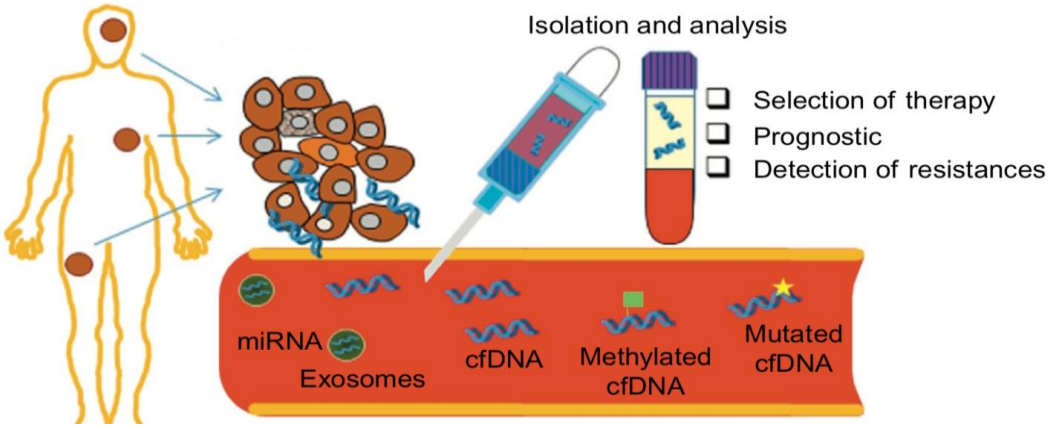

Figure 1.3: Origin of cfDNAs from primary tumors and CTCs.

cfDNAs display the tumor alterations including mutations and epigenetic alterations; they can be isolated from the blood in cancer patients and analyzed. Taken from: Estibaliz Alegre, Advances in Clinical Chemistry, Elsevier Books, 2015, 83, R47-R89, by permission of Elsevier[116].

1.3.2. Tumor-specific alterations of cfDNAs

Genetic and epigenetic profile (i.e. gene mutations, microsatellite alterations, methylation profile) of cfDNAs were shown to be similar with tumor's profile in cancer patients[117–119]. Detection of these characteristic alterations despite the presence of normal cfDNA molecules, as opposed to quantitative detection of cfDNA alone, can offer a diagnostic specificity.

An early study reported the similarity of tumor suppressor and oncogene mutations in primary tumor DNA and cfDNA in cancer patients[120]. Since the mutation frequency is high in many tumor types with their contribution to tumor progression, *TP53* and *KRAS* mutations are the most studied genes in various cancer types including breast[121], colon[122], lung[123], liver[124], bladder[125] etc. Mutations in the cfDNA *TP53* gene were reported to be positively correlated with tumor stage in colorectal[122]

and ovarian[126] cancers. *KRAS* mutation levels in plasma are correlated with the clinical outcome in metastatic colorectal cancer patients[127]. *APC*, a tumor suppressor that is frequently deregulated in early stages of various cancers[128], has been studied in cfDNA. In colorectal cancer, the serum *APC* mutation was found to be closely correlated with lymph node metastasis, tumor invasion depth and stage[129]. *BRAF*, *EGFR* and *MYC* are other frequently studied genes. In particular, *BRAF* gene mutation V600E has been detected in >70% of metastatic melanomas and advanced stages show higher mutation levels[130]. *EGFR* mutations were found to be associated with the clinical response of non-small cell lung cancer patients[131].

Necrotic and apoptotic sources result in different fragment lengths of cfDNA in circulation. Cells that have undergone necrosis release relatively longer fragments while apoptotic cells release shorter fragments. DNA integrity (cfDI), which is described as the ratio of longer to shorter fragments, is suggested for characterization of the cfDNA source in the circulation. Various gene fragments are suggested to be used for the calculation of cfDI including *ACTB*, *APP* and *ALU* elements with different long and short fragment lengths in different studies[132]. Their high stability benefits the usage of DNA integrity for diagnostic purposes. In patients with neoplastic diseases, higher ratio of longer fragments to shorter fragments was shown compared to non-neoplastic disease patients[133]. Similar results are obtained from different cancer types such as colorectal cancer[134], breast cancer[135], acute leukemia[136], melanoma[137]. However, there are studies showing opposite results in different cancer types[138–140].

Microsatellites, tandem repeated sequences, are scattered along the whole genome. Their alterations are frequently shown in various cancer types due to the malfunctioning DNA mismatch repair genes through mutations or epigenetic alterations, resulting in loss of microsatellite instability and loss of heterozygosity[141]. Alterations occurring at early stages of different cancer types can be interpreted as a potential biomarker for early diagnosis. Detection of microsatellite alterations in sera was shown to be helpful for the identification of head and neck squamous cell carcinoma patients[142]. However, there are studies reporting that microsatellite alterations in more advanced cancers decrease the sensitivity and specificity of the diagnosis. Microsatellite alterations are also considered as a biomarker for cancer prognosis and potential treatment response[143].

1.3.3. cfDNA as biomarkers and their clinical utility

Methylation signatures can be detected in extracellular body fluids in cancer[144]. It was shown that cfDNA presents an similar methylation pattern with genomic DNA isolated from tumor cells[145]. Thus, analyzing genetic and epigenetic characteristics of cfDNAs can offer a noninvasive procedure for the diagnosis and monitoring of patients. Tumor-specific DNA methylation analysis in cfDNAs has several advantages. Firstly, cfDNA can be amplified by PCR easily and with fewer false positive rates[146]. Additionally, DNA promoter methylation levels can be detected continuously without a signal loss while the levels remain stable during collection and transportation of samples[147]. Real-time PCR in gene-specific assays can be adapted easily for diagnostic purposes. Detection of hypermethylation of CpG islands is relatively easy, in contrast to genetic alterations in which it is scattered across the gene. However, only a fraction of DNA methylation markers have been identified. There are two technical challenges. Tumor-specific DNA methylation patterns are present at low levels, requiring a high signal-to-noise ratio for successful detection[148]. This challenge can be overcome by using methylation-specific primers or methylation-specific probes. The second challenge is the methylation detection of consecutive DNA sequences in individual molecules with single base-pair resolution. This challenge can be overcome by using methylation-independent PCR primers. cfDNA biomarkers are generally analyzed by conducting Receiver Operating Characteristics analysis (ROC), where diagnostic accuracy is determined by the identification of a threshold with the highest sensitivity and specificity values. Sensitivity is the true positive percentage and specificity is the true negative percentage, where the strongest biomarker would have sensitivity and specificity values above 75%. Area under ROC curve (AUC) is the strength of a parameter distinguishing between two groups. A very poor biomarker would have AUC under 0.5. As AUC gets close to 1, strength of the biomarker increases.

1.3.4. Technologies for DNA methylation biomarker discovery

Methylation signatures of cfDNAs have been investigated in various studies with different techniques (Table 1.2). Bisulfite conversion-based and affinity enrichment

techniques are the two main approaches. Affinity enrichment includes immunoprecipitation of methylated DNA (MeDIP) that uses antibodies specific to 5-methylcytosine[149]; methylated DNA fragments can thus be enriched compared to unmethylated DNA. Even though MeDIP offers reliable detection of qualitative methylation differences, it is unable to measure and differentiate single CpG methylation status[150]. Bisulfite conversion-based techniques are able to differentiate regions in heterogeneous tissues based on their methylation levels. One of the bisulfite conversion-based techniques is microarrays that enable detection of DNA methylation in population studies, which was also used in The Cancer Genome Atlas project[151]. Another technique is the direct sequencing of the bisulfite-converted DNA samples either with Direct Bisulfite Sequencing (BS) or with Next Generation Sequencing (NGS)[152]. NGS offers single base resolution with an unbiased genome-wide coverage. NGS requires relatively lower amounts of cfDNA (approx. 50-100 ng) compared to affinity enrichment techniques, indicating its high efficiency which is important for blood-based biomarker studies[153]. Methylation-specific PCR (MSP) is a qualitative method that targets a certain region by amplifying both unmethylated and methylated template by two sets of primers[154]. Then, PCR products can be analyzed by agarose gel electrophoresis. MSP is frequently combined with real-time PCR to quantify the ratio between methylated DNA and unmethylated DNA. This technique, Methylight, is highly specific, sensitive and efficient[155,156]. The Methylight technique, however, requires an additional dual-labeled fluorescent probe that differentially hybridizes to the target sequence based on the methylation status of target CpGs solely[157]. Clinical practices require rather simpler techniques for DNA methylation analyses. BS technique has been referred to as the "gold standard" of DNA methylation analysis due to its high fidelity that enables the analysis of methylation levels of each individual gene and individual cytosines[158]. The BS technique is accurate, quick, and relatively low-cost, and thus suitable for clinical sample analyses. The method involves bisulfite conversion of the sample, amplification of the target region and purification of the obtained product. At the end of the sequencing procedure, the chromatogram will display each nucleotide as peaks.

Table 1.2. Studies using different approaches for cancer diagnostic biomarkers.

Cancer	Study Design	Gene	Technique
Breast[159]	Control, 87; cancer, 101; 58 (metastatic)	*GSTP1, RASSF1A, RARβ2*	One-step MSP
Breast[160]	Control, 25; benign, 25; cancer, 25	*NBPF1*	BS
Breast[103]	Control, 14; cancer, 50	*RASSF1A, ATM*	Methylight
Breast[161]	Control, 67; cancer, 86	*EGFR, PPM1E*	BS
Ovarian[162]	Control, 80; benign, 43; cancer, 71	*OPCML*	Nested-MSP
Colorectal[163]	Control, 37; benign, 37; cancer, 47	*SFRP1, SFRP2, SDC2, PRIMA1*	Methylight
Colorectal[164]	Control, 40; cancer, 30	*MGMT*	MSP
Colorectal[165]	Control, 10; benign, 10; cancer, 51	*p16*	MeDIP
Lung[166]	Control, 20 (discovery), 188 (validation); cancer, 20 (discovery), 188 (validation)	*SHOX2*	QMSP
Prostate[167]	Benign, 10; cancer, 27	*ST6GALNAC3, HAPLN3, CCDC181*	Droplet digital MSP

1.3.5. TAGLN

The *TAGLN* gene (GenBank: NM_003186.3) is located on chromosome 11q23.3, and consists of 5 exons and 4 introns. There are two *TAGLN* transcript variants with different lengths, encoding the same protein. The encoded protein is also named as smooth muscle protein 22α, a 22kDa actin-binding protein that is highly expressed in smooth muscle cells, fibroblasts and cardiac tissues. The *TAGLN* sequence is conserved in zebrafish, mouse, rat, chicken, dog, cow, frog, and chimpanzee.

The TAGLN protein is comprised of the Calponin homology domain that enables actin binding, functions in stress fiber formation, and is correlated with smooth muscle motility and differentiation[168–171]. In embryogenesis, TAGLN is found to be an early marker for smooth muscle differentiation[172]. TAGLN is thought to have a negative role in the formation of podosomes, mediating tumor cell migration and tissue invasion[173]. TAGLN has been shown to be reduced in transformed invasive cells that generate tumors *in vivo*[174].In prostate cancer, overexpression of TAGLN has been found to repress androgen-triggered cell growth, and loss of the protein induces MMP-9 expression and tissue invasion[175,176]. Aikins et al. showed that TAGLN downregulation results in vasculogenic mimicry suppression via Interleukin-8 blockage in breast cancer cells[177]. TAGLN function was studied in different types of cancers, including breast, colorectal, prostate, lung, gastric, oral squamous cell, and esophageal squamous cell. The TAGLN protein was found to be downregulated in colorectal, breast, prostate cancer and gall bladder carcinoma[178–184]; and found to be upregulated in gastric, lung, esophageal squamous cell, oral squamous cell, pancreatic, and metastatic colon carcinoma in various studies[185–193]. The association of TAGLN expression level increase with EMT as well as cancer stem-like cells has been shown in some studies[194–196]. In addition, TAGLN has been identified as a direct target of TGF-β signaling through Smad3[197–201], and induces EMT[202–204]. The AKT and JNK pathways may alter TAGLN expression and induce the metastatic potential of colorectal cancer cell lines[186]. Some studies performed in breast cancer cell lines have shown that miR-145-stimulated TAGLN upregulation is associated with a decrease in cell motility[194,205]. However, a recent study identified TAGLN as a potential biomarker for diagnosis in TNBC[206].

TAGLN transcription has been shown to be regulated by promoter methylation in smooth muscle cells. High levels of *TAGLN* promoter methylation were found in hepatocellular and colorectal carcinoma cell lines, related with poorer prognosis of the patients[183,207,208]. Transcriptional control of *TAGLN* via chromatin structure regulation by the EZH2 polycomb repressor protein induced by IL-1β and TGFβ2 has been found in endothelial cells[209]. A recent study showed that *TAGLN* promoter was hypermethylated in 15 different breast carcinoma cell lines and its expression was downregulated as opposed to non-tumorigenic cell lines[210]. In the same study, hypermethylation of *TAGLN* promoter in 13 out of 21 and TAGLN downregulation in 19 out of 21 were shown when breast tumor tissues and paired healthy breast tissues were compared, strengthening the use of *TAGLN* promoter methylation levels in BC. Our groups' previous bioinformatic studies using two different datasets (GSE52621 and GSE58119) demonstrated that *TAGLN* promoter methylation levels could be detected from BC and normal sera[211]. Even though GSE52621 did not contain any normal sera samples, analyses using this dataset showed that BC sera possessed significantly higher *TAGLN* promoter methylation levels compared to paired healthy breast tissues. Combination of two datasets, normal sera samples from GSE58119 and BC sera from GSE52621, revealed that *TAGLN* promoter is highly methylated in BC sera. These studies indicate the potential diagnostic use of *TAGLN* promoter as a methylation biomarker in BC sera.

1.4 Aim of the study

Epigenetic alterations including upregulation of oncogenes and silencing of TSGs are frequently observed during cancer development. These genes have potential as a diagnostic or a prognostic biomarker. Discovering biomarkers are crucial for early diagnosis, prediction of therapy response, minimal residual disease detection and real-time monitoring of tumor progression. A successful biomarker should be easily identified with less invasive approaches including liquid biopsy. *TAGLN* is one of the recently identified genes that may have a role in cancer development due to its actin-binding function in healthy cells including regulating motility, differentiation and migration. In different cancer types, *TAGLN* has been shown to exhibit different characteristics either as an oncogene or a TSG. These controversial outcomes suggest *TAGLN* function should be further investigated. Our groups' previous studies

demonstrated a direct link between *TAGLN* promoter hypermethylation and TAGLN expression. Additionally, downregulation and promoter hypermethylation of *TAGLN* have been shown both in cancer cell lines as opposed to non-tumorigenic cell lines, and in breast tumor tissues as opposed to paired normal breast tissues. Moreover, bioinformatic studies have revealed that *TAGLN* promoter methylation could be detected from the serum, and TAGLN promoter was hypermethylated in BC sera compared to healthy sera in a different dataset. However, the ability to use TAGLN promoter hypermethylation as a diagnostic biomarker in BC sera was not evaluated.

The aim of this study was to identify the methylation status of *TAGLN* promoter in BC patients and healthy donor sera by bisulfite sequencing, and to determine the potential of *TAGLN* gene as a methylation biomarker that can be used in the diagnosis of BC. Moreover, we aimed to uncover the possible relationship between *TAGLN* promoter hypermethylation and BC patients' clinicopathological features in order to gain insight into roles of TAGLN in breast tumor pathogenesis.

Upon observation of elevated levels of total cfDNA in BC patients compared to healthy donors, we aimed to discover the associations between total cfDNA levels and clinicopathological parameters, as well as potential use of total cfDNA levels in order to differentiate BC patients from healthy donors.

Chapter 2. Materials and Methods

2.1 MATERIALS

2.1.1. Laboratory reagents and chemicals

Laboratory reagents and chemicals are routinely used general substances. Table 2.1 lists these substances that are used in DNA isolation, PCR, gel extraction etc. Catalog numbers and companies are provided.

Table 2.1: Chemicals, reagents, enzymes, and kits used for general laboratory purposes

Name	Catalog#	Company (Country)
Nuclease-Free Water	AM9938	Ambion
Quick-cfDNA™ Serum & Plasma Kit	D4076	Zymo Research (USA)
Quick-DNA™ Miniprep Plus Kit	D4068	Zymo Research (USA)
Carrier RNA	1081147	Qiagen (Germany)
Qubit® dsDNA HS Assay Kit	Q32854	Thermo-Fischer Scientific (USA)
EZ DNA Methylation-Lightning™ Kit	D5031	Zymo Research (USA)
ZymoTaq™ Premix	E2004	Zymo Research (USA)
Agarose	R500	Prona (Spain)
Ethidium Bromide	17898	Thermo Scientific (USA)
Gene Ruler 50 bp DNA Ladder	SM373	Thermo Scientific (USA)
QIAquick Gel Extraction Kit	28706	Qiagen (Germany)
6X DNA Loading Dye	R0611	Thermo Scientific (USA)

Table 2.1: Chemicals, reagents, enzymes, and kits used for general laboratory purposes

Name	Catalog#	Company (Country)
EpiTect Methylight PCR + ROX™ Vial Kit	59496	Qiagen (Germany)

2.1.2. Cell culture reagents and chemicals

Media, reagents and chemicals used in cell culture experiments in this study are listed in Table 2.2.

Table 2.2: Chemicals, reagents, kits and media used in cell culture

Name	Catalog #	Company (Country)
DMEM, Low Glucose	BE12-707F	Lonza
Fetal Bovine Serum	CH30160	Lonza
L-glutamine	BE17-605E	Lonza
Non-Essential Amino Acids	1114035	Thermo-Fischer Scientific (USA)
Penicillin/Streptomycin	DE17-602E	Lonza
Sodium Pyruvate (100mM)	BE13-115E	Lonza
PBS	17-516F	Lonza
Trypsin/EDTA (0.05%)	BE17-161E	Lonza
Dimethyl sulfoxide (DMSO)	A1584	Applichem (Germany)

2.1.3. PCR Primers

Primers that have been used in bisulfite sequencing have been shown in Table 2.3, including the amplicon size and optimized Tm values for the PCR annealing step. All primers were purchased from and synthesized by PRZ Biotech (Ankara). Primers were dissolved in TE upon arrival to be 100 µM and were kept at -20ºC**.**

Table 2.3: Primers used in this study

Primer Name	**Primer Sequence**	**Tm (°C)**	**Size (bp)**
TAGLN F	GGGGTTAGAGAATAGTGAAGTAGGAGTA	58	407
TAGLN R	ACACTCACAAAACTTCCTCAAAACT		
Nested TAGLN 1.1 F	GTTAGAGAATAGTGAAGTAGG	58	206
Nested TAGLN 1.2 R	CAATAACTCCACACAAACTCC		
Nested TAGLN 2.1 F	GGAGTTTGTGTGGAGTTATTG	60	217
Nested TAGLN 2.2 R	ACTCACAAAACTTCCTCAAAAC		

AGGATCTGCCACTTACCATTCACCATGTGGCCTTGAGGAAGACGCACT
CGGGGCCTCAGTTTCCTCATCTATAAAATGGGGATGTAATTACACCCTC
ACACTGTAGCTGTGAGTATTCAATGAGAGCACTGCAAAGGGCCTGGTG
TGGAGTAGGTCCTCAGGAAAGGTTGGATCCCATGTCCCATCAGAGCTA
AAAGCCCCAGGAGGAGAGGGTGGCTGGTTTGTCCCCACAAACCCCTGG
GATTCCCGGCTCCCCAGCCCCTTGCCCCTCTCTCCAGCCAGACTCTATTG
AACTCCCCCTCTTCTCAAACTCGGG**GCCAGAGAACAGTGAAGTAGG**A
GCAGCCGTAAGTCCGGGCAGGGTCCTGTCCATAAAAGGCTTTTCCCGG
GCCGGCTCCCCGCCGGCAGCGTGCCCCGCCCCGGCCCGCTCCATCTCCA
AAGCATGCAGAGAATGTCTCGGCAGCCCCGGTAGACTGCTCCAACTTG
GTGTCTTTCCCCAAATAT**GGAGCCTGTGTGGAGTCACTG**GGGGAGC
CGGGGGTGGGGAGCGGAGCCGGCTTCCTCTAGCAGGGAGGGGGCCGA
GGAGCGAGCCAGTGGGGGAGGCTGACATCACCACGGCGGCAGCCCTTT
AAACCCCTCACCCAGCCAGCGCCCCATCCTGTCTGTCCGAACCCAGACA
CAAGTCTTCACTCCTTCCTGCGA**GCCCTGAGGAAGCCTTGTGAGT**GC
ATTGGCTGGGGCTTGGAGGGAAGTTGGGCTGGAGCTGGACAGGAGCAG
TGGGTGCATTTCAGGCAGGCTCTCCTGAGGTCCCAGGCGCCAGCTCCAG
CTCCCTGGCTAGGGAAACCCACCCTCTCAGTCAGCATGGGGGCCCAAG
CTCCAGGCAGGGTGGGCTGGATCACTAGCGTCCTGGATCTCTCTCAGAC
TGGGCAGCCCCGGGCTCATTGAAATGCCCCGGATGACTTGGCTAGTGC
AGAGGAATTGATGGAAACCACCGGGGTGAGAGGGAGGCTCCCCATCTC
AGCCAGCCACATCCACAAGGTGTGTGTAAGGGTGCAGGCGCCGGCCGG

Figure 2.1: *TAGLN* promoter sequence and primers used in the nested-PCR method.

Outer primers are underlined. Nested primers spanning Section 1 (including 12 CpGs) and Section 2 (including 10 CpGs) regions are bold and underlined. CpG dinucleotides are highlighted in red. Amplified region contains total 22 CpG dinucleotides.

2.1.4. Equipment

Equipments used for experiments in this study is listed in Table 2.4.

Table 2.4: Equipment used for the experiment

Name of the instrument	Company (Country)
PCR Thermal Cycler	Applied Biosystems (USA)
Rotina 420R	Hettich (Germany)
NanoDrop ONE	Thermo Scientific (USA)
SC110 SpeedVac Concentrator	Thermo Scientific (USA)
Biofuge pico Centrifuge	Heraeus (Germany)
Chemi-Capt 2000	Vilber Lourmat (Germany)
Qubit Fluorometer	Thermo Scientific (USA)

2.2 Solutions and Media

2.2.1. Routinely used laboratory solutions

Frequently used solutions and buffers in the laboratory are listed in Table 2.5.

Table 2.5: Routinely used buffers and solutions

Buffer	For 100 ml aqueous solution (if not otherwise stated)
50X TAE	24.2 g Tris-base; 1.86 g EDTA; 5.71 ml glacial acetic acid
6X Loading Dye	0.012 g Bromophenol blue; 0.012 g Xylene cyanol; 8ml 0.5M EDTA; 80ml glycerol
3M CH_3COONa	24.6 g $NaAc.3H_2O$

2.2.2. Cell culture solutions and media

Cell lines and their appropriate media that were used in this study are listed in Table 2.6.

Table 2.6: Cell lines and their growth media

Cell Line	Medium
MCF7	Basic DMEM
MDA-MB-231	Basic DMEM

Cell lines were purchased from ATCC. Basic Media consists of; 10% FBS; 1% Non-essential amino acids; 1% L-Glutamine; 1% Penicillin/Streptomycin.

2.2.3. Patient materials

This study includes analyses of total cfDNA levels from 67 BC patients and 135 healthy donor sera samples collected before surgery and therapy. The clinicopathological characteristics of these patient samples are listed below.

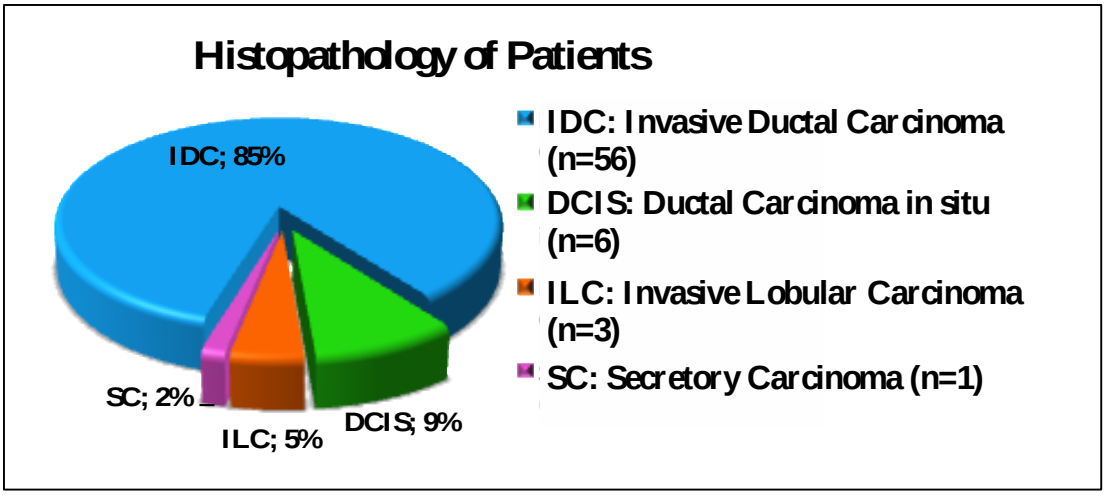

Figure 2.2: Histopathological characteristics of BC patients in this study.

The pie chart graph displays the percentage of histopathological subtypes of BC patients. IDC, DCIS, ILC and Secretory carcinoma (SC) percentages were shown in blue, green, orange and pink respectively. Sample sizes are 56 for IDC, 6 for DCIS, 3 for ILC and 1 for SC. One patient's pathological information was missing.

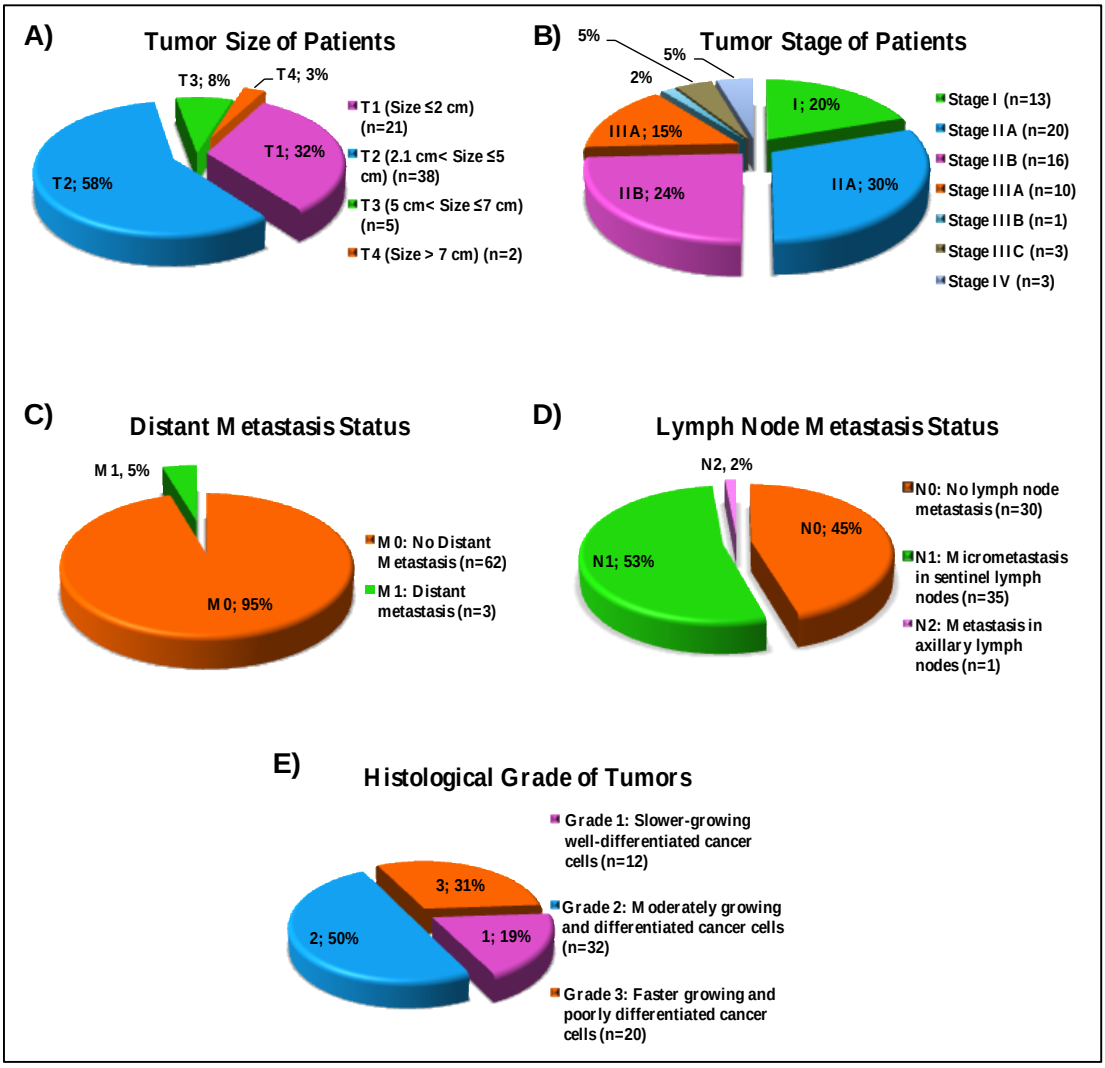

Figure 2.3: Clinical characteristics of breast cancer patients.

The pie chart graphs display the percentages of clinical subtypes of BC patients. A) Tumor size T1 (n=21), T2 (n=38), T3 (n=5) and T4 (n=2) were shown in pink, blue, green and orange respectively. B) Tumor stage I (n=13), IIA (n=20), IIB (n=16), IIIA (n=10), IIIB (n=1), IIIC (n=3) and IV (n=3) were shown in green, blue, pink, orange, light blue, brown and purple respectively. C) Distant metastasis status M0 (n=62) and M1 (n=3) were shown in orange and green, respectively. D) Lymph node metastasis status N0 (n=30), N1 (n=35) and N2 (n=1) were shown in orange and green respectively. E) Grade status 1 (n=12), 2 (n=32) and 3 (n=20) were shown in pink, blue and orange respectively. Two patients' histological grade information was missing.

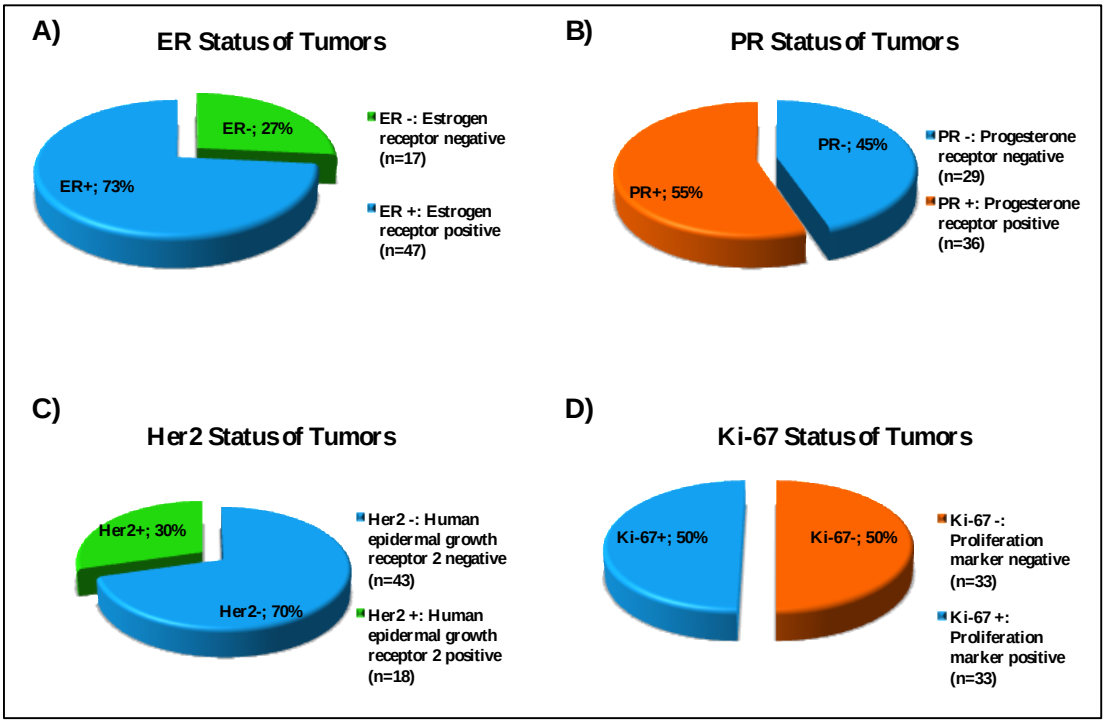

Figure 2.4: Molecular characteristics of BC patients.

The pie chart graphs display the percentages of molecular characteristics of BC patients. A) Estrogen receptor status ER-negative (ER-, n=17) and ER-positive (ER+, n=47) were shown in green and blue respectively. Two patients' ER status was missing. B) Progesterone receptor status: PR-negative (PR-, n=29) and PR-positive (PR+, n=36) were shown in blue and orange respectively. PR status of one patient was missing. C) Human epidermal growth receptor 2 status: Her2-negative (Her2-, n=43) and Her2-positive (Her2+, n=18) were shown in blue and green respectively. Five patient information of Her2 status was missing. D) Ki-67 proliferation marker status: Ki-67-negative (n=33) and Ki-67-positive (n=33) were shown in orange and blue respectively.

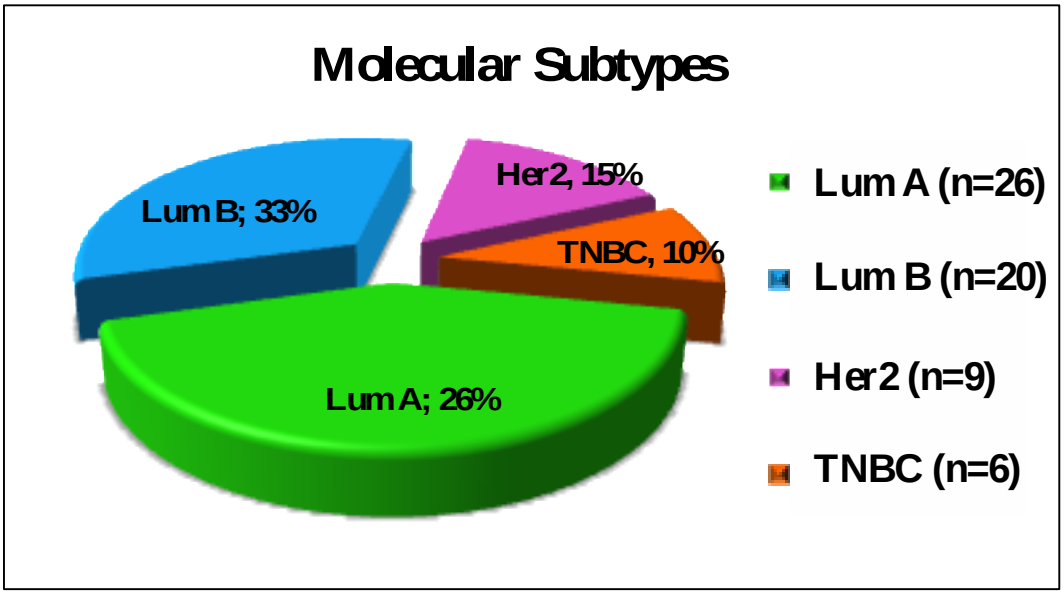

Figure 2.5: Molecular subtypes of BC patients in this study.

Percentages of each molecular subtype are shown with pie chart graphs. Lum A (n=25), Lum B (n=20), Her2 (n=9) and TNBC (n=6) were shown in green, blue, pink and orange, respectively. Five patients' Her2 status information was missing, these patients' information were not used for molecular characterization and statistical tests.

Epigenetic studies were conducted using 33 BC patient sera samples and 58 healthy donor sera samples. Clinicopathological characteristics of samples are listed in Table 2.7.

Table 2.7: Clinicopathological characteristics of BC patient samples used in epigenetic analyses

Patients	Age	ER	PR	Her2	Diagnosis	LNM	Grade	Size	Stage
P#003	36	+	+	-	IDC	N2	3	T2	3A
P#004	76	-	-	+	IDC	N0	3	T2	2A
P#005	43	+	+	-	IDC	N1	2	T2	2B
P#006	45	+	-	-	ILC	N2	2	T2	3A
P#007	61	+	+	-	IDC	N1	2	T1	2A
P#009	63	-	-	+	IDC	N1	2	T2	2B
P#010	53	NA	-	NA	IDC	N0	3	T2	2A
P#011	44	NA	-	NA	IDC	N0	3	T1	1
P#012	62	+	-	-	DCIS	N0	2	T1	1
P#013	40	-	-	NA	DCIS	N0	3	T1	1
P#014	36	+	+	+	IDC	N0	3	T2	2A
P#015	58	+	-	-	IDC	N0	2	T2	2A
P#016	58	+	+	+	IDC	N1	2	T1	2A
P#017	51	+	-	-	DCIS	N0	NA	T1	1
P#018	60	+	+	-	IDC	N0	2	T1	1A
P#022	41	+	+	+	IDC	N3	2	T2	3C
P#023	55	+	+	-	IDC	N2	2	T2	3A
P#024	46	+	+	-	IDC	N1	3	T1	2A
P#027	57	-	-	-	IDC	N0	3	T1	1
P#028	69	+	+	NA	IDC	N1	2	T2	2B
P#029	33	+	+	-	IDC	N1	1	T3	3A
P#030	82	-	-	-	IDC	N0	2	T2	2A
P#031	64	+	-	-	IDC	N1	2	T1	2A
P#032	46	+	+	-	IDC	N0	2	T1	1
P#038	43	+	+	-	ILC	N1	2	T2	2B
P#040	65	+	+	-	IDC	N2	2	T2	3A
P#041	68	+	+	-	IDC	N1	3	T2	2B
P#044	38	-	-	+	IDC	N1	3	T3	3A
P#045	71	+	+	-	IDC	N0	2	T2	2A
P#048	42	-	-	-	IDC	N0	3	T2	2A
P#050	47	+	+	-	IDC	N1	3	T2	2B
P#061	69	+	-	-	IDC	N1	2	T2	2B
P#065	49	-	+	-	IDC	N0	1	T1	1

ER: Estrogen receptor status. PR: Progesterone receptor status. Her2: Human epidermal growth receptor 2 status. IDC: Invasive ductal carcinoma, DCIS: Ductal carcinoma in situ. LNM: Lymph node metastasis status. NA: Not Assigned.

2.3 METHODS

2.3.1. General Maintenance of Human Cell Lines

Cells were stored in liquid nitrogen tanks in screw capped sterile cryovial tubes. In order to culture the frozen cell lines; the vial was placed in a 37°C water bath until a small ice ball was left and then transferred to the 37°C proper growth medium. Next, the mixture was centrifuged at 1500 rpm for five minutes at room temperature (RT). The supernatant was removed and cells were washed with growth medium and placed in a T25 tissue culture flask. Cells were maintained in incubators at a 5% CO_2 level and 37°C temperature. Cells were maintained approximately every two days depending on the cell type. When the cells required splitting or detachment, the cells were washed with sterile 1X PBS after the removal of the growth medium. Then, the cells were incubated with 0.05% Trypsin/EDTA for 5 minutes. When cells were detached from the flask, cells were collected and centrifuged at 1500 rpm at RT for 5 minutes. The supernatant was removed and cells were solved in new growth medium and transferred into a new flask. When the cell pellets required collection after the centrifugation step described above, the supernatant was removed and cells were washed with 1X ice-cold PBS and centrifuged at 1500 rpm at +4°C for 5 minutes. After supernatant removal, pellets were snap frozen and stored at -80°C. All cell culture procedures were performed in a sterile environment with sterile technique under cell culture flow hoods.

2.3.2. DNA Isolation from Breast Cancer Cell Lines

Cell lines that were grown until their confluence reached 70-80% were harvested by scraping. DNA isolation was performed with the Quick-DNA Universal DNA Isolation kit as directed by the manufacturer with elution in the kit's elution buffer. Isolated DNAs were quantified by the NanoDrop ONE spectrophotometer using 1.5 µl of DNA sample. DNA samples were kept at -20°C.

2.3.3. Collection and Preparation of Serum Samples

Blood samples from BC patients were collected from Ankara Numune Research and Teaching Hospital. Blood samples from healthy donors were also collected from

Ankara Numune Research and Teaching Hospital. The Research and Ethics Committee of Ankara Numune Research and Teaching Hospital approved the use of the collected clinical blood samples and patient consent was obtained in agreement with the Helsinki Declaration.

Blood samples from BC patients were collected before surgery, radiotherapy or chemotherapy in BD Vacutainer serum SST tubes. The samples were kept at +4°C degrees until processed. Blood samples were then centrifuged at 1400 g at +4°C for 10 minutes, and then aliquoted in 1 ml amounts and immediately snap frozen. Serum samples were stored at -80°C until cfDNA isolation.

2.3.4. Circulating Cell-free DNA Isolation from serum

Circulating cell-free DNAs were isolated from 3 ml serum with the Quick-cfDNA Serum & Plasma Kit according to the manufacturer instructions, with the following modification: 1 µg carrier RNA addition for every 1 ml serum. Columns were eluted in two steps with 40 µl 60°C pre-warmed DNA Elution Buffer. Columns were incubated with 20 µl elution buffer for 5 minutes and then centrifuged at RT at 13000 rpm; incubation was then performed with the remaining 20 µl elution buffer for 3 minutes and the samples were centrifuged at RT at 13000 rpm. Isolated DNA samples were quantified by Qubit® 1.0 (ThermoFisher) according to the manufacturer's instructions. Isolated DNAs were stored at -20°C.

2.3.5. Bisulfite modification of DNA and two step nested-PCR

0.18 µg of BC cell line genomic DNA and cfDNA was used for bisulfite modification; conversion of unmethylated cytosine residues to uracil was performed with EZ DNA Methylation-Lightning Kit (Zymo Research) following the user protocol. Samples with lower cfDNA levels (<0.18 µg) were used for bisulfite modification. Bisulfite-modified DNAs were eluted in 15 µl of the kit's elution buffer. Primers for PCR of bisulfite-converted cfDNA (BSP) were designed for bisulfite sequencing (Table 2.3) using the Methyl Primer Express v1.0 software (Applied Biosystems). The bisulfite-modified MCF7 cell line DNA was used as a positive control. 2 µl of Bisulfite-converted DNA was amplified with ZymoTaq Premix (Zymo Research, USA) using bisulfite DNA specific primers targeting the

TAGLN promoter (Table 2.3). The TAGLN BSP PCR reaction mixture was prepared as follows:

ZymoTaq Premix (2X)	12.5 µl
TAGLN BSP Forward Primer (10mM)	1 µl
TAGLN BSP Reverse Primer (10mM)	1 µl
Bisulfite-converted DNA	2 µl
ddH_2O	8.5 µl

Thermal cycler conditions for the TAGLN BSP primers were as follows: initial denaturation at 95°C for 10 minutes, 45 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 58°C) and extension (30 seconds at 72°C). The PCR was finished with a final extension at 72°C for 7 minutes. Amplified PCR product was diluted 1:50 in nuclease-free water and 2 µl of the diluted PCR product was used as a template for the second PCR amplification. Thermal cycler conditions for the second amplification were as follows: initial denaturation at 95°C for 10 minutes, 45 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 58°C for Section 1 and 60°C for Section 2) and extension (30 seconds at 72°C). PCR was finished with a final extension at 72°C for 7 minutes. After BSP nested-PCR, 5 µl of 6X Loading Dye (Thermo Scientific) was added to the PCR products and all samples were loaded into 1% agarose gel. The agarose gel was visualized with the Chemi-Capt 2000 image capturing system.

2.3.6. Gel extraction and sequencing of the bisulfite samples

TAGLN BSP nested-PCR product bands at correct size (206bp for Section 1, 217bp for Section 2) were excised from the gel. PCR products were extracted from the gel with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. PCR products were eluted in 40 µl pre-warmed 60°C kit's elution buffer. Purified products were sequenced with TAGLN BSP primers for each section using the dideoxy chain termination method (by PRZ Biotech Company, Turkey).

2.3.7. Statistical Analysis of Methylation Data

Raw bisulfite sequencing data were aligned with reference genomic data of the amplified region between -286 to -80 bp for Section 1 and -102 to +115 bp for Section 2 with respect to TSS with the Quantification of Methylation Analysis (QUMA) tool[212]. The QUMA tool calculates the bisulfite conversion efficiency by analyzing the unconverted cytosine residues in non-CG sites within the sequence. Samples from BC patients and healthy donors were displayed as lollipop graphs. The Mann-Whitney U test was used to test unpaired methylation differences between BC patients and healthy donor sera.

2.3.8. Correlation analyses in patient and healthy samples

Methylation levels (%) and cfDNA levels of the BC patient and healthy samples were used to test the association with the following patient characteristics: age, pathology, molecular subtypes, tumor stage, histological grade, tumor size, lymph node and metastasis status. For patient and healthy samples, associations were tested with Spearman correlation, and difference between groups was tested with Mann-Whitney U test.

Chapter 3. Results

3.1 Categorization of female breast cancer patients

Epigenetic analyses were conducted with 33 out of 67 BC patient sera and 58 out of 135 healthy donor sera. Serum samples with pathological and clinical results were used to categorize the BC patients into different histopathological, clinical and molecular groups (Table 2.7).

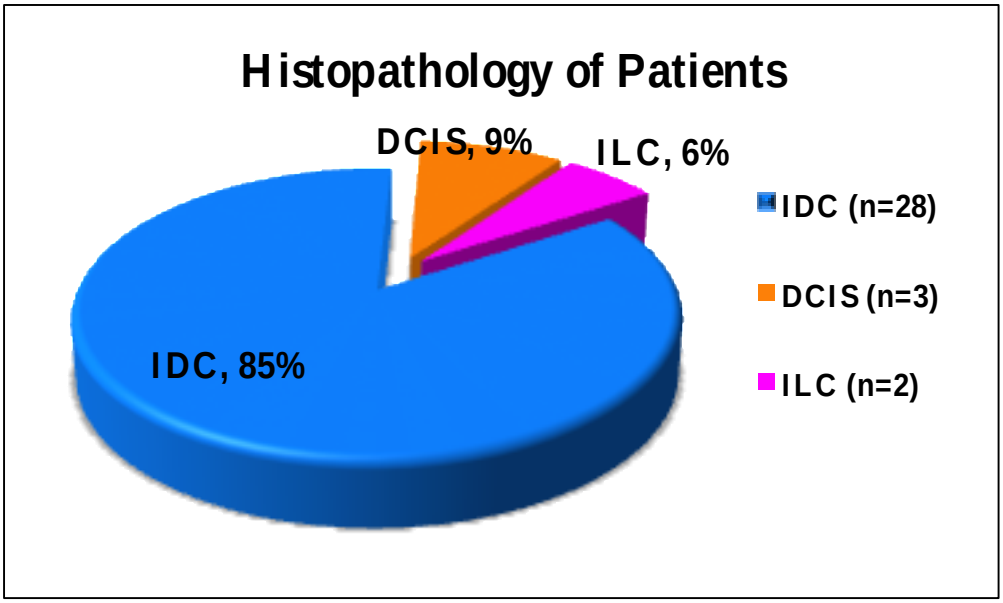

Figure 3.1: Histopathological characteristics of BC patients in this study.

The pie chart graph displays the percentage of histopathological subtypes of BC patients. IDC, DCIS, and ILC percentages were shown in blue, orange and pink. Sample sizes are 28 for IDC, 3 for DCIS and 2 for ILC.

Histopathological characteristics of the tumors of BC patients were determined based on the infiltration of cancer cells to fatty tissues of the breast (Figure 3.1). IDC tumors, in which cancer cells in milk ducts have invaded the surrounding breast tissue, correspond to 85% of the patient cohort. DCIS tumors, which are contained in the milk ducts, correspond to 9% of the patient cohort. ILC tumors, in which cancer cells in lobules have started to invade the breast tissue, correspond to 6%.

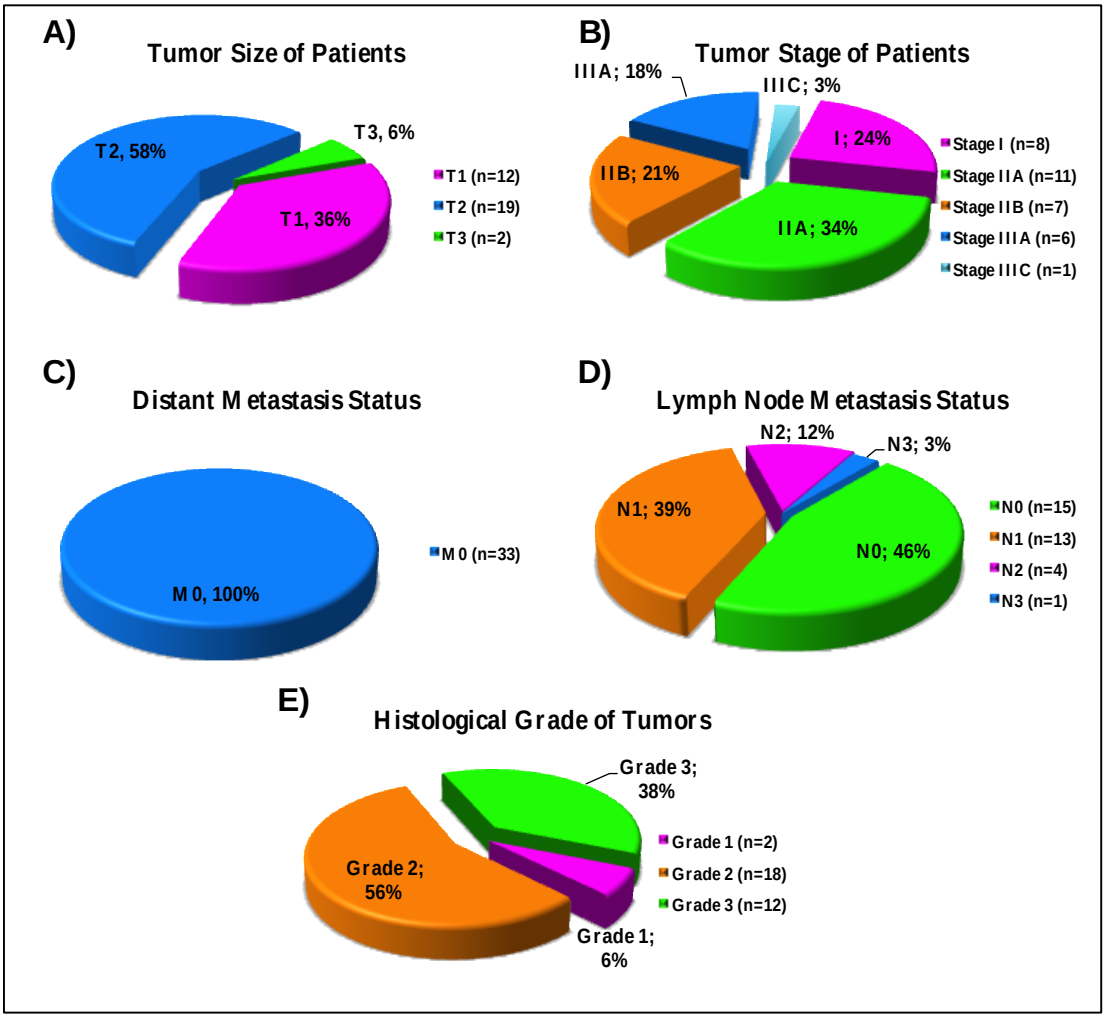

Figure 3.2: Clinical characteristics of breast cancer patients.

The pie chart graphs display the percentages of the clinical subtypes of BC patients. A) Tumor size T1 (n=12), T2 (n=19) and T3 (n=2) were shown in pink, blue and green respectively. B) Tumor stage I (n=8), IIA (n=11), IIB (n=7), IIIA (n=6) and IIIC (n=1), were shown in pink, green, orange, blue and light blue respectively. C) Distant metastasis (M0, n=33) was shown in blue. D) Lymph node metastasis status N0 (n=15), N1 (n=13), N2 (n=4) and N3 (n=1) were shown in green, orange, pink and blue respectively. E) Grade 1 (n=2), 2 (n=18) and 3 (n=12) were shown in pink, orange and green respectively.

Clinical characteristics of the BC patients were determined based on the tumor size, stage, grade, distant metastasis status and the lymph node metastasis status (Figure 3.2). T1 (tumor size $\leq$ 2 cm) corresponds to 36%, T2 (2 cm < tumor size $\leq$ 5 cm) corresponds to 58% and T3 (5 cm < tumor size $\leq$ 7 cm) corresponds to 6% (Figure

3.2A). Stage I corresponds to 24%, Stage IIA to 34%, Stage IIB to 21%, Stage IIIA to 18% and Stage IIIC to 3% respectively (Figure 3.2B). None of the BC patients in the cohort have distant metastasis in secondary locations (Figure 3.2C). 46% of the patients do not have lymph node metastasis, whereas 39% of the patients have micrometastasis in their sentinel lymph nodes (Figure 3.2D). 12% of patients have metastasis in 4-9 axillary lymph nodes and 3% of patients have metastasis in 10 or more axillary lymph nodes. Grade 1 (well-differentiated cancer cells with slow growing power) corresponds to 6%, Grade 2 (moderate differentiation and growth rate) corresponds to 56% and Grade 3 (poorly-differentiated cancer cells with faster growth rate) corresponds to 38% of the cohort (Figure 3.2E). One of the BC patients' tumor grade information was missing and this patient was therefore not included in the grade correlation statistical tests.

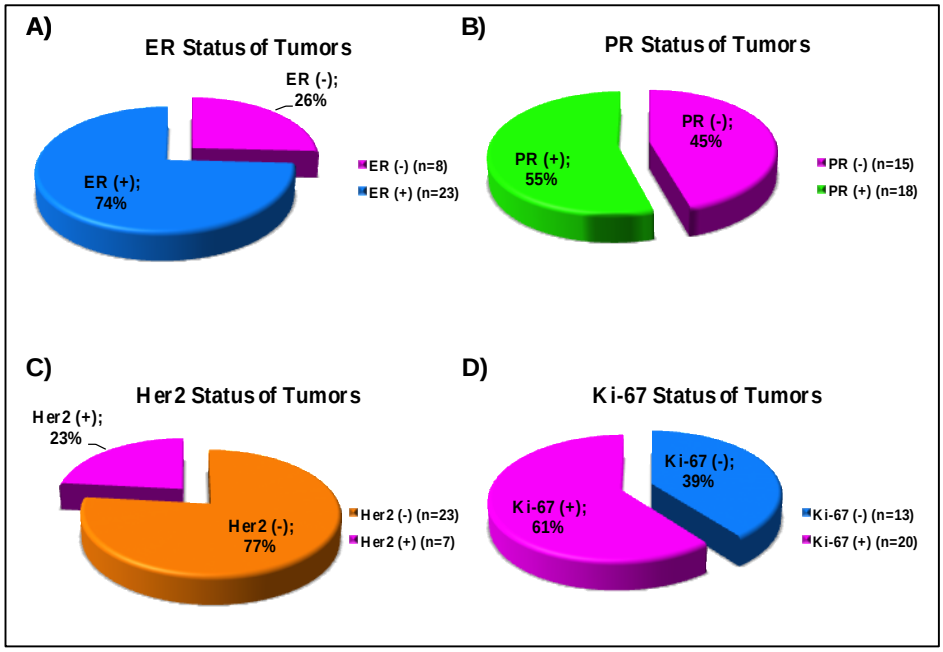

Figure 3.3: Molecular characteristics of BC patients.

The pie chart graphs display the percentages of molecular characteristics of BC patients. A) Estrogen receptor status: ER-negative (ER-, n=8) and ER-positive (ER+, n=23) were shown in pink and blue respectively. B) Progesterone receptor status: PR-negative (PR-, n=15) and PR-positive (PR+, n=18) were shown in pink and green respectively. C) Human epidermal growth receptor 2 status: Her2-negative (Her2-, n=23) and Her2-positive (Her2+, n=7) were shown in orange and pink respectively. D) Ki-67 proliferation marker status: Ki-67-negative (n=13) and Ki-67-positive (n=20) were shown in blue and pink respectively.

Molecular characteristics and subtypes of tumors were determined by IHC based on hormone receptor (ER, PR, Her2) and proliferation marker Ki-67 expressions (Figure 3.3). 26% of BC patients in this cohort were ER-negative whereas 74% were ER-positive (Figure 3.3A). Two patients' ER information was missing and therefore these samples were not included in the statistical tests. 45% of patients were PR-negative whereas 55% were PR-positive (Figure 3.3B). Patients who were Her2-negative correspond to 77% whereas Her2-positive patients correspond to 23% of the cohort (Figure 3.3C). Three patients' Her2 information was missing and these samples were not included in the statistical tests. Ki-67 was found to be negative in 39% and positive in 61% of the cohort (Figure 3.3D).

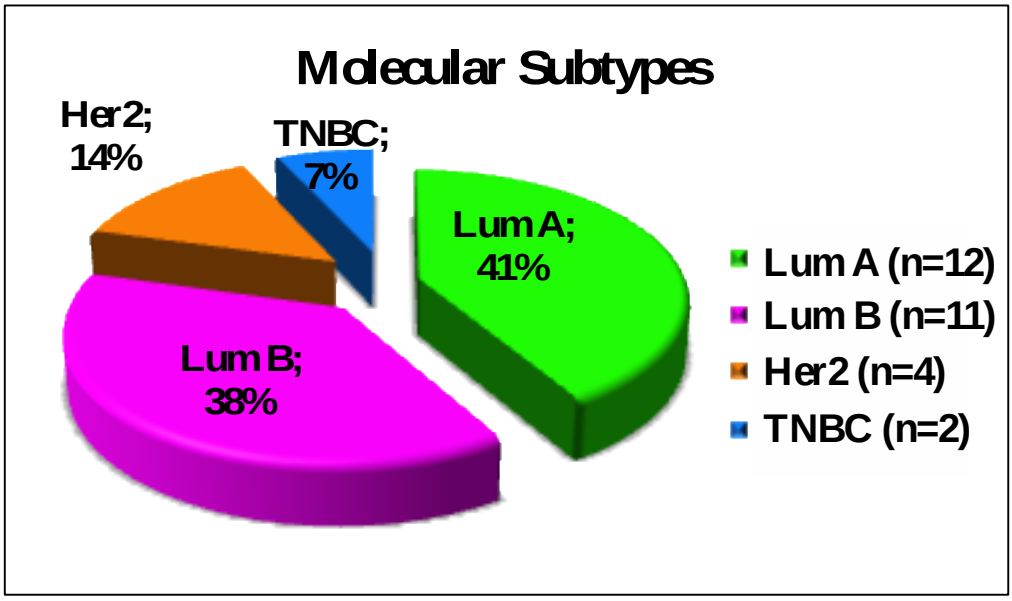

Figure 3.4: Molecular subtypes of BC patients in this study.

Percentages of each molecular subtype are shown with pie chart graphs. Lum A (n=12), Lum B (n=11), Her2 (n=4) and TNBC (n=2) were shown in green, pink, orange and blue, respectively.

In the 33 BC patient samples, patients who were Lum A corresponded to 41% of the study cohort (Figure 3.4), 38% of patients were found to be Lum B, 14% to be Her2-enriched, and 7% to be TNBC. ER status of two BC patients and Her2 status of three patients were missing due to technical problems at the pathology department of the hospital and these patients were therefore not used for molecular categorization of the tumors.

3.2 Correlation of *TAGLN* promoter methylation to clinicopathological parameters in serum samples

The demonstration of hypermethylation of *TAGLN* promoter region in breast cancer cell lines and breast tumor tissues compared to non-tumorigenic cell lines and normal tissues[210] indicated potential characterization of the *TAGLN* gene as a tumor suppressor in BC. In order to assess whether *TAGLN* hypermethylation is also present in cfDNA originating from the primary tumor in BC patients, *TAGLN* hypermethylation levels were compared between 58 healthy donors and 33 BC patients (Table 2.7). The average age of each group was calculated as 45.5 and 53 years for healthy donors and BC patients, respectively (Figure 3.5).

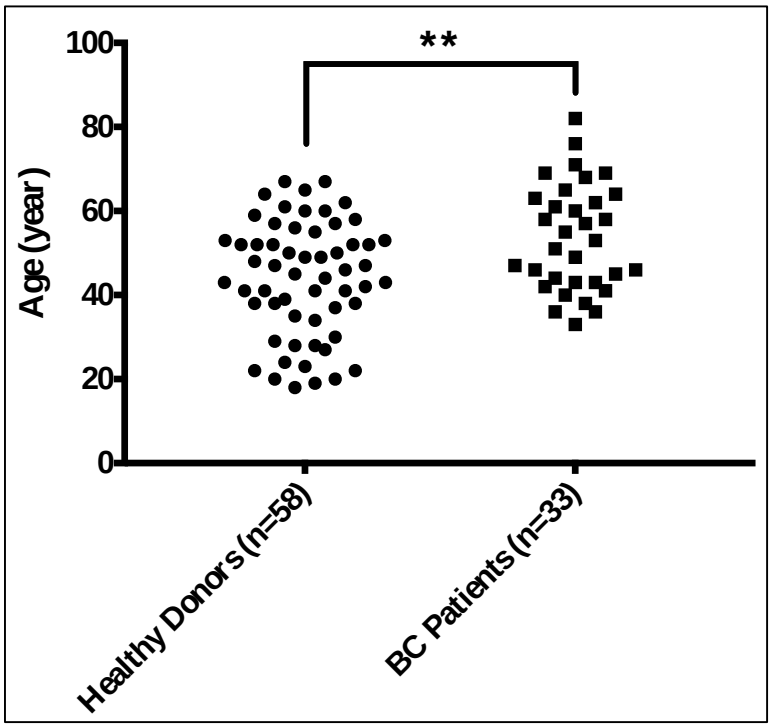

Figure 3.5: Age status of healthy donors and BC patients used for *TAGLN* promoter methylation study.

*Scatter dot plots showing the average age of healthy donors (n=58) and BC patients (n=33) are 45.5 and 53, respectively. **P < 0.001. Mann Whitney test, Whiskers show 5-95 percentiles.*

cfDNAs, isolated from sera samples of healthy donors and BC patients, underwent bisulfite treatment where unmethylated cytosine residues are converted into uracil, whereas methylated cytosine residues are protected. Then, nested-PCR primers targeting non-CpG sites and spanning 206 bp (Section 1) and 217 bp (Section 2) regions in *TAGLN* gene promoter including 22 CpG dinucleotides (Figure 3.6A) were used to amplify bisulfite treated cfDNA from 33 BC patients and 58 healthy donors. PCR products were then sequenced using the nested-PCR primer pairs for each section. Finally, obtained raw data was aligned and analyzed using the QUMA[212]. Average *TAGLN* promoter methylation was found to be significantly higher in BC patient sera (77.3%) compared to healthy donor sera (68.2%, P = 0.0023, (Figure 3.6B). *TAGLN* promoter methylation levels were found to be higher than the average *TAGLN* promoter methylation levels of healthy donors in 22 out of 33 (66.6%) BC patients.

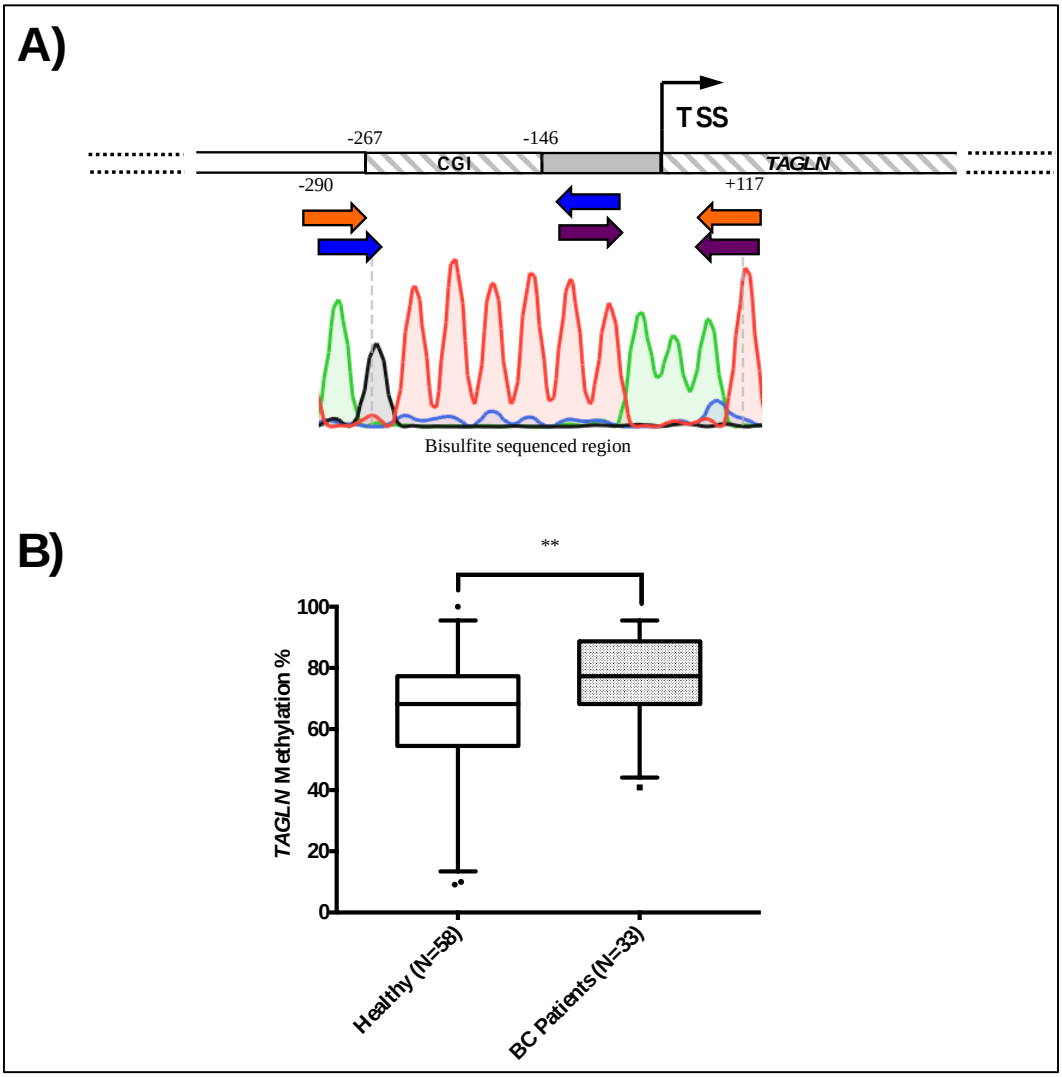

Figure 3.6: Methylation of the *TAGLN* promoter region in healthy donors and BC patients.

*A) Schematic representation of nested-PCR primers targeting a total of 22 CpGs. Outer primer, nested-PCR primers for Section 1 and nested-PCR primers for Section 2 were shown in orange, blue and purple, respectively. TSS: Transcription start site. CGI: CpG island. B) Box-plots showing significantly higher TAGLN promoter methylation in BC patient sera (n=33) compared to healthy donor sera (n=58). ** P < 0.01, Mann-Whitney test. Whiskers show 5-95 percentiles*

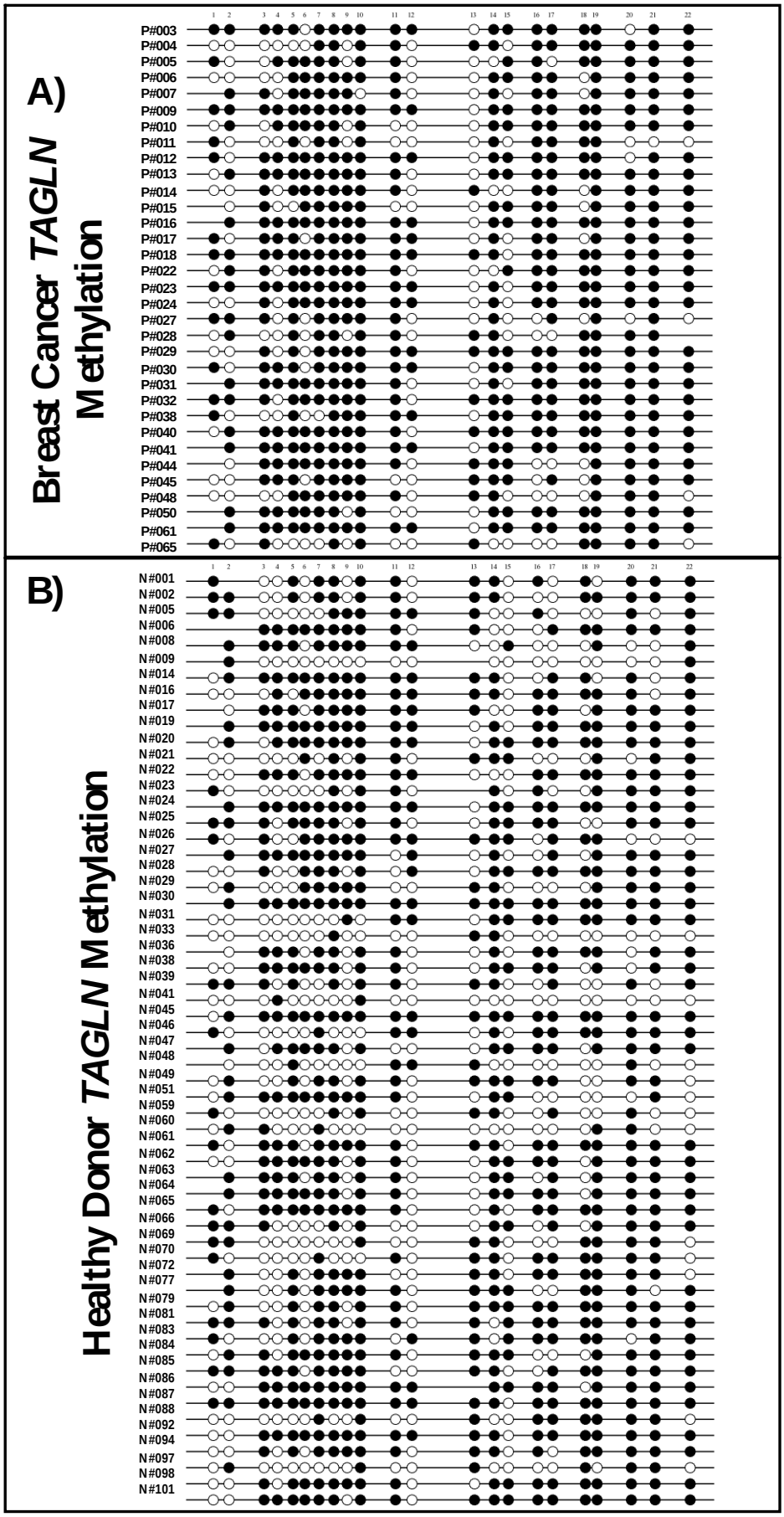
A)
Breast Cancer TAGLN Methylation
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
P#003
P#004
P#005
P#006
P#007
P#009
P#010
P#011
P#012
P#013
P#014
P#015
P#016
P#017
P#018
P#022
P#023
P#024
P#027
P#028
P#029
P#030
P#031
P#032
P#038
P#040
P#041
P#044
P#045
P#048
P#050
P#061
P#065
B)
Healthy Donor TAGLN Methylation
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
N#001
N#002
N#005
N#006
N#008
N#009
N#014
N#016
N#017
N#019
N#020
N#021
N#022
N#023
N#024
N#025
N#026
N#027
N#028
N#029
N#030
N#031
N#033
N#036
N#038
N#039
N#041
N#045
N#046
N#047
N#048
N#049
N#051
N#059
N#060
N#061
N#062
N#063
N#064
N#065
N#066
N#069
N#070
N#072
N#077
N#079
N#081
N#083
N#084
N#085
N#086
N#087
N#088
N#092
N#094
N#097
N#098
N#101

Figure 3.7: Overall *TAGLN* methylation in healthy donor and BC patient sera.

TAGLN was hypermethylated in the A) 33 BC patients' sera compared to B) 58 healthy donors' sera (P = 0.0053) analyzed using QUMA. Mann-Whitney test. Empty circles: Unmethylated CpGs, Full circles: Methylated CpGs. Numbers above each circle represents the order of the CpG in the promoter region.

TAGLN promoter methylation maps of 22 CpGs both in BC patients and healthy donors were created using the QUMA tool (Figure 3.7). CpG methylation states in BC patients and healthy donors were analyzed using Fisher's exact test. Significantly methylated CpGs were indicated with "*". Statistical analysis revealed significant methylation differences in certain CpG loci (Figure 3.8). Seven CpGs including CpG5, CpG8, CpG9, CpG13, CpG16, CpG19 and CpG21 were found to be significantly differentially methylated between BC patients and healthy donors. Interestingly, CpG13 was significantly less methylated in BC patients. CpG1, CpG2, CpG3, CpG4, CpG6, CpG7, CpG10, CpG11, CpG12, CpG14, CpG15, CpG17, CpG18, CpG20, and CpG22 were not significantly methylated in either group.

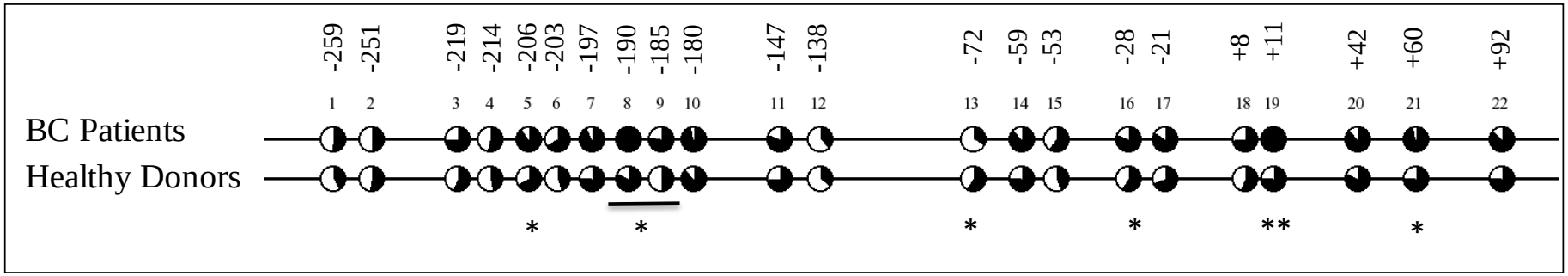

Figure 3.8: *TAGLN* methylation in BC patients and healthy donors

Methylation levels of every CpG in the sera of BC patients (n=33) and healthy donors (n=58) were compared in order to discover differentially methylated CpGs. Methylation percentage of every CpG was represented as a pie chart. Methylation levels were analyzed with the QUMA tool. Fisher's exact test was used. Methylation significance was designated with "". Empty circles: Unmethylated CpGs, Full circles: Methylated CpGs. Numbers above every circle indicate the location of each CpG related to the TSS.*

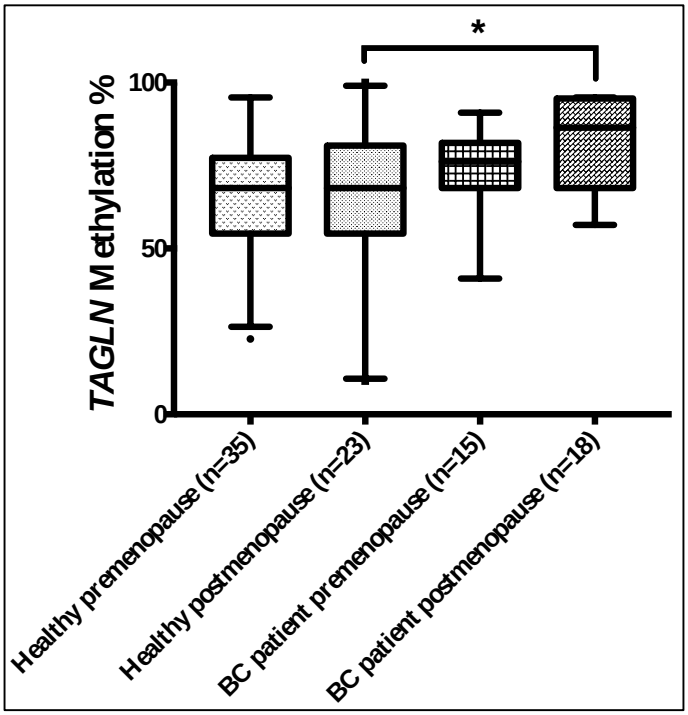

Figure 3.9: Evaluation of *TAGLN* promoter methylation levels in different age groups.

*Comparison of TAGLN promoter methylation levels in healthy donor age groups (Age≤50 n=35, Age>50, n=23) and BC patient samples (Age≤50 n=15, Age>50 n=18). *P < 0.05. Mann-Whitney test. Whiskers show 5-95 percentiles*

cfDNA *TAGLN* promoter methylation levels were tested for their difference in various clinicopathological groups including age, molecular subtypes, tumor size, stage and grade. In order to examine whether *TAGLN* promoter methylation differs among pre- and post-menopausal status, *TAGLN* promoter methylation levels were compared within different age groups in BC patients and healthy donors. 50 years of age is the common age for menopause[213], and thus 50 years was used as a threshold for pre- and post-menopausal status of healthy donors and BC patients[213]. There was no significant difference in *TAGLN* promoter methylation levels in pre- (68.2% methylation) and post-menopausal (68.2% methylation) healthy donors (Figure 3.9, *P*=0.7017). Among BC patients, older patients (n=18) (86.4% methylation) showed higher methylation status of *TAGLN* promoter region compared to younger patients' (n=15) (76.2% methylation) levels (*P* = 0.0687). BC patients over 50 years of age displayed significantly higher *TAGLN* promoter methylation levels than healthy

donors over 50 years of age (*P = 0.0211)*. In addition, the correlation between sera *TAGLN* promoter methylation levels and the individual's age was not statistically significant in healthy donors (Figure 3.10A, *Spearman r=0.1474, P = 0.2695*) or in BC patients (Figure 3.10B, *Spearman r = -0.2009, P = 0.2623)*.

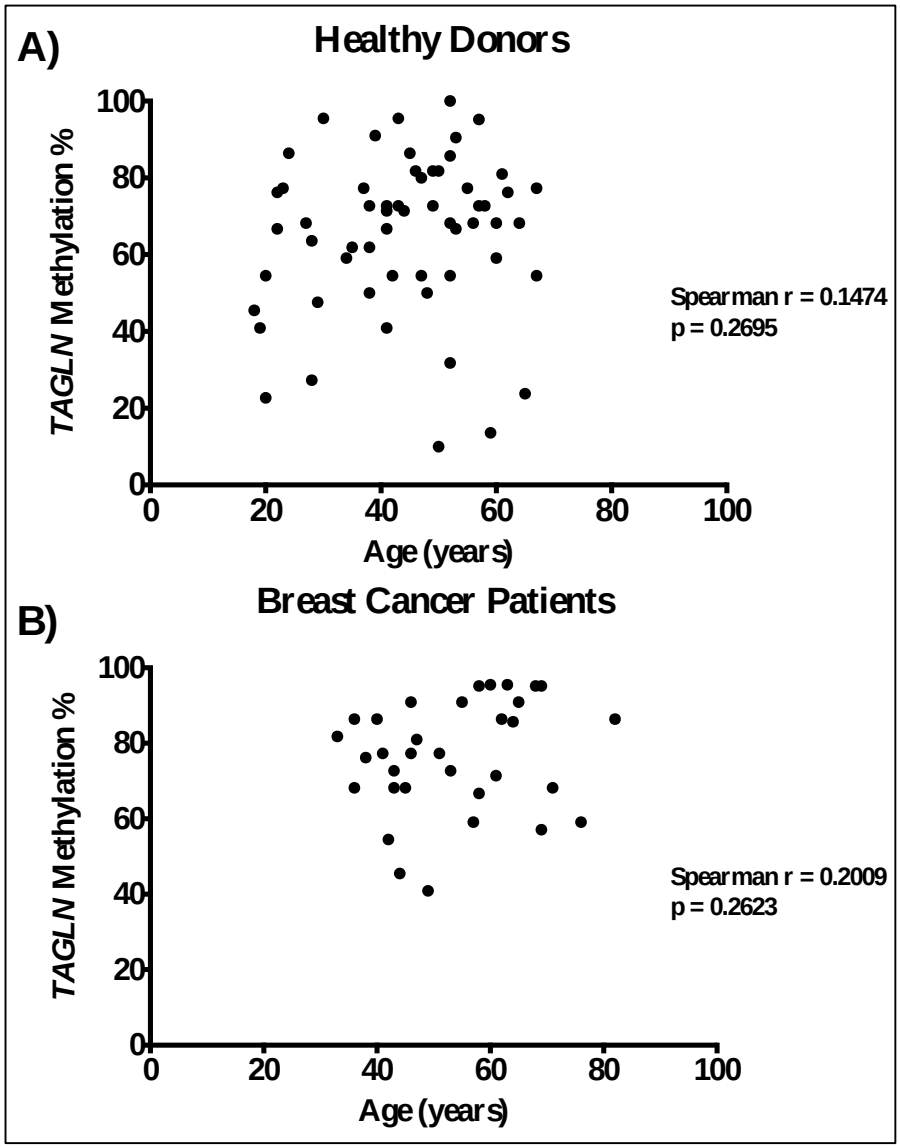

Figure 3.10: Correlation of TAGLN promoter methylation with age status in healthy donors and BC patients.

TAGLN promoter methylation was not significantly correlated with age in A) healthy donors (Spearman r=0.1474, p=0.2695, n=58) and B) BC patients (Spearman r=0.2009, p=0.2623, n=33).

In (Table 2.7) molecular subtypes of BC were tested for association with *TAGLN* promoter methylation levels. *TAGLN* promoter was not significantly methylated in any group, while Lum A showed slightly higher *TAGLN* promoter methylation compared to Lum B. Moreover, the Lum A molecular subtype displayed the highest *TAGLN* promoter methylation levels among all subtypes. Since the sample size of TNBC (n=3) subtype was small, TNBC was not included in the graph and the statistical tests. Furthermore, the Lum B subtype shows the largest deviation in methylation levels among its samples.

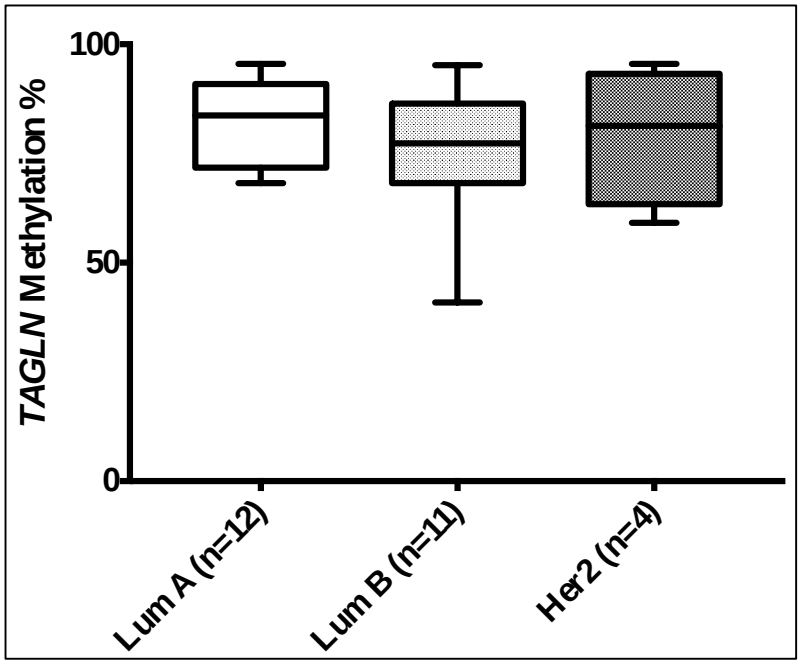

Figure 3.11: Evaluation of TAGLN methylation levels with molecular subtypes.

TAGLN promoter methylation levels did not differ among molecular subtypes Lum A (n=12), Lum B (n=11), Her2 (n=4) and TNBC (n=2). Mann-Whitney test is used for comparisons. Due to small samples size TNBC subtype (n=3) was not included in statistical tests. Mann Whitney test. Whiskers show 5-95 percentiles.

Sample sizes of DCIS (n=3) and ILC (n=2) were small and these groups were therefore not included in the graph and the statistical tests (Figure 3.12A) Tumor sizes of T1 and T2 did not statistically differ in terms of *TAGLN* promoter

methylation (P = 0.8177, Figure 3.12B). Samples with T1 tumor size exhibited the highest and the lowest *TAGLN* promoter methylation levels with the largest deviation. Samples with T3 tumor size were not included in the graph and the statistical test due to the small sample size (n=2). *TAGLN* promoter methylation levels did not statistically differ between different tumor grades (P =0.1049, Figure 3.12D). Grade 1 samples were not included in the graph and the statistical tests due to the small sample size (n=2). The tumor histological grade information was missing for one patient.

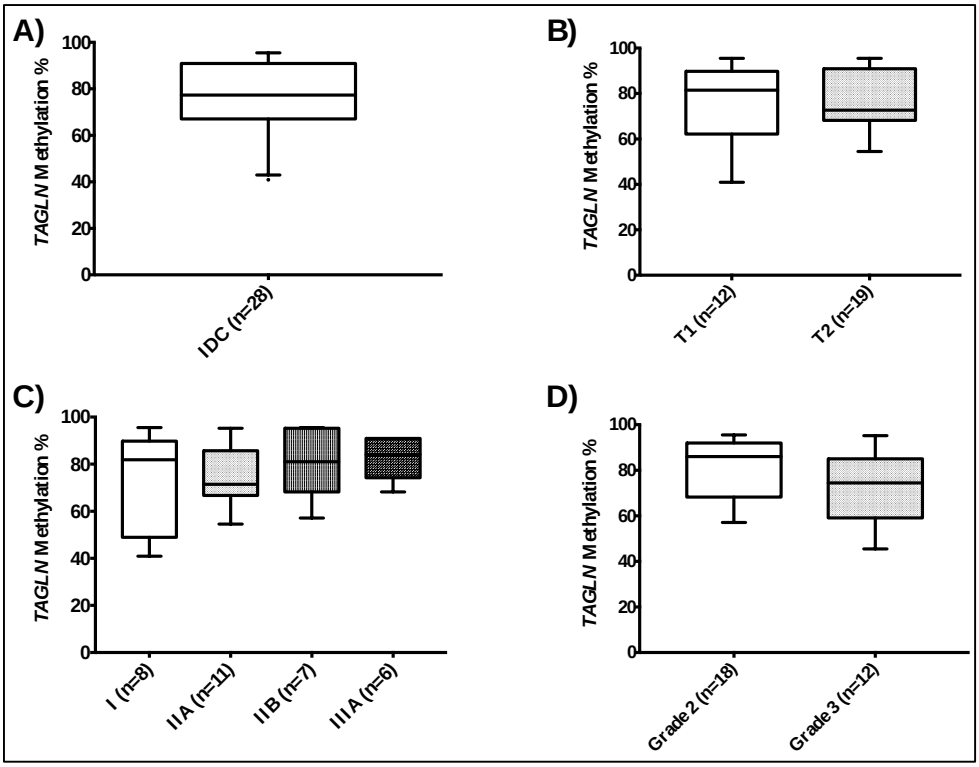

Figure 3.12: Evaluation of TAGLN promoter methylation levels with clinical and pathological parameters.

A) Box-plots shows TAGLN promoter methylation levels in IDC (n=28). DCIS (n=3) and ILC (n=2) samples were not included in the graph and the statistical test. B) Comparison of TAGLN promoter methylation levels in various tumor sizes T1 (n=12) and T2 (n=19) shows no significant difference. P = 0.8177. Sample with T3 (n=2) size was not included in the graph and the statistical test. Mann-Whitney test. C) Box-plots shows no significant difference of TAGLN promoter methylation levels in different tumor stages I (n=8), IIA (n=11), IIB (n=7) and IIIA (n=6). P = 0.4803. Kruskal-Wallis Test. Sample with IIIC stage (n=1) was not included in the graph and the statistical test. D) Comparison of TAGLN promoter methylation levels in tumor

Grade 2 (n=18) and 3 (n=12) shows no significant difference. P = 0.1049. Sample with Grade 1 (n=2) was not included in the graph and the statistical test. One of the patient's information is missing. Mann-Whitney test. Whiskers show 5-95 percentiles.

Considering the pathological subtype, tumor size and histological grade data, *TAGLN* promoter methylation was not expected to significantly differ in different tumor stages. Statistical significance in terms of TAGLN promoter methylation between different tumor stages was not found. (P = 0.4803, Figure 3.12C). Stage IIIC was not included in the statistical test due to its small sample size (n=1).

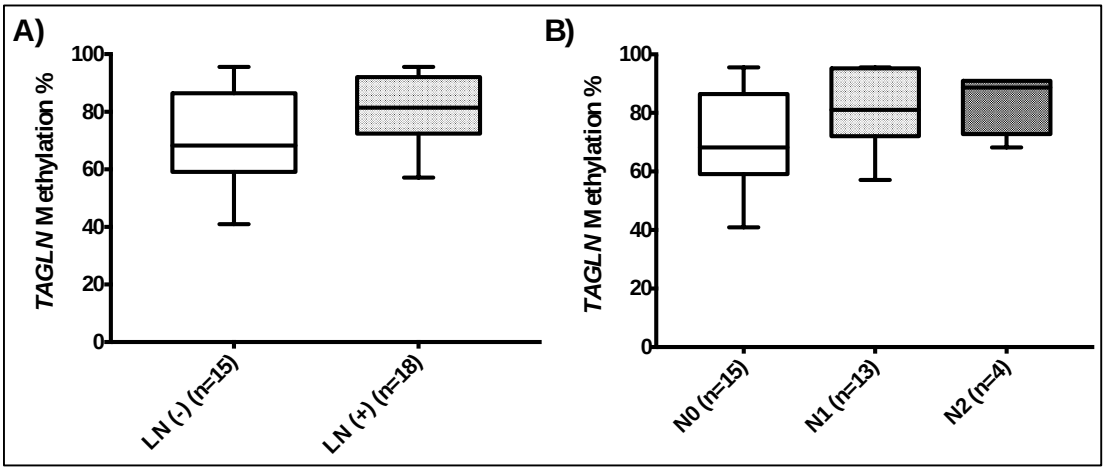

Figure 3.13: Evaluation of TAGLN promoter methylation levels with lymph node metastasis status.

A) Box-plots show higher methylation level of TAGLN promoter LN (+) BC patients (n=18) compared to LN (-) patients (n=15). P = 0.0625. Mann-Whitney test. B) Comparison of TAGLN promoter methylation levels between different lymph node metastasis states N0 (n=15), N1 (n=13) and N2 (n=4). N3 (n=1) samples were not included in the graph and the statistical tests. P = 0.1600. Kruskal-Wallis test.

When lymph node metastasis status was tested for significance, the LN (+) and LN (-) BC patient groups did not exhibit statistical difference in terms of *TAGLN* methylation levels (Figure 3.13A). Although a significant difference in *TAGLN* promoter methylation was not observed, LN (+) patients exhibited slightly higher levels of promoter methylation (*P* = 0.0625). The LN status also did not show any

statistically significant difference in promoter methylation levels (Figure 3.13B). The sample size of N3 (n=1) was small, and this group was therefore not included in the statistical test.

The significant difference in *TAGLN* promoter methylation levels in the sera of BC patients compared to healthy donors may indicate a possible use as a methylation biomarker for breast cancer diagnosis. When receiver-operator characteristics (ROC) analyses were conducted with *TAGLN* promoter methylation levels to address this topic, 74.45% was determined as the threshold that can be used for the identification BC patients with 57.58% sensitivity and 67.24% specificity among the cancer and healthy population. The area under the curve (AUC) was calculated as 0.6748 with 95% confidence interval (CI) of 0.5613 to 0.7882 ($P < 0.01$,Figure 3.14). It can be expected that when the sample size increases the specificity and sensitivity of *TAGLN* promoter methylation as a serum diagnostic biomarker can be much stronger. These results indicated that *TAGLN* promoter methylation levels could be a potential marker for breast cancer diagnosis.

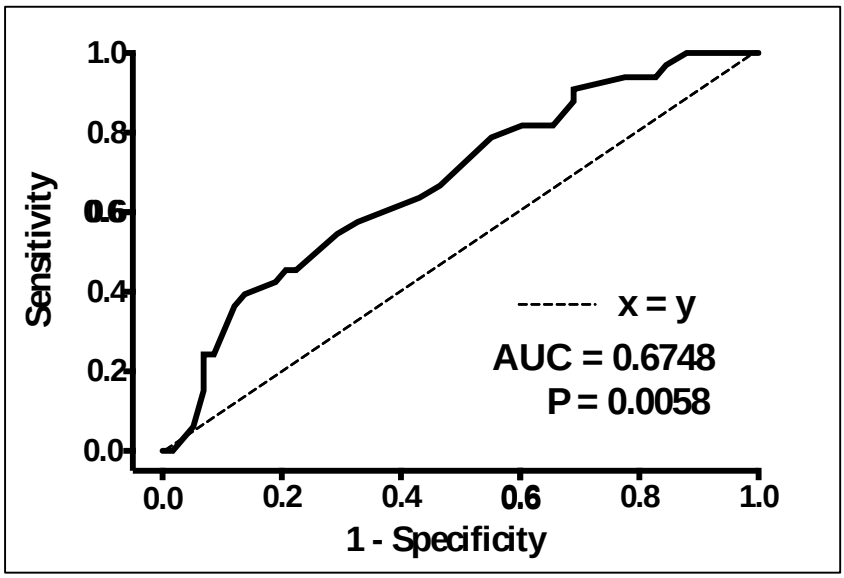

Figure 3.14: *TAGLN* promoter methylation as a diagnostic serum methylation biomarker to differentiate BC patients from healthy donors.

TAGLN promoter methylation levels could identify BC patients among the healthy donors with 57.58% sensitivity and 67.24% specificity, when 74.45% threshold was used. AUC = 0.6748, P = 0.0058

3.3 Correlation analyses of cfDNA amount with clinicopathological parameters in derived serum samples

Elevation of cfDNA levels originating from apoptotic, necrotic as well as circulating tumor cells could be a marker for presence of a malignant disease. In order to see whether patients who were diagnosed with BC possess higher cfDNA levels, cfDNAs were isolated from 135 healthy donor and 67 BC patient sera and measured.

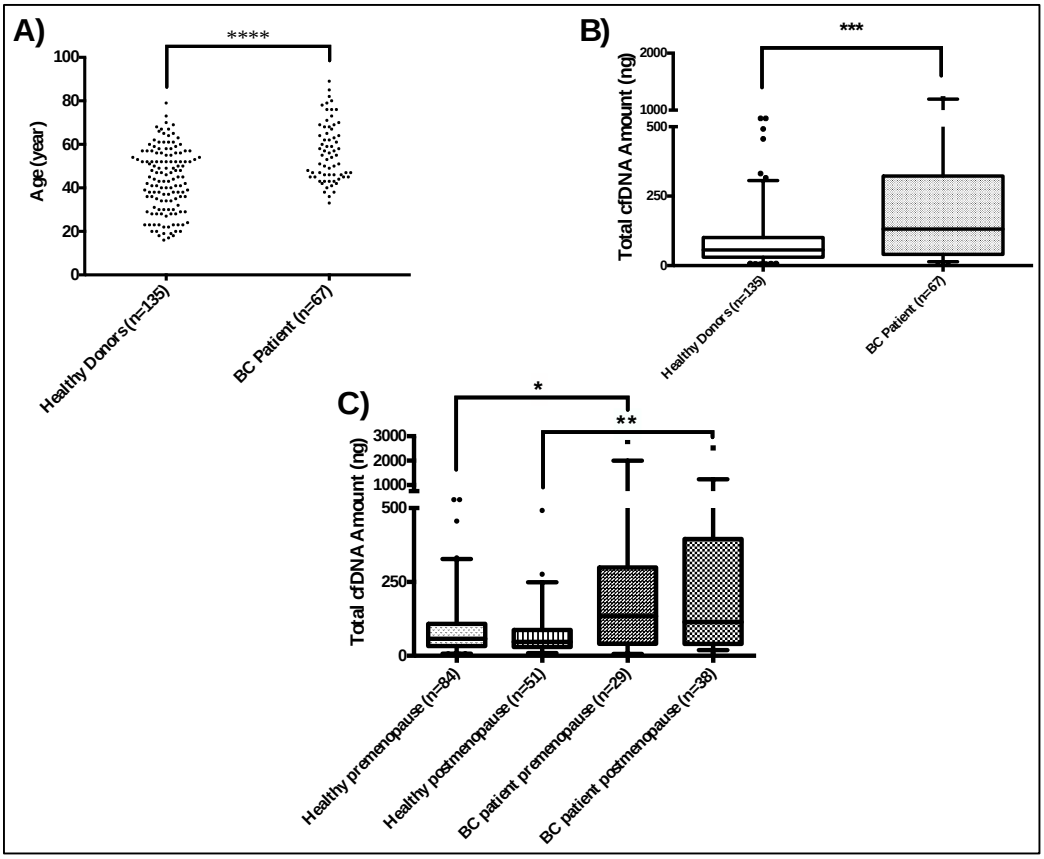

Figure 3.15: Age status and total cfDNA amounts of healthy donors and BC patients.

*A) Scatter dot plots showing that the average age of healthy donors (n=135) and BC patients (n=67) is 44 and 55 years, respectively. B) Comparison of total cfDNA amounts in healthy donors and BC patients. cfDNA levels were higher in the sera from BC patients compared to healthy donors. *** P < 0.001, Mann-Whitney test. C) Comparison of total cfDNA amount in healthy donor age groups (premenopausal Age≤50 n=84, postmenopausal Age>50 n=51 and comparison of total cfDNA amount in patient age groups (Age≤50 n=29, Age>50 n=38). **P < 0.01, Kruskal-Wallis test. Whiskers show 5-95 percentiles.*

Average age of healthy donors and BC patients were found to be 44 and 55 years, respectively (Figure 3.15A). BC patients possess significantly higher levels of total cfDNA in their sera (avg. 131.6 ng) compared to healthy donors (avg. 56.4 ng) (Figure 3.15B, $P < 0.001$). 50 years of age is the common age for menopause[213] and thus 50 was used as a threshold for pre- and post-menopausal status of healthy donors and BC patients. However, there was no significant difference in sera cfDNA levels among healthy pre- (avg. 57.6 ng) and post-menopausal (avg. 47.2 ng) groups of women (Figure 3.15C, $P = 0.5572$). cfDNA levels also did not differ significantly among BC patients who were pre- (avg. 133.6 ng) and post-menopausal (avg. 114.2 ng) women ($P = 0.7201$). Premenopausal BC patients possessed higher levels of total cfDNA than premenopausal healthy donors ($P = 0.0265$). Additionally, postmenopausal BC patients showed higher total cfDNA levels compared to postmenopausal healthy donors ($P = 0.0025$). A statistically significant correlation was not found between cfDNA levels and the individual's age in healthy donors (Figure 3.16A, *Spearman r = -0.01785, P = 0.8372)* or in BC patients (Figure 3.16B, *Spearman r = -0.09625, P = 0.4384).*

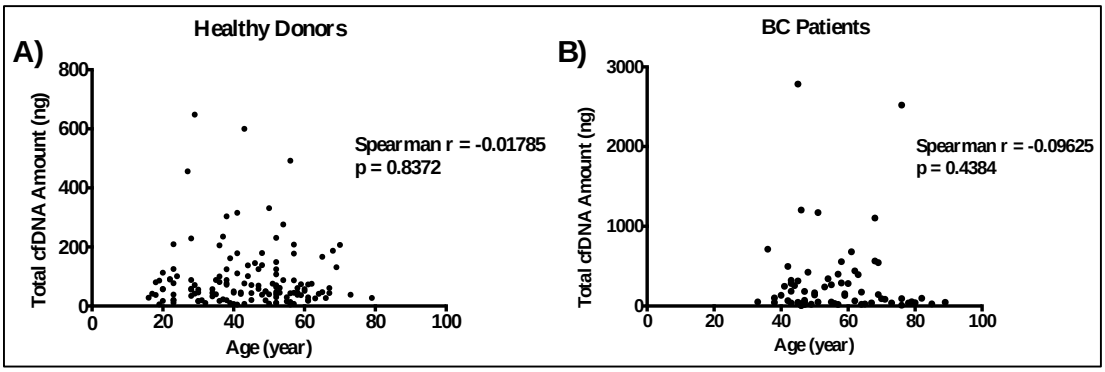

Figure 3.16: Correlation graph of age with cfDNA amount in healthy donors and BC patients.

Scatter plots show no correlations of total cfDNA amounts with age in A) healthy donors (Spearman r = -0.01785, P = 0.8372, n=135); and B) BC patients (Spearman r = -0.09625, P = 0.4384, n=67).

cfDNA levels can be associated with the clinical and pathological variables[214]. These associations can be statistically tested to see whether certain subtypes possess higher

cfDNA levels. Molecular subtypes of BC were tested for association with cfDNA levels (Figure 2.5). The TNBC subtype is the most aggressive type of BC compared to Lum A, Lum B and Her2-enriched due to its high mitotic index and higher tumor grade. TNBC samples displayed the lowest median serum cfDNA, whereas Lum B showed the highest median (Figure 3.17). Although the difference between Lum B and TNBC cfDNA levels was the largest (P = 0.1744), no statistical significance was found among all subtypes, possibly due to the small sample size.

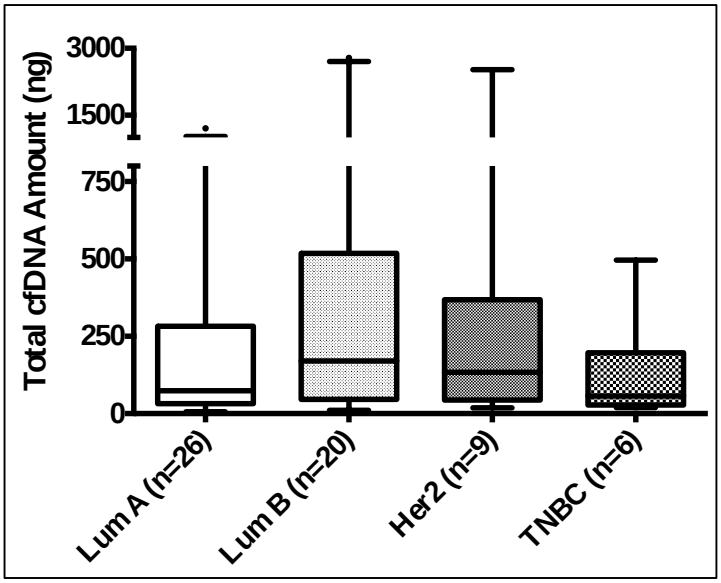

Figure 3.17: Evaluation of total cfDNA amount with molecular subtypes.

Total cfDNA amounts did not differ among molecular subtypes Lum A (n=26), Lum B (n=20), Her2 (n=9) and TNBC (n=6). P = 0.3744. Kruskal-Wallis test was used for comparisons. Whiskers show 5-95 percentiles.

In order to examine the association between cfDNA levels and clinicopathological parameters, pathological subtypes (IDC and DCIS), tumor size, tumor stage and histological grades were tested. Since IDC subtype is more invasive than DCIS, cfDNA levels were expected to be significantly higher in this subtype. However, IDC did not show any significant difference compared to other subtypes (P = *0.3318*, Figure 3.18A). ILC and SC samples were not included in the statistical test due to small sample size (n=3 and n=1, respectively). Even though tumor size is not an independent factor for tumor aggressiveness, it can be considered as a potential

indicator. Thus, larger tumor size could indicate a more aggressive phenotype. As a result, larger tumors may secrete more cfDNA into the bloodstream due to their potential aggressiveness as opposed to smaller tumors. However, a statistically significant difference in cfDNA levels between different tumor sizes was not observed (*P = 0.7928*, Figure 3.18B). Total cfDNA medians of T1 and T2 were highly similar even though patients with T2 size tumors possessed higher levels of total cfDNA. Samples with T4 (n=2) sizes were not included in the graph and the statistical tests due to their small sample size. Tumor stage is composed of tumor size, lymph nodes and metastasis and is therefore an important indicator of tumor aggressiveness. Thus, higher tumor stages may be expected to possess higher cfDNA levels in the bloodstream as opposed to lower tumor stages. There are also some studies demonstrating that cfDNA levels do not always correlate with tumor stage[215]. Due to small sample sizes, higher-stage tumors IIIB (n=1), IIIC (n=3) and IV (n=3) were not included in the graph and the statistical tests. Significant difference in total cfDNA levels were not detected among stage I, IIA, IIB and IIIA tumors (*P = 0.8461*, Figure 3.18C). Total cfDNA levels were highly similar among tumor stages. Histological grade of a tumor is one of the most important aspects of tumor aggressiveness and its ability to spread to secondary locations[216]. Higher-grade tumors exhibit relatively less differentiated phenotype compared to lower grade tumors, which show a more differentiated and normal-like phenotype. Grade 2 BC patients display elevated cfDNA levels in sera compared to Grade 1 BC patients (*P* = 0.0707). Grade 3 did not exhibit significant cfDNA levels compared to Grade 1 and Grade 2 (Figure 3.18D).

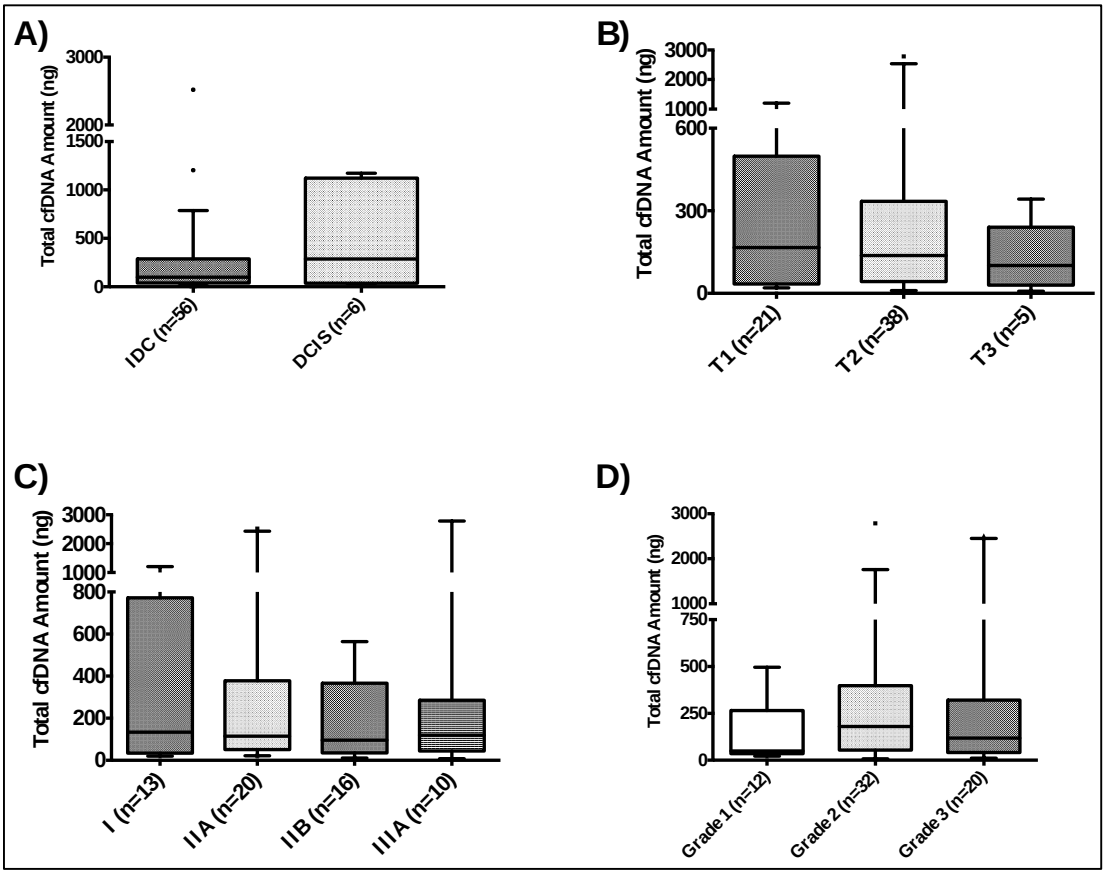

Figure 3.18: Evaluation of total cfDNA amount with clinical and pathological parameters.

A) The box-plots shows no significant difference of total cfDNA amount in different pathological groups IDC (n=56), DCIS (n=6). Mann-Whitney test P = 0.3318. Due to their small sample size, ILC (n=3) and SC (n=1) samples were not included in the graph and the statistical tests. B) Comparison of total cfDNA amount in various tumor sizes T1 (n=21), T2 (n=38) and T3 (n=5) shows no significant difference. The T4 (n=2) sample was not included in the graph and the statistical test due to its small sample size. Kruskal-Wallis test. P = 0.7928. C) The box-plots show no significant difference of total cfDNA amount in different tumor stages I (n=13), IIA (n=20), IIB (n=16), IIIA (n=10). IIIB (n=1), IIIC (n=3) and IV (n=3) samples were not included in the graph and the statistical tests due to their small sample size. Kruskal-Wallis test. P = 0.8461. D) Grade 2 (n=32) showed higher cfDNA levels compared to Grade 1 (n=12) Kruskal-Wallis test. P = 0.1809. Whiskers show 5-95 percentiles.

Lymph node (LN) metastasis is a process where the cancer cells spread to sentinel or axillary lymph nodes[217]. cfDNA levels can therefore be expected to be higher in LN (+) patients as opposed to non-metastatic patients. Hence, cfDNA levels were tested for significant difference between LN (+) and LN (-) BC patients (Figure 3.19A). However, a statistically significant difference was not found between these groups (p=0.4694). The LN status also did not demonstrate any statistically significant difference (P =0.5213, Figure 3.19B). N3 samples were not included in the graph and the statistical test due to its small sample size (n=3). Since the sample size of the clinicopathological groups of BC patients were small and some of the clinicopathological data was missing, reliable significant associations could not be detected among the clinical variables and serum cfDNA levels.

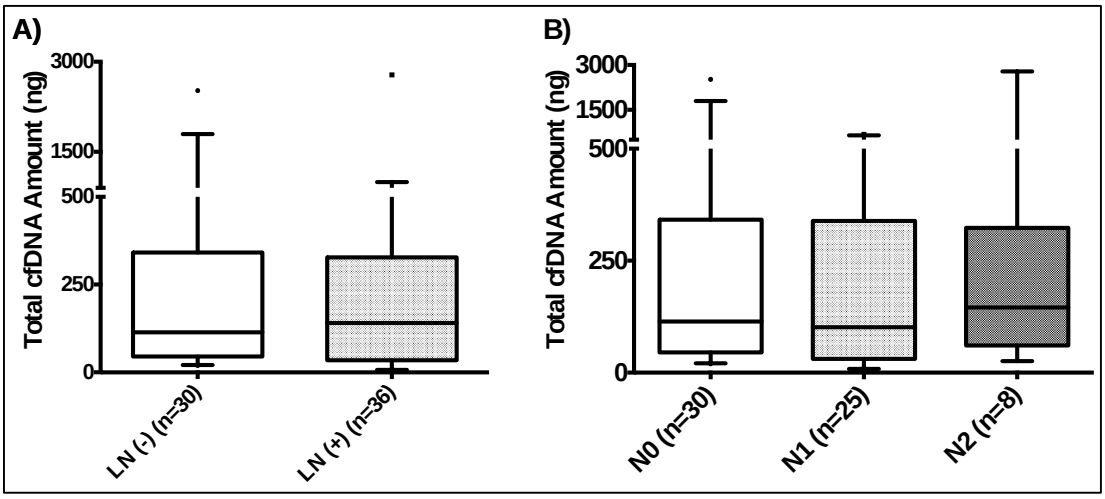

Figure 3.19: Evaluation of total cfDNA amount with lymph node metastasis status.

A) The box-plots show no significant difference of total cfDNA amount among LN (-) (n=30) and LN (+) (n=36) BC patients. P = 0.4694. Mann-Whitney test. B) Comparison of total cfDNA amount between different lymph node metastasis states N0 (n=28), N1 (n=24) and N2 (n=7) did not show any significance. The Mann-Whitney test was used for comparisons. N3 samples (n=3) were not included in the graph and the statistical test due to the small sample size. P = 0.5213. Kruskal-Wallis test.

The significant difference of the cfDNA levels in the sera of BC patients compared to healthy donors indicates a possible use of cfDNA levels as a biomarker for BC. When receiver-operator characteristics (ROC) analyses were conducted with total cfDNA levels of both groups to address this topic, 91.4 ng was obtained as a threshold that can be used for the identification BC patients with 56.72% sensitivity and 73.33% specificity among the cancer and healthy population. Area under the curve (AUC) was calculated as 0.6608 with 95% confidence interval (CI) of 0.5757 to 0.7458 ($P < 0.001$, Figure 3.20). These results indicated that cfDNA levels could be potentially used as a biomarker for BC but it is much better to identify the malignancy with strong biomarkers (i.e *RASSF1A*[60], *APC*[218] etc.) prior to cfDNA level detection. Then, cfDNA levels can be analyzed to further support diagnosis.

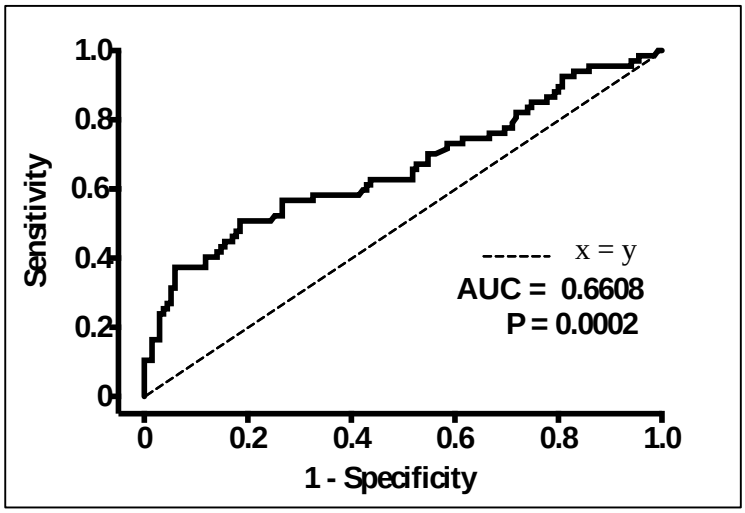

Figure 3.20: Total cfDNA amount as a marker to differentiate BC patients from healthy donors.

Total cfDNA amounts could identify BC patients (n=67) from the healthy donors (n=135) with 56.72% sensitivity and 73.33% specificity when the 91.4ng threshold was used (P = 0.002).

Chapter 4. Discussion

The transgelin protein is known for its actin-binding ability due to its Calponin homology domain which is critical for actin dynamics. Involvement of transgelin was shown in muscle fiber contraction, stress fiber formation, smooth muscle cell migration and differentiation[168]. Previous studies have demonstrated the TAGLN gene's potential involvement in cancer development and progression. Several studies showed decreased expression of TAGLN in various cancers including colorectal, prostate, gall bladder and BC[178–184]. On the other hand, some other studies showed TAGLN upregulation in gastric, lung, esophageal squamous cell, oral squamous cell, pancreatic and metastatic colon carcinoma[185–193]. A recent study has suggested that TAGLN expression is higher in lymph node positive breast tumors while another study has demonstrated that miR-145-stimulated TAGLN upregulation is associated with a decrease in cell motility in breast cancer cell lines[194,205]. Additionally, *TAGLN* promoter hypermethylation has been shown in breast tumors compared to paired normal breast tissues[210]. Bioinformatic analysis performed previously at our lab has revealed that *TAGLN* promoter is hypermethylated in BC sera compared to healthy sera in two different datasets[211]. Although BC sera and normal sera samples reside in two different data sets, this result suggests that TAGLN promoter hypermethylation can be detected from serum samples. In spite of these studies, the *TAGLN* gene has not been fully characterized in terms of its role in cancer. Additionally, samples used in those studies mostly consist of cancer cell lines and tumor tissues. Cell lines and tumor tissues are convenient for identification of a biomarker, but are inadequate for diagnostic purposes. Liquid biopsy taken from bodily fluids to analyse of circulating nucleic acids (CNAs) is a less-invasive procedure used for early detection, minimal residual disease detection, prediction of therapy response, and real-time monitoring of tumor progression. Liquid biopsy can be performed frequently in advanced stage cancer patients in whom biopsies are not performable, both practically and ethically. CfDNA was first mentioned in 1948 with a distinct feature of having a lower molecular weight than genomic DNA, and it was later demonstrated that cfDNA can possess both genetic and epigenetic alterations that are originally found in the malignant primary tumor[73,117,118,219]. Thus, liquid biopsy is a suitable approach for cancer diagnostic or prognostic biomarker studies.

We have investigated sera from 33 BC patients and 58 healthy donors via the gold standard method "bisulfite sequencing". *TAGLN* promoter hypermethylation in BC patient sera as opposed to healthy donor sera was shown as a first time in this study. The investigated regions of *TAGLN* gene (-286 bp to -80 bp for Section 1 and -102 bp to +115 bp for Section 2) were frequently methylated (40.9%-95.5% methylation) in BC patients.In accordance with our findings, Sayar *et al*. showed *TAGLN* promoter hypermethylation and protein downregulation in breast cancer cell lines compared to non-tumorigenic cell lines, and in tumor tissues compared to paired normal breast tissues[210]. In our study, we have noticed that sample size can directly affect the diagnostic strength of a possible biomarker. Differences in sensitivity and specificity between different studies may be due to using healthy controls with different backgrounds.

Methylation at certain CpG sites in *TAGLN* promoter may affect the probability of transcription factor binding to the promoter. Six out of twenty-two CpGs were differentially hypermethylated in BC patients, and one CpG was differentially hypomethylated in the patients (Figure 3.8). It would be beneficial to investigate the molecular control mechanism of *TAGLN* promoter in order to understand the effect of promoter hypermethylation. Further studies can be performed to investigate the role of transcription factors targeting the *TAGLN* promoter (such as SRF, YY1)[220,221]. Additionally, factors that affect *TAGLN* promoter methylation, as well as the prognostic strength of *TAGLN* promoter hypermethylation can be studied.

Menopausal status of BC patients and healthy donors were categorized using the age of 50 as the threshold[213]. In order to test the relationship between *TAGLN* promoter methylation and the individual's age, Spearman correlation was conducted. However, *TAGLN* promoter methylation in pre- and postmenopausal individuals did not display a significant difference (Figure 3.15). In the postmenopausal group, TAGLN promoter methylation levels were significantly higher in BC patients compared to healthy donors. There was no correlation between age and *TAGLN* promoter methylation levels in either group (Figure 3.16).Studies performed in colorectal cancer including our findings may suggest that cfDNA *TAGLN* promoter methylation levels do not differ with patient age[96].

TAGLN expression levels were implied to be associated with different clinicopathological characteristics in breast cancer[210]. The information about

TAGLN's role in tumor progression was limited and we therefore investigated an association between *TAGLN* promoter methylation levels and clinicopathological features of BC sera used in this study. We used the Mann-Whitney test to compare continuous data in the context of pathological and molecular groups, tumor size, stage, grade and lymph node status. Even though this approach failed to demonstrate a significant association between different clinicopathological features and *TAGLN* promoter methylation levels, there was an upward trend in terms of lymph node states since lymph node positive patients possess higher *TAGLN* promoter methylation levels, indicating a potential regulator role of TAGLN in terms of metastasis (Figure 3.13). Actin filament disintegration was found to correlate with neoplasia[222], and TAGLN may therefore regulate the invasion capacity due to its actin-binding ability. In prostate cancer, overexpression of TAGLN has been found to repress androgen-triggered cell growth, and loss of the protein induces MMP-9 expression and tissue invasion[175,176]. Our results may suggest the importance of *TAGLN* promoter methylation status on invasion capability of the primary breast tumor.

In this study, the clinical parameters such as stage, size and grade did not correlate with *TAGLN* promoter methylation. Similarly, in colorectal cancer, *TAGLN* promoter methylation levels were not correlated with patient clinical parameters (stage, size and grade), which supports our findings[183,223]. The sample size of some clinicopathological subgroups was insufficient to conduct a reliable statistical test. Thus, large sample sizes may be able to assist the identification of a possible association between *TAGLN* promoter methylation levels and clinical variables in breast cancer. The findings in this study with this patient group showed that *TAGLN* promoter methylation levels in breast cancer patients can discriminate patients from the population, with 57.58% sensitivity and 67.24% specificity (Figure 3.14, AUC=0.6748). Sayar *et al*. showed that *TAGLN* promoter hypermethylation can be used to distinguish breast tumors tissues among healthy tissues with 83.14% sensitivity and 100% specificity[210]. A study showed RUNX3, a tumor suppressor in gastric cancer, promoter methylation in 91% of gastric cancers and in 29% of patient serum samples[224]. *TAGLN* promoter methylation can be used in a gene panel with other previously published powerful biomarkers for diagnostic purposes. These strong biomarkers could be *RASSF1A*[60] (75% sensitivity and 100% specificity) or

RAR1β[225] (87% sensitivity and 94% specificity). There is no study evaluating *TAGLN* promoter methylation using patient serum. But there are two datasets from methylation array studies that give us an indication of *TAGLN* methylation in breast cancer patient sera compare to normal sera. Dataset GSE56621 showed that in BC patient sera *TAGLN* promoter methylation was significantly higher than paired normal breast tissues. When breast cancer sera in GSE56621 and healthy sera in GSE58119 were analyzed together, *TAGLN* promoter hypermethylation was shown in breast cancer sera[211]. These studies indicate the potential use of *TAGLN* promoter as a methylation biomarker in breast cancer sera. In this study, we investigated total cfDNA levels in BC patients and healthy donors with their relation to clinicopathological features of the patients. We have collected blood and separated the sera from 67 BC patients and 135 healthy donors. BC patients possessed higher total cfDNA amounts in their sera compared to healthy donors (Figure 3.15B). There are other studies that have demonstrated that cfDNA levels are associated with the malignancy in different cancer types including prostate, breast, colorectal etc[86,102,108,226–229]. A recent study has shown that colorectal cancer patients who have undergone systemic therapy had significantly lower levels of total cfDNA compared to off-therapy colorectal cancer patients[230]. However, BC patient samples were collected before surgery and therapy in our study. It is generally speculated that cfDNA originates from apoptotic cells in the bloodstream in a healthy individual but can originate from necrotic cells as well as the malignant primary tumor in cancer patients[111]. The primary tumor itself or CTCs released from the primary tumor are the main source of the tumorigenic origin of cfDNA[231]. Thus the elevated levels of total cell free DNA resource can be the primary breast tumor.

The menopausal status of BC patients and healthy donors were categorized using the age of 50 as the threshold[213]. Total cfDNA amount in pre- and postmenopausal individuals did not display any significant difference (Figure 3.15C). Patients display higher total cfDNA levels compared to healthy donors in the same age group. There was no correlation between age and total cfDNA amount in either group (Figure 3.16). This finding correlated with the studies in the literature in different types of cancer including non-small cell lung carcinoma, thyroid and ovarian cancers[226,232–234]. On the other hand, another study has reported a positive correlation between age and cfDNA levels[235], but the sample size was small and controls were imbalanced,

requiring further studies on the association between age and total cfDNA levels. Total cfDNA yield and age have not shown any correlation in BC in the literature[236]. Luminal breast cancer subtypes were expected to possess the lowest levels of total cfDNA due to their low proliferation rates compared to Her2-enriched and triple negative (TN) subtypes, which are more aggressive[237]. Total cfDNA levels in sera did not exhibit any significance within different molecular subtypes in our study (Figure 3.17). Lum B displayed the highest total cfDNA levels. Sample size of Her2-enriched and TN subtypes were small and thus these subtypes could not reflect a reliable significant result. Total cfDNA levels did not show any significant difference in different clinicopathological features (Figure 3.18). In the literature, total cfDNA level correlations with clinicopathological features of patients with different cancer types are highly contradictory. In BC, some studies have reported that cfDNA levels were linked with tumor stage and size but not with hormone-receptor status[83,100,101]. On the other hand, one study did not find any relationship between total cfDNA levels and tumor stage, size or histological grades[102]. This contradiction may be due to different sample sizes or cfDNA origin may be beyond tumor lysis and actually belong to a more complex mechanism. Since higher-grade tumors exhibit a relatively less differentiated phenotype with rapid cellular proliferation, Grade 3 was expected to display the highest total cfDNA levels. However, histological grades did not show any difference in terms of total cfDNA (Figure 3.18B). Grade 2 tumors possessed the highest median levels of total cfDNA. Lymph node (LN) metastasis is a process where the cancer cells invade sentinel or axillary lymph nodes, thus LN (+) patients were expected to possess higher total cfDNA levels than LN (-) patients. Lymph node status did not show a correlation pattern with total cfDNA levels (Figure 3.19). In the literature, the lymph node status of patients has been shown to correlate with total cfDNA levels in some studies[102,238–241], while another study did not verify such findings[104]. These contradictions are possibly due to small size of a particular study sample and the lack of a standardized protocol for liquid biopsy studies.

Studies have shown that total cfDNA levels were able to discriminate patients in a population of various cancer types with different sensitivity and specificity values[214,232,242,243]. In breast cancer, Agassi *et al*. have shown the diagnostic power of total cfDNA levels in patients with 72% sensitivity and 75% specificity (AUC=0.83)[238]. These differences in sensitivity and specificity between different

studies may be due to using healthy controls with different backgrounds. Two recent studies have conducted meta-analyses of retrospective studies in BC, and have demonstrated the increasing strength of total cfDNA levels for discriminating patients from healthy individuals[244,245]. Total cfDNA levels in our patient cohort can discriminate between healthy donors and breast cancer patients with 56.72% sensitivity and 73.33% specificity. Sample size directly affects the evaluation of the discrimination strength of a potential biomarker.

Chapter 5. Conclusion and Future Perspectives

In conclusion, this study demonstrated that sera can be used to identify the tumor profile in BC patients and that *TAGLN* promoter methylation levels were significantly higher in BC patients compared to healthy individuals. *TAGLN* promoter methylation levels were increased with lymph node status of the patients. Patients who were over 50 years of age displayed higher *TAGLN* promoter methylation levels than patients who were below 50 years and healthy donors who were above 50 years of age. Additionally, total cfDNA levels were significantly elevated in patients diagnosed with BC as opposed to healthy donors. ROC curves showed the possibility of using total cfDNA and *TAGLN* promoter methylation levels for early detection of BC. Higher number of sample size would be better to establish the potential use of *TAGLN* promoter hypermethylation as a methylation biomarker in BC sera.

Age distributions among breast cancer patients and healthy donors were statistically significant in epigenetic analyses and total cfDNA analyses. However, we have showed that breast cancer patients had significantly higher TAGLN promoter methylation levels compared to healthy donors below and above 50 years of age, suggesting that TAGLN promoter methylation levels are independent of age. Nevertheless, increasing the healthy donor cohort with donors who are above 50 years of age would increase the diagnostic potential of TAGLN methylation.

Although the amount of cfDNA obtained from patients was enough to perform the bisulfite sequencing, cfDNA from some of the serum samples were degraded and were not amplified with PCR. There are other methods including vacuum manifold and magnetic beads to isolate cfDNA from sera in order to increase the cfDNA yield and quality from samples.

Bisulfite sequencing is the golden method for discovering methylation profiles of genes prior to any other test. Upon identification, high-throughput techniques such as multiplex quantitative methylation-specific PCR (qMSP) can be used to discover the methylation status of multiple genes. Droplet Digital PCR (ddPCR) is another technique that can be used for direct quantification of methylation of a single DNA

molecule even at low abundance. In this study, we only demonstrated *TAGLN* methylation levels in serum. The formalin fixed paraffin embedded breast tumor tissue sections that have been obtained from Pathology department are currently in use to investigate whether sera *TAGLN* promoter methylation levels correlate with paired tissue samples. Additionally, CpG methylation patterns of TAGLN both in sera and paired tumor tissues can be identified. Since tumors consist of cells of various origins, tumor heterogeneity can be a problem for biomarker studies. In order to overcome this situation, CTCs can be isolated from blood and the single cell sequencing technique can be performed to investigate the methylation levels in each cell type.

We have demonstrated *TAGLN* promoter hypermethylation in BC patients. In order to investigate whether promoter hypermethylation is specific to BC, serum samples collected from patients with different cancer types as well as patients with benign breast disease can be used as additional controls. Moreover, a gene panel consisting of strong biomarkers including *TAGLN* promoter hypermethylation can be established for BC in order to increase the sensitivity and specificity of the diagnosis.

We have shown increased methylation levels of certain CpG sites in BC patients compared to healthy donors. It would be beneficial to investigate the transcription factors targeting the *TAGLN* promoter (SRF, YY1 etc.) with the chromatin immunoprecipitation (ChIP) technique with unmethylated and methylated controls in order to investigate whether methylation status affect the binding efficiencies of transcription factors. Additionally, each sample can be cloned into a cloning vector and 5 of these clones can be sent for bisulfite sequencing. Multivariant analyses can be conducted to identify preferentially methylated CpG sites in each group.

Finally, overall and relapse-free survival of the patients in this study can be followed from 6 months to 5 years in order to investigate the biomarker ability of *TAGLN* promoter hypermethylation regarding disease progression and the detection of disease stage at the time of breast cancer diagnosis.

References

(1) Hanahan, D.; Weinberg, R. A. Hallmarks of Cancer: The next Generation. *Cell*. 2011, pp 646–674.

(2) Toriola, A. T.; Colditz, G. A. Trends in Breast Cancer Incidence and Mortality in the United States: Implications for Prevention. *Breast Cancer Res. Treat.* **2013**, *138* (3), 665–673.

(3) Stingl, J.; Caldas, C. Molecular Heterogeneity of Breast Carcinomas and the Cancer Stem Cell Hypothesis. *Nature Reviews Cancer*. 2007, pp 791–799.

(4) What is breast cancer? | beBRCAware https://www.bebrcaware.com/breast-cancer-the-brca-link/what-is-breast-cancer.html (accessed Jul 29, 2018).

(5) Hassiotou, F.; Geddes, D. Anatomy of the Human Mammary Gland: Current Status of Knowledge. *Clin. Anat.* **2013**, *26* (1), 29–48.

(6) Love, S. M.; Barsky, S. H. Anatomy of the Nipple and Breast Ducts Revisited. *Cancer* **2004**, *101* (9), 1947–1957.

(7) Venta, L. A.; Dudiak, C. M.; Salomon, C. G.; Flisak, M. E. Sonographic Evaluation of the Breast. *Radiographics* **1994**, *14* (1), 29–50.

(8) Li, C. I.; Moe, R. E.; Daling, J. R. Risk of Mortality by Histologic Type of Breast Cancer among Women Aged 50 to 79 Years. *Arch Intern Med* **2003**, *163* (18), 2149–2153.

(9) Perou, C. M.; Sørile, T.; Eisen, M. B.; Van De Rijn, M.; Jeffrey, S. S.; Ress, C. A.; Pollack, J. R.; Ross, D. T.; Johnsen, H.; Akslen, L. A.; et al. Molecular Portraits of Human Breast Tumours. *Nature* **2000**, *406* (6797), 747–752.

(10) Tavassoli, F. a; Schnitt, S. J.; Hoefler, H.; Boecker, W.; Rosai, J.; Heywang-Kobrunner, S. H.; Holland, R.; Moinfar, F.; Ellis, I. O.; Lakhani, S. R. Intraductal Proliferative Lesions. *World Heal. Organ. Classif. Tumours. Pathol. Genet. Tumours Breast Female Genit. Organs.* **2003**, 63–74.

(11) Dereere, E.; Papadimitriou, K.; Tjalma, W.; Altintas, S. Ductal Carcinoma in Situ: A Disease Entity That Merits More Recognition. *Minerva Chir.* **2015**, *70*

(4), 231–239.

(12) Mitchell, K. B.; Kuerer, H. Ductal Carcinoma In Situ: Treatment Update and Current Trends. *Current Oncology Reports*. 2015.

(13) Mallon, E.; Osin, P.; Nasiri, N.; Blain, I.; Howard, B.; Gusterson, B. The Basic Pathology of Human Breast Cancer. *J. Mammary Gland Biol. Neoplasia* **2000**, *5* (2), 139–163.

(14) Sharma, G. N.; Dave, R.; Sanadya, J.; Sharma, P.; Sharma, K. K. Various Types and Management of Breast Cancer: An Overview. *J. Adv. Pharm. Technol. Res.* **2010**, *1* (2), 109–126.

(15) Yoder, B. J.; Wilkinson, E. J.; Massoll, N. A. Molecular and Morphologic Distinctions between Infiltrating Ductal and Lobular Carcinoma of the Breast. *Breast Journal*. 2007, pp 172–179.

(16) Vuong, D.; Simpson, P. T.; Green, B.; Cummings, M. C.; Lakhani, S. R. Molecular Classification of Breast Cancer. *Virchows Arch.* **2014**, *465* (1), 1–14.

(17) Yoder, B. J.; Wilkinson, E. J.; Massoll, N. A. Molecular and Morphologic Distinctions between Infiltrating Ductal and Lobular Carcinoma of the Breast. *Breast J.* **2007**, *13* (2), 172–179.

(18) Rakha, E. A.; Reis-Filho, J. S.; Baehner, F.; Dabbs, D. J.; Decker, T.; Eusebi, V.; Fox, S. B.; Ichihara, S.; Jacquemier, J.; Lakhani, S. R.; et al. Breast Cancer Prognostic Classifi Cation in the Molecular Era : The Role of Histological Grade. *Rakha al. Breast Cancer Res. 2010*, **2010**, *133* (6), 860–868.

(19) Bombardieri, E.; Bonadonna, G.; Gianni, L. *Breast Cancer*; Bombardieri, E., Bonadonna, G., Gianni, L., Eds.; Springer-Verlag Berlin Heidelberg, 2008.

(20) Veronesi, U.; Viale, G.; Rotmensz, N.; Goldhirsch, A. Rethinking TNM: Breast Cancer TNM Classification for Treatment Decision-Making and Research. *Breast*. 2006, pp 3–8.

(21) Benson, J. R. The TNM Staging System and Breast Cancer. *Lancet Oncology*. 2003, pp 56–57.

(22) Wang, M.; Chen, H.; Wu, K.; Ding, A.; Zhang, M.; Zhang, P. Evaluation of

the Prognostic Stage in the 8th Edition of the American Joint Committee on Cancer in Locally Advanced Breast Cancer: An Analysis Based on SEER 18 Database. *Breast* **2018**, *37*, 56–63.

(23) Atoum, M.; Hourani, H.; Shoter, A.; Al-Raheem, S.; Al Muhrib, T. TNM Staging and Classification (Familial and Nonfamilial) of Breast Cancer in Jordanian Females. *Indian J. Cancer* **2010**, *47* (2), 194.

(24) Dai, X.; Li, T.; Bai, Z.; Yang, Y.; Liu, X.; Zhan, J.; Shi, B. Breast Cancer Intrinsic Subtype Classification, Clinical Use and Future Trends. *Am J Cancer Res* **2015**, *5* (10), 2929–2943.

(25) Cho, N. Molecular Subtypes and Imaging Phenotypes of Breast Cancer. *Ultrasonography* **2016**, *35* (4), 281–288.

(26) Haybaeck, J. *Mechanisms of Molecular Carcinogenesis – Volume 1*; 2017; Vol. 2.

(27) Cianfrocca, M.; Gradishar, W. New Molecular Classifications of Breast Cancer. *CA. Cancer J. Clin.* **2009**, *59* (5), 303–313.

(28) Sorlie, T.; Perou, C. M.; Tibshirani, R.; Aas, T.; Geisler, S.; Johnsen, H.; Hastie, T.; Eisen, M. B.; van de Rijn, M.; Jeffrey, S. S.; et al. Gene Expression Patterns of Breast Carcinomas Distinguish Tumor Subclasses with Clinical Implications. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (19), 10869–10874.

(29) Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M, Thürlimann B, Senn HJ, P. M. Tailoring Therapies—Improving the Management of Early Breast Cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. *Ann. Oncol.* **2015**, *26* (8), 1533–1546.

(30) Brenton, J. D.; Carey, L. A.; Ahmed, A.; Caldas, C. Molecular Classification and Molecular Forecasting of Breast Cancer: Ready for Clinical Application? *Journal of Clinical Oncology*. 2005, pp 7350–7360.

(31) Arteaga, C. L.; Sliwkowski, M. X.; Osborne, C. K.; Perez, E. A.; Puglisi, F.; Gianni, L. Treatment of HER2-Positive Breast Cancer: Current Status and Future Perspectives. *Nat. Rev. Clin. Oncol.* **2011**, *9* (1), 16–32.

(32) Sotiriou, C.; Neo, S.; McShane, L. Breast Cancer Classification and Prognosis

Based on Gene Expression Profiles from a Population-Based Study. *Proc. ...* **2003**, *100* (18), 10393–10398.

(33) Pareja, F.; Geyer, F. C.; Marchiò, C.; Burke, K. A.; Weigelt, B.; Reis-Filho, J. S. Triple-Negative Breast Cancer: The Importance of Molecular and Histologic Subtyping, and Recognition of Low-Grade Variants. *npj Breast Cancer* **2016**, *2* (1), 16036.

(34) Mehrgou, A.; Akouchekian, M. The Importance of BRCA1 and BRCA2 Genes Mutations in Breast Cancer Development. *Med. J. Islam. Repub. Iran* **2016**, 1–12.

(35) Siegel, R.; Naishadham, D.; Jemal, A. Cancer Statistics, 2013. *CA. Cancer J. Clin.* **2013**, *63* (1), 11–30.

(36) Weber, B. L.; Nathanson, K. L. Low Penetrance Genes Associated with Increased Risk for Breast Cancer. *Eur. J. Cancer* **2000**, *36* (10), 1193–1199.

(37) Menendez, D.; Inga, A.; Resnick, M. A. The Expanding Universe of P53 Targets. *Nat. Rev. Cancer* **2009**, *9* (10), 724–737.

(38) Olivier M, Goldgar DE, Sodha N, Ohgaki H, Kleihues P, H. P. Li-Fraumeni and Related Syndromes : Correlation between Tumor Type , Family. **2003**, *Cancer Res*, 6643–6650.

(39) Thompson, D.; Duedal, S.; Kirner, J.; McGuffog, L.; Last, J.; Reiman, A.; Byrd, P.; Taylor, M.; Easton, D. F. Cancer Risks and Mortality in Heterozygous ATM Mutation Carriers. *J. Natl. Cancer Inst.* **2005**, *97* (11), 813–822.

(40) Herman, J. G.; Baylin, S. B. Gene Silencing in Cancer in Association with Promoter Hypermethylation. *http://dx.doi.org/10.1056/NEJMra023075* **2009**.

(41) Schuebel, K.; Gitik, M.; Domschke, K.; Goldman, D. Making Sense of Epigenetics. *International Journal of Neuropsychopharmacology*. 2016.

(42) Bird, A. DNA Methylation Patterns and Epigenetic Memory. *Genes Dev* **2002**, *16*, 6–21.

(43) Gardiner-Garden, M.; Frommer, M. CpG Islands in Vertebrate Genomes. *J. Mol. Biol.* **1987**, *196* (2), 261–282.

(44) Inbar-Feigenberg, M.; Choufani, S.; Butcher, D. T.; Roifman, M.; Weksberg, R. Basic Concepts of Epigenetics. *Fertil. Steril.* **2013**, *99* (3), 607–615.

(45) Esteller, M. Epigenetic Gene Silencing in Cancer: The DNA Hypermethylome. *Hum. Mol. Genet.* **2007**, *16* (R1), 50–59.

(46) Esteller, M.; Herman, J. G. Cancer as an Epigenetic Disease: DNA Methylation and Chromatin Alterations in Human Tumours. *J. Pathol.* **2002**, *196* (1), 1–7.

(47) Pfeifer, G. P.; Rauch, T. A. DNA Methylation Patterns in Lung Carcinomas. *Seminars in Cancer Biology*. 2009, pp 181–187.

(48) Rauch, T. a; Zhong, X.; Wu, X.; Wang, M.; Kernstine, K. H.; Wang, Z.; Riggs, A. D.; Pfeifer, G. P. High-Resolution Mapping of DNA Hypermethylation and Hypomethylation in Lung Cancer. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (1), 252–257.

(49) Grunau, C.; Brun, M. E.; Rivals, I.; Selves, J.; Hindermann, W.; Favre-Mercuret, M.; Granier, G.; De Sario, A. BAGE Hypomethylation, a New Epigenetic Biomarker for Colon Cancer Detection. *Cancer Epidemiol. Biomarkers Prev.* **2008**, *17* (6), 1374–1379.

(50) Ortmann, C. A.; Eisele, L.; Nückel, H.; Klein-Hitpass, L.; Führer, A.; Dührsen, U.; Zeschnigk, M. Aberrant Hypomethylation of the Cancer - Testis Antigen PRAME Correlates with PRAME Expression in Acute Myeloid Leukemia. *Ann. Hematol.* **2008**, *87* (10), 809–818.

(51) Novak, P.; Jensen, T.; Oshiro, M. M.; Watts, G. S.; Kim, C. J.; Futscher, B. W. Agglomerative Epigenetic Aberrations Are a Common Event in Human Breast Cancer. *Cancer Res.* **2008**, *68* (20), 8616–8625.

(52) Pakneshan, P.; Têtu, B.; Rabbani, S. A. Demethylation of Urokinase Promoter as a Prognostic Marker in Patients with Breast Carcinoma. *Clin. Cancer Res.* **2004**, *10* (9), 3035–3041.

(53) Ateeq, B.; Unterberger, A.; Szyf, M.; Rabbani, S. A. Pharmacological Inhibition of DNA Methylation Induces Proinvasive and Prometastatic Genes In Vitro and In Vivo. *Neoplasia* **2008**, *10* (3), 266–278.

(54) Lopez-Serra, L.; Esteller, M. Proteins That Bind Methylated DNA and Human

Cancer: Reading the Wrong Words. *British Journal of Cancer*. 2008, pp 1881–1885.

(55) Nephew, K. P.; Huang, T. H. M. Epigenetic Gene Silencing in Cancer Initiation and Progression. *Cancer Letters*. 2003, pp 125–133.

(56) Greger, V.; Passarge, E.; Höpping, W.; Messmer, E.; Horsthemke, B. Epigenetic Changes May Contribute to the Formation and Spontaneous Regression of Retinoblastoma. *Hum. Genet.* **1989**, *83* (2), 155–158.

(57) Wang, J.; Wang, B.; Chen, X.; Bi, J. The Prognostic Value of RASSF1A Promoter Hypermethylation in Non-Small Cell Lung Carcinoma: A Systematic Review and Meta-Analysis. *Carcinogenesis* **2011**, *32* (3), 411–416.

(58) Balgkouranidou, I.; Matthaios, D.; Karayiannakis, A.; Bolanaki, H.; Michailidis, P.; Xenidis, N.; Amarantidis, K.; Chelis, L.; Trypsianis, G.; Chatzaki, E.; et al. Prognostic Role of APC and RASSF1A Promoter Methylation Status in Cell Free Circulating DNA of Operable Gastric Cancer Patients. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* **2015**, *778*, 46–51.

(59) Dworkin, A. M.; Huang, T. H. M.; Toland, A. E. Epigenetic Alterations in the Breast: Implications for Breast Cancer Detection, Prognosis and Treatment. *Seminars in Cancer Biology*. 2009, pp 165–171.

(60) Shukla, S.; Mirza, S.; Sharma, G.; Parshad, R.; Gupta, S. D.; Ralhan, R. Detection of RASSF1A and RARβ Hypermethylation in Serum DNA from Breast Cancer Patients. *Epigenetics* **2006**, *1* (2), 88–93.

(61) Jerónimo, C.; Costa, I.; Martins, M. C.; Monteiro, P.; Lisboa, S.; Palmeira, C.; Henrique, R.; Teixeira, M. R.; Lopes, C. Detection of Gene Promoter Hypermethylation in Fine Needle Washings from Breast Lesions. *Clin. Cancer Res.* **2003**, *9* (9), 3413–3417.

(62) Terry, M. B.; McDonald, J. A.; Wu, H. C.; Eng, S.; Santella, R. M. Epigenetic Biomarkers of Breast Cancer Risk: Across the Breast Cancer Prevention Continuum. In *Advances in Experimental Medicine and Biology*; 2016; Vol. 882, pp 33–68.

(63) Jeronimo, C.; Monteiro, P.; Henrique, R.; Dinis-Ribeiro, M.; Costa, I.; Costa,

V. L.; Filipe, L.; Carvalho, A. L.; Hoque, M. O.; Pais, I.; et al. Quantitative Hypermethylation of a Small Panel of Genes Augments the Diagnostic Accuracy in Fine-Needle Aspirate Washings of Breast Lesions. *Breast Cancer Res. Treat.* **2008**, *109* (1), 27–34.

(64) Sunami, E.; Shinozaki, M.; Sim, M. S.; Nguyen, S. L.; Vu, A. T.; Giuliano, A. E.; Hoon, D. S. B. Estrogen Receptor and HER2/Neu Status Affect Epigenetic Differences of Tumor-Related Genes in Primary Breast Tumors. *Breast Cancer Res.* **2008**, *10* (3), 1–11.

(65) Widschwendter, M.; Siegmund, K. D.; Müller, H. M.; Fiegl, H.; Marth, C.; Müller-Holzner, E.; Jones, P. A.; Laird, P. W. Association of Breast Cancer DNA Methylation Profiles with Hormone Receptor Status and Response to Tamoxifen. *Cancer Res.* **2004**, *64* (11), 3807–3813.

(66) Nimmrich, I.; Sieuwerts, A. M.; Meijer-Van Gelder, M. E.; Schwope, I.; Bolt-De Vries, J.; Harbeck, N.; Koenig, T.; Hartmann, O.; Kluth, A.; Dietrich, D.; et al. DNA Hypermethylation of PITX2 Is a Marker of Poor Prognosis in Untreated Lymph Node-Negative Hormone Receptor-Positive Breast Cancer Patients. *Breast Cancer Res. Treat.* **2008**, *111* (3), 429–437.

(67) Li, L.; Lee, K.-M.; Han, W.; Choi, J.-Y.; Lee, J.-Y.; Kang, G. H.; Park, S. K.; Noh, D.-Y.; Yoo, K.-Y.; Kang, D. Estrogen and Progesterone Receptor Status Affect Genome-Wide DNA Methylation Profile in Breast Cancer. *Hum. Mol. Genet.* **2010**, *19* (21), 4273–4277.

(68) Schilsky, R. L. Implementing Personalized Cancer Care. *Nature Reviews Clinical Oncology*. 2014, pp 432–438.

(69) Gingras, I.; Salgado, R.; Ignatiadis, M. Liquid Biopsy. *Curr. Opin. Oncol.* **2015**, *27* (6), 560–567.

(70) Loughran, C. F.; Keeling, C. R. Seeding of Tumour Cells Following Breast Biopsy: A Literature Review. *Br. J. Radiol.* **2011**, *84* (1006), 869–874.

(71) Bedard, P. L.; Hansen, A. R.; Ratain, M. J.; Siu, L. L. Tumour Heterogeneity in the Clinic. *Nature*. 2013, pp 355–364.

(72) Brooks, J. D. Translational Genomics: The Challenge of Developing Cancer Biomarkers. *Genome Research*. 2012, pp 183–187.

(73) Mandel, P.; Metais, P. Les Acides Nucléiques Du Plasma Sanguin Chez l'homme. *C. R. Seances Soc. Biol. Ses Fil.* 1948, pp 241–243.

(74) Stroun, M.; Anker, P.; Lyautey, J.; Lederrey, C.; Maurice, P. A. Isolation and Characterization of DNA from the Plasma of Cancer Patients. *Eur J Cancer Clin Oncol* **1987**, *23* (6), 707–712.

(75) Jahr, S.; Hentze, H.; Englisch, S.; Hardt, D.; Fackelmayer, F. O.; Hesch, R. D.; Knippers, R. DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells. *Cancer Res.* **2001**, *61* (4), 1659–1665.

(76) Skvortsova, T. E.; Rykova, E. Y.; Tamkovich, S. N.; Bryzgunova, O. E.; Starikov, A. V.; Kuznetsova, N. P.; Vlassov, V. V.; Laktionov, P. P. Cell-Free and Cell-Bound Circulating DNA in Breast Tumours: DNA Quantification and Analysis of Tumour-Related Gene Methylation. *Br. J. Cancer* **2006**, *94* (10), 1492–1495.

(77) Shapiro, B.; Chakrabarty, M.; Cohn, E. M.; Leon, S. a. Determination of Circulating DNA Levels in Patients with Benign or Malignant Gastrointestinal Disease. *Cancer* **1983**, *51* (11), 2116–2120.

(78) Tan, E. M.; Schur, P. H.; Carr, R. I.; Kunkel, H. G. Deoxybonucleic Acid (DNA) and Antibodies to DNA in the Serum of Patients with Systemic Lupus Erythematosus. *J. Clin. Invest.* **1966**, *45* (11), 1732–1740.

(79) Koffler, D.; Agnello, V.; Winchester, R.; Kunkel, H. G. The Occurrence of Single-Stranded DNA in the Serum of Patients with Systemic Lupus Erythematosus and Other Diseases. *J. Clin. Invest.* **1973**, *52* (1), 198–204.

(80) Sands, J.; Li, Q.; Hornberger, J. Urine Circulating-Tumor DNA (CtDNA) Detection of Acquired EGFR T790M Mutation in Non-Small-Cell Lung Cancer: An Outcomes and Total Cost-of-Care Analysis. *Lung Cancer* **2017**, *110*, 19–25.

(81) Husain, H.; Nykin, D.; Bui, N.; Quan, D.; Gomez, G.; Woodward, B.; Venkatapathy, S.; Duttagupta, R.; Fung, E.; Lippman, S. M.; et al. Cell-Free DNA from Ascites and Pleural Effusions: Molecular Insights into Genomic Aberrations and Disease Biology. *Mol. Cancer Ther.* **2017**, *16* (5), 948–955.

(82) Pan, W.; Gu, W.; Nagpal, S.; Gephart, M. H.; Quake, S. R. Brain Tumor Mutations Detected in Cerebral Spinal Fluid. *Clin. Chem.* **2015**, *61* (3), 514–522.

(83) Umetani, N.; Giuliano, A. E.; Hiramatsu, S. H.; Amersi, F.; Nakagawa, T.; Martino, S.; Hoon, D. S. B. Prediction of Breast Tumor Progression by Integrity of Free Circulating DNA in Serum. *J. Clin. Oncol.* **2006**, *24* (26), 4270–4276.

(84) Wang, J.-Y.; Hsieh, J.-S.; Chang, M.-Y.; Huang, T.-J.; Chen, F.-M.; Cheng, T.-L.; Alexandersen, K.; Huang, Y.-S.; Tzou, W.-S.; Lin, S.-R. Molecular Detection of APC, K- Ras, and P53 Mutations in the Serum of Colorectal Cancer Patients as Circulating Biomarkers. *World J. Surg.* **2004**, *28* (7), 721–726.

(85) Chen, X. Q.; Bonnefoi, H.; Pelte, M. F.; Lyautey, J.; Lederrey, C.; Movarekhi, S.; Schaeffer, P.; Mulcahy, H. E.; Meyer, P.; Stroun, M.; et al. Telomerase RNA as a Detection Marker in the Serum of Breast Cancer Patients. *Clin Cancer Res* **2000**, *6* (10), 3823–3826.

(86) Leon, S. A.; Shapiro, B.; Sklaroff, D. M.; Yaros, M. J. Free DNA in the Serum of Cancer Patients and the Effect of Therapy. *Cancer Res.* **1977**, *37* (3), 646–650.

(87) Ag, C. C.; Care, P.; Wang, J.-C.; Sahoo, T.; Schonberg, S.; Kopita, K. A.; Ross, L.; Patek, K.; Strom, C. M.; Jiang, P.; et al. The Long and Short of Circulating Cell-Free DNA and the Ins and Outs of Molecular Diagnostics. *Prenat. Diagn.* **2016**, *32* (3), 360–371.

(88) Xu, S.; Lou, F.; Wu, Y.; Sun, D.-Q.; Zhang, J.-B.; Chen, W.; Ye, H.; Liu, J.-H.; Wei, S.; Zhao, M.-Y.; et al. Circulating Tumor DNA Identified by Targeted Sequencing in Advanced-Stage Non-Small Cell Lung Cancer Patients. *Cancer Lett.* **2016**, *370* (2), 324–331.

(89) Stroun, M.; Lyautey, J.; Lederrey, C.; Olson-Sand, A.; Anker, P. About the Possible Origin and Mechanism of Circulating DNA: Apoptosis and Active DNA Release. In *Clinica Chimica Acta*; 2001; Vol. 313, pp 139–142.

(90) Van Der Vaart, M.; Pretorius, P. J. The Origin of Circulating Free DNA [1].

Clinical Chemistry. 2007, p 2215.

(91) Nagata, S.; Nagase, H.; Kawane, K.; Mukae, N.; Fukuyama, H. Degradation of Chromosomal DNA during Apoptosis. *Cell Death and Differentiation*. 2003, pp 108–116.

(92) Choi, J. J.; Reich, C. F.; Pisetsky, D. S. The Role of Macrophages in the in Vitro Generation of Extracellular DNA from Apoptotic and Necrotic Cells. *Immunology* **2005**, *115* (1), 55–62.

(93) Jung, K.; Fleischhacker, M.; Rabien, A. Cell-Free DNA in the Blood as a Solid Tumor Biomarker-A Critical Appraisal of the Literature. *Clinica Chimica Acta*. 2010, pp 1611–1624.

(94) Wang, W.; Kong, P.; Ma, G.; Li, L.; Zhu, J.; Xia, T.; Xie, H.; Zhou, W.; Wang, S. Characterization of the Release and Biological Significance of Cell-Free DNA from Breast Cancer Cell Lines. *Oncotarget* **2017**, *8* (26).

(95) Lowry, M. C.; Gallagher, W. M.; O'Driscoll, L. The Role of Exosomes in Breast Cancer. *Clinical Chemistry*. 2015, pp 1457–1465.

(96) Bhangu, J. S.; Taghizadeh, H.; Braunschmid, T.; Bachleitner-Hofmann, T.; Mannhalter, C. Circulating Cell-Free DNA in Plasma of Colorectal Cancer Patients - A Potential Biomarker for Tumor Burden. *Surg. Oncol.* **2017**, *26* (4), 395–401.

(97) Salvi, S.; Gurioli, G.; De Giorgi, U.; Conteduca, V.; Tedaldi, G.; Calistri, D.; Casadio, V. Cell-Free DNA as a Diagnostic Marker for Cancer: Current Insights. *OncoTargets and Therapy*. 2016, pp 6549–6559.

(98) Fournié, G. J.; Courtin, J. P.; Laval, F.; Chalé, J. J.; Pourrat, J. P.; Pujazon, M. C.; Lauque, D.; Carles, P. Plasma DNA as a Marker of Cancerous Cell Death. Investigations in Patients Suffering from Lung Cancer and in Nude Mice Bearing Human Tumours. *Cancer Lett.* **1995**, *91* (2), 221–227.

(99) Paci, M.; Maramotti, S.; Bellesia, E.; Formisano, D.; Albertazzi, L.; Ricchetti, T.; Ferrari, G.; Annessi, V.; Lasagni, D.; Carbonelli, C.; et al. Circulating Plasma DNA as Diagnostic Biomarker in Non-Small Cell Lung Cancer. *Lung Cancer* **2009**, *64* (1), 92–97.

(100) Kohler, C.; Radpour, R.; Barekati, Z.; Asadollahi, R.; Bitzer, J.; Wight, E.;

Bürki, N.; Diesch, C.; Holzgreve, W.; Zhong, X. Y. Levels of Plasma Circulating Cell Free Nuclear and Mitochondrial DNA as Potential Biomarkers for Breast Tumors. *Mol. Cancer* **2009**, *8*.

(101) Zhong, X. Y.; Ladewig, A.; Schmid, S.; Wight, E.; Hahn, S.; Holzgreve, W. Elevated Level of Cell-Free Plasma DNA Is Associated with Breast Cancer. *Arch. Gynecol. Obstet.* **2007**, *276* (4), 327–331.

(102) Catarino, R.; Ferreira, M. M.; Rodrigues, H.; Coelho, A.; Nogal, A.; Sousa, A.; Medeiros, R. Quantification of Free Circulating Tumor DNA as a Diagnostic Marker for Breast Cancer. *DNA Cell Biol.* **2008**, *27* (8), 415–421.

(103) Papadopoulou, E.; Davilas, E.; Sotiriou, V.; Georgakopoulos, E.; Georgakopoulou, S.; Koliopanos, A.; Aggelakis, F.; Dardoufas, K.; Agnanti, N. J.; Karydas, I.; et al. Cell-Free DNA and RNA in Plasma as a New Molecular Marker for Prostate and Breast Cancer. In *Annals of the New York Academy of Sciences*; 2006; Vol. 1075, pp 235–243.

(104) Schwarzenbach, H.; Muller, V.; Stahmann, N.; Pantel, K. Detection and Characterization of Circulating Microsatellite-DNA in Blood of Patients with Breast Cancer. *Circ. Nucleic Acids PlasmaSerum Iii Serum Proteomics* **2004**, *1022*, 25–32.

(105) Oakman, C.; Pestrin, M.; Bessi, S.; Galardi, F.; di Leo, A. Significance of Micrometastases: Circulating Tumor Cells and Disseminated Tumor Cells in Early Breast Cancer. *Cancers*. 2010, pp 1221–1235.

(106) Dasgupta, A.; Lim, A. R.; Ghajar, C. M. Circulating and Disseminated Tumor Cells: Harbingers or Initiators of Metastasis? *Mol. Oncol.* **2017**, *11* (1), 40–61.

(107) Riva, F.; Dronov, O. I.; Khomenko, D. I.; Huguet, F.; Louvet, C.; Mariani, P.; Stern, M. H.; Lantz, O.; Proudhon, C.; Pierga, J. Y.; et al. Clinical Applications of Circulating Tumor DNA and Circulating Tumor Cells in Pancreatic Cancer. *Molecular Oncology*. 2016, pp 481–493.

(108) Schwarzenbach, H.; Alix-Panabières, C.; Müller, I.; Letang, N.; Vendrell, J. P.; Rebillard, X.; Pantel, K. Cell-Free Tumor DNA in Blood Plasma as a Marker for Circulating Tumor Cells in Prostate Cancer. *Clin. Cancer Res.* **2009**, *15* (3), 1032–1038.

(109) De Mattos-Arruda, L.; Olmos, D.; Tabernero, J. Prognostic and Predictive Roles for Circulating Biomarkers in Gastrointestinal Cancer. *Futur. Oncol.* **2011**, *7* (12), 1385–1397.

(110) Van Der Auwera, I.; Elst, H. J.; Van Laere, S. J.; Maes, H.; Huget, P.; Van Dam, P.; Van Marck, E. A.; Vermeulen, P. B.; Dirix, L. Y. The Presence of Circulating Total DNA and Methylated Genes Is Associated with Circulating Tumour Cells in Blood from Breast Cancer Patients. *Br. J. Cancer* **2009**, *100* (8), 1277–1286.

(111) Schwarzenbach, H.; Hoon, D. S. B.; Pantel, K. Cell-Free Nucleic Acids as Biomarkers in Cancer Patients. *Nature Reviews Cancer*. 2011, pp 426–437.

(112) Minchin, R. F.; Carpenter, D.; Orr, R. J. Polyinosinic Acid and Polycationic Liposomes Attenuate the Hepatic Clearance of Circulating Plasmid DNA. *J Pharmacol Exp Ther* **2001**, *296* (3), 1006–1012.

(113) Korabecna, M.; Opatrna, S.; Wirth, J.; Rulcova, K.; Eiselt, J.; Sefrna, F.; Horinek, A. Cell-Free Plasma DNA during Peritoneal Dialysis and Hemodialysis and in Patients with Chronic Kidney Disease. In *Annals of the New York Academy of Sciences*; 2008; Vol. 1137, pp 296–301.

(114) Tamkovich, S. N.; Vlassov, V. V.; Laktionov, P. P. Circulating DNA in the Blood and Its Application in Medical Diagnosis. *Mol. Biol.* **2008**, *42* (1), 9–19.

(115) Puszyk, W. M.; Crea, F.; Old, R. W. Unequal Representation of Different Unique Genomic DNA Sequences in the Cell-Free Plasma DNA of Individual Donors. *Clin. Biochem.* **2009**, *42* (7–8), 736–738.

(116) Alegre, E.; Sammamed, M.; Fernández-Landázuri, S.; Zubiri, L.; González, Á. Circulating Biomarkers in Malignant Melanoma. *Adv. Clin. Chem.* **2015**, *69*, 47–88.

(117) Nawroz, H.; Koch, W.; Anker, P.; Stroun, M.; Sidransky, D. Microsatellite Alterations in Serum DNA of Head and Neck Cancer Patients. *Nat. Med.* **1996**, *2* (9), 1035–1037.

(118) Chiang, P. W.; Beer, D. G.; Wei, W. L.; Orringer, M. B.; Kurnit, D. M. Detection of ErbB-2 Amplifications in Turners and Sera from Esophageal

Carcinoma Patients. *Clin. Cancer Res.* **1999**, *5* (6), 1381–1386.

(119) Physiology, P.; Isotopes, P.; Science, F. Point Mutations of the N-Ras Gene in the Blood Plasma DNA of Patients with Myelodysplastic Syndrome or Acute Myelogenous Leukaemia. **1994**, 774–779.

(120) Stroun, M; Anker, P; Maurice, P; Lyautey, J; Lederrey, C; Beljanski, M. Neoplastic Characteristics of the DNA Found in Plasma of Cancer Patients. *Oncology* **1989**, No. 46, 318–322.

(121) Silva, J. M.; Garcia, J. M.; Dominguez, G.; Silva, J.; Miralles, C.; Cantos, B.; Coca, S.; Provencio, M.; Espana, P.; Bonilla, F. Persistence of Tumor DNA in Plasma of Breast Cancer Patients after Mastectomy 3626. *Ann. Surg. Oncol.* **11AD**, *Annals-of-*, 1–76.

(122) Ito, T.; Kaneko, K.; Makino, R.; Konishi, K.; Kurahashi, T.; Ito, H.; Katagiri, A.; Kushima, M.; Kusano, M.; Mitamura, K.; et al. Clinical Significance in Molecular Detection of P53 Mutation in Serum of Patients with Colorectal Carcinoma. *Oncol Rep* **2003**, *10* (6), 1937–1942.

(123) Kimura, T.; Holland, W. S.; Kawaguchi, T.; Williamson, S. K.; Chansky, K.; Crowley, J. J.; Doroshow, J. H.; Lenz, H. J.; Gandara, D. R.; Gumerlock, P. H. Mutant DNA in Plasma of Lung Cancer Patients: Potential for Monitoring Response to Therapy. *Ann. N. Y. Acad. Sci.* **2004**, *1022*, 55–60.

(124) Szymañska, K.; Chen, J. G.; Cui, Y.; Yun, Y. G.; Turner, P. C.; Villar, S.; Wild, C. P.; Parkin, D. M.; Hainaut, P. TP53 R249S Mutations, Exposure to Aflatoxin, and Occurrence of Hepatocellular Carcinoma in a Cohort of Chronic Hepatitis B Virus Carriers from Qidong, China. *Cancer Epidemiol. Biomarkers Prev.* **2009**, *18* (5), 1638–1643.

(125) Gormally, E.; Vineis, P.; Matullo, G.; Veglia, F.; Caboux, E.; Le Roux, E.; Peluso, M.; Garte, S.; Guarrera, S.; Munnia, A.; et al. TP53 and KRAS2 Mutations in Plasma DNA of Healthy Subjects and Subsequent Cancer Occurrence: A Prospective Study. *Cancer Res.* **2006**, *66* (13), 6871–6876.

(126) Otsuka, J.; Okuda, T.; Sekizawa, a; Amemiya, S.; Saito, H.; Okai, T.; Kushima, M. Detection of P53 Mutations in the Plasma DNA of Patients with Ovarian Cancer. *Int. J. Gynecol. Cancer* **2004**, *14* (3), 459–464.

(127) Spindler, K.-L. G.; Pallisgaard, N.; Vogelius, I. S.; Jakobsen, A. Quantitative Cell-Free DNA, KRAS, and BRAF Mutations in Plasma from Patients with Metastatic Colorectal Cancer during Treatment with Cetuximab and Irinotecan. *Clin. Cancer Res.* **2012**, *18* (4), 1177–1185.

(128) Fodde, R. The APC Gene in Colorectal Cancer. *Eur. J. Cancer* **2002**, *38* (7), 867–871.

(129) Hsieh, J.-S.; Lin, S.-R.; Chang, M.-Y.; Chen, F.-M.; Lu, C.-Y.; Huang, T.-J.; Huang, Y.-S.; Huang, C.-J.; Wang, J.-Y. APC, K-Ras, and P53 Gene Mutations in Colorectal Cancer Patients: Correlation to Clinicopathologic Features and Postoperative Surveillance. *Am. Surg.* **2005**, *71* (4), 336–343.

(130) Shinozaki, M.; O'Day, S. J.; Kitago, M.; Amersi, F.; Kuo, C.; Kim, J.; Wang, H. J.; Hoon, D. S. B. Utility of Circulating B-RAF DNA Mutation in Serum for Monitoring Melanoma Patients Receiving Biochemotherapy. *Clin. Cancer Res.* **2007**, *13* (7), 2068–2074.

(131) MacK, P. C.; Holland, W. S.; Burich, R. A.; Sangha, R.; Solis, L. J.; Li, Y.; Beckett, L. A.; Lara, P. N.; Davies, A. M.; Gandara, D. R. EGFR Mutations Detected in Plasma Are Associated with Patient Outcomes in Erlotinib plus Docetaxel-Treated Non-Small Cell Lung Cancer. *J. Thorac. Oncol.* **2009**, *4* (12), 1466–1472.

(132) Cheng, J.; Tang, Q.; Cao, X.; Burwinkel, B. Cell-Free Circulating DNA Integrity Based on Peripheral Blood as a Biomarker for Diagnosis of Cancer: A Systematic Review. *Cancer Epidemiol. Biomarkers Prev.* **2017**, cebp.0502.2017.

(133) Wang, B. G.; Huang, H.; Chen, Y.; Bristow, R. E.; Kassauei, K.; Cheng, C.; Roden, R.; Sokoll, L. J.; Chan, D. W.; Shih, I. Increased Plasma DNA Integrity in Cancer Patients Increased Plasma DNA Integrity in Cancer Patients 1. **2003**, No. 410, 3966–3968.

(134) Umetani, N.; Kim, J.; Hiramatsu, S.; Reber, H. A.; Hines, O. J.; Bilchik, A. J.; Hoon, D. S. B. Increased Integrity of Free Circulating DNA in Sera of Patients with Colorectal or Periampullary Cancer: Direct Quantitative PCR for ALU Repeats. *Clin. Chem.* **2006**, *52* (6), 1062–1069.

(135) Madhavan, D.; Wallwiener, M.; Bents, K.; Zucknick, M.; Nees, J.; Schott, S.; Cuk, K.; Riethdorf, S.; Trumpp, A.; Pantel, K.; et al. Plasma DNA Integrity as a Biomarker for Primary and Metastatic Breast Cancer and Potential Marker for Early Diagnosis. *Breast Cancer Res. Treat.* **2014**, *146* (1), 163–174.

(136) Gao, Y. J.; He, Y. J.; Yang, Z. L.; Shao, H. Y.; Zuo, Y.; Bai, Y.; Chen, H.; Chen, X. C.; Qin, F. X.; Tan, S.; et al. Increased Integrity of Circulating Cell-Free DNA in Plasma of Patients with Acute Leukemia. *Clin. Chem. Lab. Med.* **2010**, *48* (11), 1651–1656.

(137) Salvianti, F.; Pinzani, P.; Verderio, P.; Ciniselli, C. M.; Massi, D.; de Giorgi, V.; Grazzini, M.; Pazzagli, M.; Orlando, C. Multiparametric Analysis of Cell-Free DNA in Melanoma Patients. *PLoS One* **2012**, *7* (11), 1–9.

(138) Mead, R.; Duku, M.; Bhandari, P.; Cree, I. A. Circulating Tumour Markers Can Define Patients with Normal Colons, Benign Polyps, and Cancers. *Br. J. Cancer* **2011**, *105* (2), 239–245.

(139) Huang, A.; Zhang, X.; Zhou, S.-L.; Cao, Y.; Huang, X.-W.; Fan, J.; Yang, X.-R.; Zhou, J. Plasma Circulating Cell-Free DNA Integrity as a Promising Biomarker for Diagnosis and Surveillance in Patients with Hepatocellular Carcinoma. *J. Cancer* **2016**, *7* (13), 1798–1803.

(140) Maltoni, R.; Casadio, V.; Ravaioli, S.; Foca, F.; Tumedei, M.; Salvi, S.; Martignano, F.; Calistri, D.; Rocca, A.; Schirone, A.; et al. Cell-Free DNA Detected by " Liquid Biopsy " as a Potential Prognostic Biomarker in Early Breast Cancer. *Oncotarget* **2017**, *8* (10), 16642–16649.

(141) Vilar, E.; Gruber, S. B. Microsatellite Instability in Colorectal Cancerthe Stable Evidence. *Nat. Rev. Clin. Oncol.* **2010**, *7* (3), 153–162.

(142) Nawroz-Danish, H.; Eisenberger, C. F.; Yoo, G. H.; Wu, L.; Koch, W.; Black, C.; Ensley, J. F.; Wei, W. Z.; Sidransky, D. Microsatellite Analysis of Serum DNA in Patients with Head and Neck Cancer. *Int. J. Cancer* **2004**, *111* (1), 96–100.

(143) Fleischhacker, M.; Schmidt, B. Circulating Nucleic Acids (CNAs) and Cancer-A Survey. *Biochim. Biophys. Acta - Rev. Cancer* **2007**, *1775* (1), 181–232.

(144) An, J. H.; Choi, A.; Shin, K. J.; Yang, W. I.; Lee, H. Y. DNA Methylation-Specific Multiplex Assays for Body Fluid Identification. *Int. J. Legal Med.* **2013**, *127* (1), 35–43.

(145) Swarup, V.; Rajeswari, M. R. Circulating (Cell-Free) Nucleic Acids - A Promising, Non-Invasive Tool for Early Detection of Several Human Diseases. *FEBS Lett.* **2007**, *581* (5), 795–799.

(146) Takai, E.; Totoki, Y.; Nakamura, H.; Morizane, C.; Nara, S.; Hama, N.; Suzuki, M.; Furukawa, E.; Kato, M.; Hayashi, H.; et al. Clinical Utility of Circulating Tumor DNA for Molecular Assessment in Pancreatic Cancer. *Sci. Rep.* **2015**, *5*, 1–10.

(147) Bulla, A.; De Witt, B.; Ammerlaan, W.; Betsou, F.; Lescuyer, P. Blood DNA Yield but Not Integrity or Methylation Is Impacted After Long-Term Storage. *Biopreserv. Biobank.* **2016**, *14* (1), 29–38.

(148) Weisenberger, D. J.; Trinh, B. N.; Campan, M.; Sharma, S.; Long, T. I.; Ananthnarayan, S.; Liang, G.; Esteva, F. J.; Hortobagyi, G. N.; McCormick, F.; et al. DNA Methylation Analysis by Digital Bisulfite Genomic Sequencing and Digital MethyLight. *Nucleic Acids Res.* **2008**, *36* (14), 4689–4698.

(149) Weber, M.; Davies, J. J.; Wittig, D.; Oakeley, E. J.; Haase, M.; Lam, W. L.; Schübeler, D. Chromosome-Wide and Promoter-Specific Analyses Identify Sites of Differential DNA Methylation in Normal and Transformed Human Cells. *Nat. Genet.* **2005**, *37* (8), 853–862.

(150) Bock, C. Analysing and Interpreting DNA Methylation Data. *Nature reviews. Genetics*. 2012, pp 705–719.

(151) Juneau, K.; Bogard, P. E.; Huang, S.; Mohseni, M.; Wang, E. T.; Ryvkin, P.; Kingsley, C.; Struble, C. A.; Oliphant, A.; Zahn, J. M. Microarray-Based Cell-Free DNA Analysis Improves Noninvasive Prenatal Testing. *Fetal Diagn. Ther.* **2014**, *36* (4), 282–286.

(152) Bennett, C. W.; Berchem, G.; Kim, Y. J.; El-Khoury, V. Cell-Free DNA and next-Generation Sequencing in the Service of Personalized Medicine for Lung Cancer. *Oncotarget* **2015**.

(153) Gu, H.; Bock, C.; Mikkelsen, T. S.; Jäger, N.; Smith, Z. D.; Tomazou, E.;

Gnirke, A.; Lander, E. S.; Meissner, A. Genome-Scale DNA Methylation Mapping of Clinical Samples at Single-Nucleotide Resolution. *Nat. Methods* **2010**, *7* (2), 133–136.

(154) Herman, J. G.; Graff, J. R.; Myohanen, S.; Nelkin, B. D.; Baylin, S. B. Methylation-Specific PCR: A Novel PCR Assay for Methylation Status of CpG Islands. *Proc. Natl. Acad. Sci.* **1996**, *93* (18), 9821–9826.

(155) Eads, C. A.; Danenberg, K. D.; Kawakami, K.; Saltz, L. B.; Danenberg, P. V; Laird, P. W. CpG Island Hypermethylation in Human Colorectal Tumors Is Not Associated with DNA Methyltransferase Overexpression Advances in Brief CpG Island Hypermethylation in Human Colorectal Tumors Is Not Associated With. *Cancer Res.* **1999**, *59*, 2302–2306.

(156) Eads, C. A.; Danenberg, K. D.; Kawakami, K.; Saltz, L. B.; Blake, C.; Shibata, D.; Danenberg, P. V; Laird, P. W. MethyLight: A High-Throughput Assay to Measure DNA Methylation. *Nucleic Acids Res* **2000**, *28* (8), E32.

(157) Fink, L.; Seeger, W.; Ermert, L.; Hänze, J.; Stahl, U.; Grimminger, F.; Kummer, W.; Bohle, R. M. Real-Time Quantitative RT-PCR after Laser-Assisted Cell Picking. *Nat. Med.* **1998**, *4* (11), 1329–1333.

(158) Lister, R.; Ecker, J. R. Finding the Fifth Base: Genome-Wide Sequencing of Cytosine Methylation. *Genome Research*. 2009, pp 959–966.

(159) Yamamoto, N.; Nakayama, T.; Kajita, M.; Miyake, T.; Iwamoto, T.; Kim, S. J.; Sakai, A.; Ishihara, H.; Tamaki, Y.; Noguchi, S. Detection of Aberrant Promoter Methylation of GSTP1, RASSF1A, and RARβ2 in Serum DNA of Patients with Breast Cancer by a Newly Established One-Step Methylation-Specific PCR Assay. *Breast Cancer Res. Treat.* **2012**, *132* (1), 165–173.

(160) Li, D.; Li, P.; Wu, J.; Yi, J.; Dou, Y.; Guo, X.; Yin, Y.; Wang, D.; Ma, C.; Qiu, L. Methylation of NBPF1 as a Novel Marker for the Detection of Plasma Cell-Free DNA of Breast Cancer Patients. *Clin. Chim. Acta* **2018**, *484*, 81–86.

(161) Li, Z.; Guo, X.; Tang, L.; Peng, L.; Chen, M.; Luo, X.; Wang, S.; Xiao, Z.; Deng, Z.; Dai, L.; et al. Methylation Analysis of Plasma Cell-Free DNA for Breast Cancer Early Detection Using Bisulfite next-Generation Sequencing. *Tumor Biol.* **2016**, *37* (10), 13111–13119.

(162) Wang, B.; Yu, L.; Luo, X.; Huang, L.; Li, Q. S.; Shao, X. S.; Liu, Y.; Fan, Y.; Yang, G. Z. Detection of OPCML Methylation, a Possible Epigenetic Marker, from Free Serum Circulating DNA to Improve the Diagnosis of Early-Stage Ovarian Epithelial Cancer. *Oncol. Lett.* **2017**, *14* (1), 217–223.

(163) Barták, B. K.; Kalmár, A.; Péterfia, B.; Patai, Á. V.; Galamb, O.; Valcz, G.; Spisák, S.; Wichmann, B.; Nagy, Z. B.; Tóth, K.; et al. Colorectal Adenoma and Cancer Detection Based on Altered Methylation Pattern of SFRP1, SFRP2, SDC2, and PRIMA1 in Plasma Samples. *Epigenetics* **2017**, *12* (9), 751–763.

(164) Naini, M. A.; Kavousipour, S.; Hasanzarini, M.; Nasrollah, A.; Monabati, A.; Mokarram, P. O6-Methyguanine-DNA Methyl Transferase (MGMT) Promoter Methylation in Serum DNA of Iranian Patients with Colorectal Cancer. *Asian Pacific J. Cancer Prev.* **2018**, *19* (5).

(165) Jacinto, F. V.; Ballestar, E.; Ropero, S.; Esteller, M. Discovery of Epigenetically Silenced Genes by Methylated DNA Immunoprecipitation in Colon Cancer Cells. *Cancer Res.* **2007**, *67* (24), 11481–11486.

(166) Kneip, C.; Schmidt, B.; Seegebarth, A.; Weickmann, S.; Fleischhacker, M.; Liebenberg, V.; Field, J. K.; Dietrich, D. SHOX2 DNA Methylation Is a Biomarker for the Diagnosis of Lung Cancer in Plasma. *J. Thorac. Oncol.* **2011**, *6* (10), 1632–1638.

(167) Haldrup, C.; Pedersen, A. L.; Øgaard, N.; Strand, S. H.; Høyer, S.; Borre, M.; Ørntoft, T. F.; Sørensen, K. D. Biomarker Potential of ST6GALNAC3 and ZNF660 Promoter Hypermethylation in Prostate Cancer Tissue and Liquid Biopsies. *Mol. Oncol.* **2018**.

(168) Assinder, S. J.; Stanton, J. A. L.; Prasad, P. D. Transgelin: An Actin-Binding Protein and Tumour Suppressor. *International Journal of Biochemistry and Cell Biology*. 2009, pp 482–486.

(169) Lees-Miller, J. P.; Heeley, D. H.; Smillie, L. B.; Kay, C. M. Isolation and Characterization of an Abundant and Novel 22-KDa Protein (SM22) from Chicken Gizzard Smooth Muscle. *J. Biol. Chem.* **1987**, *262* (7), 2988–2993.

(170) Robin, Y. M.; Penel, N.; Pérot, G.; Neuville, A.; Vélasco, V.; Ranchère-

Vince, D.; Terrier, P.; Coindre, J. M. Transgelin Is a Novel Marker of Smooth Muscle Differentiation That Improves Diagnostic Accuracy of Leiomyosarcomas: A Comparative Immunohistochemical Reappraisal of Myogenic Markers in 900 Soft Tissue Tumors. *Mod. Pathol.* **2013**, *26* (4), 502–510.

(171) Pérot, G.; Derré, J.; Coindre, J. M.; Tirode, F.; Lucchesi, C.; Mariani, O.; Gibault, L.; Guillou, L.; Terrier, P.; Aurias, A. Strong Smooth Muscle Differentiation Is Dependent on Myocardin Gene Amplification in Most Human Retroperitoneal Leiomyosarcomas. *Cancer Res.* **2009**, *69* (6), 2269–2278.

(172) Li, L.; Miano, J. M.; Cserjesi, P.; Olson, E. N. SM22α, a Marker of Adult Smooth Muscle, Is Expressed in Multiple Myogenic Lineages during Embryogenesis. *Circ. Res.* **1996**, *78* (2), 188–195.

(173) Gimona, M. Calponin Repeats Regulate Actin Filament Stability and Formation of Podosomes in Smooth Muscle Cells. *Mol. Biol. Cell* **2003**, *14* (6), 2482–2491.

(174) Lawson, D.; Harrison, M.; Shapland, C. Fibroblast Transgelin and Smooth Muscle SM22alpha Are the Same Protein, the Expression of Which Is down-Regulated in Many Cell Lines. *Cell Motil. Cytoskeleton* **1997**, *38* (3), 250–257.

(175) Yang, Z.; Chang, Y.-J.; Miyamoto, H.; Ni, J.; Niu, Y.; Chen, Z.; Chen, Y.-L.; Yao, J. L.; di Sant'Agnese, P. A.; Chang, C. Transgelin Functions as a Suppressor via Inhibition of ARA54-Enhanced Androgen Receptor Transactivation and Prostate Cancer Cell Growth. *Mol. Endocrinol.* **2007**, *21* (2), 343–358.

(176) Nair, R. R.; Solway, J.; Boyd, D. D. Expression Cloning Identifies Transgelin (SM22) as a Novel Repressor of 92-KDa Type IV Collagenase (MMP-9) Expression. *J. Biol. Chem.* **2006**, *281* (36), 26424–26436.

(177) Aikins, A. R.; Kim, M. J.; Raymundo, B.; Kim, C. W. Downregulation of Transgelin Blocks Interleukin-8 Utilization and Suppresses Vasculogenic Mimicry in Breast Cancer Cells. *Exp. Biol. Med.* **2017**, *242* (6), 573–583.

(178) Prasad, P. D.; Stanton, J. A. L.; Assinder, S. J. Expression of the Actin-Associated Protein Transgelin (SM22) Is Decreased in Prostate Cancer. *Cell Tissue Res.* **2010**, *339* (2), 337–347.

(179) Li, Q.; Shi, R.; Wang, Y.; Niu, X. TAGLN Suppresses Proliferation and Invasion, and Induces Apoptosis of Colorectal Carcinoma Cells. *Tumor Biol.* **2013**, *34* (1), 505–513.

(180) Shields, J. M.; Rogers-Graham, K.; Der, C. J. Loss of Transgelin in Breast and Colon Tumors and in RIE-1 Cells by Ras Deregulation of Gene Expression through Raf-Independent Pathways. *J. Biol. Chem.* **2002**, *277* (12), 9790–9799.

(181) Chunhua, L.; Donglan, L.; Xiuqiong, F.; Lihua, Z.; Qin, F.; Yawei, L.; Liang, Z.; Ge, W.; Linlin, J.; Ping, Z.; et al. Apigenin Up-Regulates Transgelin and Inhibits Invasion and Migration of Colorectal Cancer through Decreased Phosphorylation of AKT. *J. Nutr. Biochem.* **2013**, *24* (10), 1766–1775.

(182) Yeo, M.; Park, H. J.; Kim, D. K.; Kim, Y. B.; Cheong, J. Y.; Lee, K. J.; Cho, S. W. Loss of SM22 Is a Characteristic Signature of Colon Carcinogenesis and Its Restoration Suppresses Colon Tumorigenicity in Vivo and in Vitro. *Cancer* **2010**, *116* (11), 2581–2589.

(183) Zhao, L.; Wang, H.; Deng, Y.-J.; Wang, S.; Liu, C.; Jin, H.; Ding, Y.-Q. Transgelin as a Suppressor Is Associated with Poor Prognosis in Colorectal Carcinoma Patients. *Mod. Pathol. an Off. J. United States Can. Acad. Pathol. Inc* **2009**, *22* (6), 786–796.

(184) Sahasrabuddhe, N. A.; Barbhuiya, M. A.; Bhunia, S.; Subbannayya, T.; Gowda, H.; Advani, J.; Shrivastav, B. R.; Navani, S.; Leal, P.; Roa, J. C.; et al. Identification of Prosaposin and Transgelin as Potential Biomarkers for Gallbladder Cancer Using Quantitative Proteomics. *Biochem. Biophys. Res. Commun.* **2014**, *446* (4), 863–869.

(185) Zhou, L.; Zhang, R.; Zhang, L.; Sun, Y.; Yao, W.; Zhao, A.; Li, J.; Yuan, Y. Upregulation of Transgelin Is an Independent Factor Predictive of Poor Prognosis in Patients with Advanced Pancreatic Cancer. *Cancer Sci.* **2013**, *104* (4), 423–430.

(186) Zhou, H.; Zhang, Y.; Chen, Q.; Lin, Y. AKT and JNK Signaling Pathways Increase the Metastatic Potential of Colorectal Cancer Cells by Altering Transgelin Expression. *Dig. Dis. Sci.* **2016**, *61* (4), 1091–1097.

(187) Zhou, H.; Fang, Y.; Weinberger, P. M.; Ding, L.; Cowell, J. K.; Hudson, F. Z.; Ren, M.; Lee, J. R.; Chen, Q.; Su, H.; et al. Transgelin Increases Metastatic Potential of Colorectal Cancer Cells in Vivo and Alters Expression of Genes Involved in Cell Motility. *BMC Cancer* **2016**, *16* (1), 55.

(188) Yu, B.; Chen, X.; Li, J.; Qu, Y.; Su, L.; Peng, Y.; Huang, J.; Yan, J.; Yu, Y.; Gu, Q.; et al. Stromal Fibroblasts in the Microenvironment of Gastric Carcinomas Promote Tumor Metastasis via Upregulating TAGLN Expression. *BMC Cell Biol.* **2013**, *14* (1).

(189) Wu, X.; Dong, L.; Zhang, R.; Ying, K.; Shen, H. Transgelin Overexpression in Lung Adenocarcinoma Is Associated with Tumor Progression. *Int. J. Mol. Med.* **2014**, *34* (2), 585–591.

(190) Huang, Q.; Huang, Q.; Chen, W.; Wang, L.; Lin, W.; Lin, J.; Lin, X. Identification of Transgelin as a Potential Novel Biomarker for Gastric Adenocarcinoma Based on Proteomics Technology. *J. Cancer Res. Clin. Oncol.* **2008**, *134* (11), 1219–1227.

(191) Rho, J. H.; Roehrl, M. H. A.; Wang, J. Y. Tissue Proteomics Reveals Differential and Compartment-Specific Expression of the Homologs Transgelin and Transgelin-2 in Lung Adenocarcinoma and Its Stroma. *J. Proteome Res.* **2009**, *8* (12), 5610–5618.

(192) Zhang, Y.; Liao, R.; Li, H.; Liu, L.; Chen, X.; Chen, H. Expression of Cofilin-1 and Transgelin in Esophageal Squamous Cell Carcinoma. *Med. Sci. Monit.* **2015**, *21*, 2659–2665.

(193) Bu, J.; Bu, X.; Liu, B.; Chen, F.; Chen, P. Increased Expression of Tissue/Salivary Transgelin MRNA Predicts Poor Prognosis in Patients with Oral Squamous Cell Carcinoma (OSCC). *Med. Sci. Monit.* **2015**, *21*, 2275–2281.

(194) Thompson, O.; Moghraby, J. S.; Ayscough, K. R.; Winder, S. J. Depletion of the Actin Bundling Protein SM22/Transgelin Increases Actin Dynamics and

Enhances the Tumourigenic Phenotypes of Cells. *BMC Cell Biol.* **2012**, *13*.

(195) Gröger, C. J.; Grubinger, M.; Waldhör, T.; Vierlinger, K.; Mikulits, W. Meta-Analysis of Gene Expression Signatures Defining the Epithelial to Mesenchymal Transition during Cancer Progression. *PLoS One* **2012**, *7* (12).

(196) Lee, E.-K.; Han, G.-Y.; Park, H. W.; Song, Y.-J.; Kim, C.-W. Transgelin Promotes Migration and Invasion of Cancer Stem Cells. *J. Proteome Res.* **2010**, *9* (10), 5108–5117.

(197) Adam, P. J.; Regan, C. P.; Hautmann, M. B.; Owens, G. K. Positive- and Negative-Acting Kruppel-like Transcription Factors Bind a Transforming Growth Factor Beta Control Element Required for Expression of the Smooth Muscle Cell Differentiation Marker SM22alpha in Vivo. *J. Biol. Chem.* **2000**, *275* (48), 37798–37806.

(198) Chen, S. Smad Proteins Regulate Transcriptional Induction of the SM22alpha Gene by TGF-Beta. *Nucleic Acids Res.* **2003**, *31* (4), 1302–1310.

(199) Qiu, P.; Ritchie, R. P.; Fu, Z.; Cao, D.; Cumming, J.; Miano, J. M.; Wang, D. Z.; Li, H. J.; Li, L. Myocardin Enhances Smad3-Mediated Transforming Growth Factor- B1signaling in a CArG Box-Independent Manner: Smad-Binding Element Is an Important Cis Element for SM22α Transcription in Vivo. *Circ. Res.* **2005**, *97* (10), 983–991.

(200) Deaton, R. A.; Su, C.; Valencia, T. G.; Grant, S. R. Transforming Growth Factor-Beta1-Induced Expression of Smooth Muscle Marker Genes Involves Activation of PKN and P38 MAPK. *J. Biol. Chem.* **2005**, *280* (35), 31172–31181.

(201) Yu, H.; Königshoff, M.; Jayachandran, A.; Handley, D.; Seeger, W.; Kaminski, N.; Eickelberg, O. Transgelin Is a Direct Target of TGF-Beta/Smad3-Dependent Epithelial Cell Migration in Lung Fibrosis. *FASEB J.* **2008**, *22* (6), 1778–1789.

(202) Oft, M.; Peli, J.; Rudaz, C.; Schwarz, H.; Beug, H.; Reichmann, E. TGF-B1 and Ha-Ras Collaborate in Modulating the Phenotypic Plasticity and Invasiveness of Epithelial Tumor Cells. *Genes Dev.* **1996**, *10* (19), 2462–2477.

(203) Christiansen, J. J.; Rajasekaran, A. K. Reassessing Epithelial to Mesenchymal Transition as a Prerequisite for Carcinoma Invasion and Metastasis. *Cancer Research*. 2006, pp 8319–8326.

(204) Lin, Y.; Buckhaults, P. J.; Lee, J. R.; Xiong, H.; Farrell, C.; Podolsky, R. H.; Schade, R. R.; Dynan, W. S. Association of the Actin-Binding Protein Transgelin with Lymph Node Metastasis in Human Colorectal Cancer. *Neoplasia* **2009**, *11* (9), 864-IN5.

(205) Götte, M.; Mohr, C.; Koo, C. Y.; Stock, C.; Vaske, A. K.; Viola, M.; Ibrahim, S. A.; Peddibhotla, S.; Teng, Y. H. F.; Low, J. Y.; et al. MiR-145-Dependent Targeting of Junctional Adhesion Molecule A and Modulation of Fascin Expression Are Associated with Reduced Breast Cancer Cell Motility and Invasiveness. *Oncogene* **2010**, *29* (50), 6569–6580.

(206) Rao, D.; Kimler, B. F.; Nothnick, W. B.; Davis, M. K.; Fan, F.; Tawfik, O. Transgelin: A Potentially Useful Diagnostic Marker Differentially Expressed in Triple-Negative and Non-Triple-Negative Breast Cancers. *Hum. Pathol.* **2015**, *46* (6), 876–883.

(207) Hirasawa, Y.; Arai, M.; Imazeki, F.; Tada, M.; Mikata, R.; Fukai, K.; Miyazaki, M.; Ochiai, T.; Saisho, H.; Yokosuka, O. Methylation Status of Genes Upregulated by Demethylating Agent 5-Aza-2′-Deoxycytidine in Hepatocellular Carcinoma. *Oncology* **2007**, *71* (1–2), 77–85.

(208) Yamamura, H.; Masuda, H.; Ikeda, W.; Tokuyama, T.; Takagi, M.; Shibata, N.; Tatsuta, M.; Takahashi, K. Structure and Expression of the Human SM22alpha Gene, Assignment of the Gene to Chromosome 11, and Repression of the Promoter Activity by Cytosine DNA Methylation. *J. Biochem.* **1997**, *122* (1), 157–167.

(209) Maleszewska, M.; Gjaltema, R. A. F.; Krenning, G.; Harmsen, M. C. Enhancer of Zeste Homolog-2 (EZH2) Methyltransferase Regulates Transgelin/Smooth Muscle-22α Expression in Endothelial Cells in Response to Interleukin-1β and Transforming Growth Factor-B2. *Cell. Signal.* **2015**, *27* (8), 1589–1596.

(210) Sayar, N.; Karahan, G.; Konu, O.; Bozkurt, B.; Bozdogan, O.; Yulug, I. G. Transgelin Gene Is Frequently Downregulated by Promoter DNA

Hypermethylation in Breast Cancer. *Clin. Epigenetics* **2015**, 1–16.

(211) Sayar, N. Unmasking of Epigenetically Silenced Genes and Identification of Transgelin as a Potential Methylation Biomarker in Breast Cancer, Bilkent University, 2015.

(212) Kumaki, Y.; Oda, M.; Okano, M. QUMA: Quantification Tool for Methylation Analysis. *Nucleic Acids Res.* **2008**, *36* (Web Server issue).

(213) Cramer, D. W.; Xu, H. Predicting Age at Menopause. *Maturitas* **1996**, *23* (3), 319–326.

(214) Chen, K.; Zhang, H.; Zhang, L. N.; Ju, S. Q.; Qi, J.; Huang, D. F.; Li, F.; Wei, Q.; Zhang, J. Value of Circulating Cell-Free DNA in Diagnosis of Hepatocelluar Carcinoma. *World J. Gastroenterol.* **2013**, *19* (20), 3143–3149.

(215) Bratman, S. V.; Newman, A. M.; Alizadeh, A. A.; Diehn, M. Potential Clinical Utility of Ultrasensitive Circulating Tumor DNA Detection with CAPP-Seq. *Expert Rev Mol Diagn* **2015**, *15* (6), 715–719.

(216) Dalton, L. W.; Pinder, S. E.; Elston, C. E.; Ellis, I. O.; Page, D. L.; Dupont, W. D.; Blamey, R. W. Histologic Grading of Breast Caner: Linkage of Patient Outcome with Level of Pathologist Agreement. *Mod. Pathol.* **2000**, *13* (7), 730–735.

(217) Association of Breast Surgery. Management of the Malignant Axilla in Early Breast Cancer. *ABS Multidiscip. Consens. Meet.* **2015**, No. March, 1–2.

(218) Qian, X.; Ruan, L. APC Gene Promoter Aberrant Methylation in Serum as a Biomarker for Breast Cancer Diagnosis: A Meta-Analysis. *Thorac. Cancer* **2018**, *9*, 284–290.

(219) Vasioukhin, V.; Anker, P.; Maurice, P.; Lyautey, J.; Lederrey, C.; Stroun, M. Point Mutations of the N-Ras Gene in the Blood Plasma DNA of Patients with Myelodysplastic Syndrome or Acute Myelogenous Leukaemia. *Br. J. Haematol.* **1994**, *86* (4), 774–779.

(220) Chang, P. S.; Li, L.; McAnally, J.; Olson, E. N. Muscle Specificity Encoded by Specific Serum Response Factor-Binding Sites. *J. Biol. Chem.* **2001**, *276* (20), 17206–17212.

(221) Ellis, P. D.; Martin, K. M.; Rickman, C.; Metcalfe, J. C.; Kemp, P. R. Increased Actin Polymerization Reduces the Inhibition of Serum Response Factor Activity by Yin Yang 1. *Biochem. J.* **2002**, *364* (Pt 2), 547–554.

(222) Bar-Sagi, D.; Hall, A. Ras and Rho GTPases: A Family Reunion. *Cell*. 2000, pp 227–238.

(223) Y., L.; E., W.; J., Z.; D., K.; B., L. Downregulation of SM22α Protein by Hypermethylation of Its Promoter in Colorectal Cancer. *Oncol. Lett.* **2018**, *15* (5), 7675–7680.

(224) Sakakura, C.; Hamada, T.; Miyagawa, K.; Nishio, M.; Miyashita, A.; Nagata, H.; Ida, H.; Yazumi, S.; Otsuji, E.; Chiba, T.; et al. Quantitative Analysis of Tumor-Derived Methylated RUNX3 Sequences in the Serum of Gastric Cancer Patients. *Anticancer Res.* **2009**, *29* (7), 2619–2625.

(225) Kim, J. H.; Shin, M. H.; Kweon, S. S.; Park, M. H.; Yoon, J. H.; Lee, J. S.; Choi, C.; Fackler, M. J.; Sukumar, S. Evaluation of Promoter Hypermethylation Detection in Serum as a Diagnostic Tool for Breast Carcinoma in Korean Women. *Gynecol. Oncol.* **2010**, *118* (2), 176–181.

(226) Elshimali, Y. I.; Khaddour, H.; Sarkissyan, M.; Wu, Y.; Vadgama, J. V. The Clinical Utilization of Circulating Cell Free DNA (CCFDNA) in Blood of Cancer Patients. *International Journal of Molecular Sciences*. 2013, pp 18925–18958.

(227) Figg, W. D.; Reid, J. Monitor Tumor Burden with Circulating Tumor DNA. *Cancer Biol. Ther.* **2013**, *14* (8), 697–698.

(228) Suqing Xie, D. J. Circulating DNA-Important Biomarker of Cancer. *J. Mol. Biomark. Diagn.* **2012**, *s2* (01).

(229) Perkins, G.; Yap, T. A.; Pope, L.; Cassidy, A. M.; Dukes, J. P.; Riisnaes, R.; Massard, C.; Cassier, P. A.; Miranda, S.; Clark, J.; et al. Multi-Purpose Utility of Circulating Plasma DNA Testing in Patients with Advanced Cancers. *PLoS One* **2012**, *7* (11).

(230) Liu, L.; Toung, J. M.; Jassowicz, A. F.; Vijayaraghavan, R.; Kang, H.; Zhang, R. Targeted Methylation Sequencing of Plasma Cell-Free DNA for Cancer Detection and Classification. *Ann. Oncol.* **2018**, No. April 2018.

(231) Ilié, M.; Hofman, P. Pros: Can Tissue Biopsy Be Replaced by Liquid Biopsy? *Transl. Lung Cancer Res.* **2016**, *5* (4), 420–423.

(232) Szpechcinski, A.; Chorostowska-Wynimko, J.; Struniawski, R.; Kupis, W.; Rudzinski, P.; Langfort, R.; Puscinska, E.; Bielen, P.; Sliwinski, P.; Orlowski, T. Cell-Free DNA Levels in Plasma of Patients with Non-Small-Cell Lung Cancer and Inflammatory Lung Disease. *Br. J. Cancer* **2015**, *113* (3), 476–483.

(233) Salvianti, F.; Pinzani, P. The Diagnostic Potential of Mutation Detection from Single Circulating Tumor Cells in Cancer Patients. *Expert Review of Molecular Diagnostics*. 2017, pp 975–981.

(234) Kamat, A. A.; Baldwin, M.; Urbauer, D.; Dang, D.; Han, L. Y.; Godwin, A.; Karlan, B. Y.; Simpson, J. L.; Gershenson, D. M.; Coleman, R. L.; et al. Plasma Cell-Free DNA in Ovarian Cancer: An Independent Prognostic Biomarker. *Cancer* **2010**, *116* (8), 1918–1925.

(235) Jylhävä, J.; Kotipelto, T.; Raitala, A.; Jylhä, M.; Hervonen, A.; Hurme, M. Aging Is Associated with Quantitative and Qualitative Changes in Circulating Cell-Free DNA: The Vitality 90+ Study. *Mech. Ageing Dev.* **2011**, *132* (1–2), 20–26.

(236) Wu, X.; Tanaka, H. Aberrant Reduction of Telomere Repetitive Sequences in Plasma Cell-Free DNA for Early Breast Cancer Detection. *Oncotarget* **2015**, *6* (30).

(237) Uehiro, N.; Sato, F.; Pu, F.; Tanaka, S.; Kawashima, M.; Kawaguchi, K.; Sugimoto, M.; Saji, S.; Toi, M. Circulating Cell-Free DNA-Based Epigenetic Assay Can Detect Early Breast Cancer. *Breast Cancer Res.* **2016**, *18* (1).

(238) Agassi, R.; Czeiger, D.; Shaked, G.; Avriel, A.; Sheynin, J.; Lavrenkov, K.; Ariad, S.; Douvdevani, A. Measurement of Circulating Cell-Free DNA Levels by a Simple Fluorescent Test in Patients with Breast Cancer. *Am. J. Clin. Pathol.* **2015**, *143* (1), 18–24.

(239) Hashad, D.; Sorour, A.; Ghazal, a; Talaat, I. Free Circulating Tumor DNA as a Diagnostic Marker for Breast Cancer. *J Clin Lab Anal* **2012**, *26* (6), 467–472.

(240) Zanetti-Dällenbach, R. A.; Schmid, S.; Wight, E.; Holzgreve, W.; Ladewig, A.; Hahn, S.; Zhong, X. Y. Levels of Circulating Cell-Free Serum DNA in Benign and Malignant Breast Lesions. *Int. J. Biol. Markers* **2007**, *22* (2), 95–99.

(241) Agostini, M.; Enzo, M. V.; Bedin, C.; Belardinelli, V.; Goldin, E.; Del Bianco, P.; Maschietto, E.; D'Angelo, E.; Izzi, L.; Saccani, A.; et al. Circulating Cell-Free DNA: A Promising Marker of Regional Lymphonode Metastasis in Breast Cancer Patients. *Cancer Biomarkers* **2012**, *11* (2–3), 89–98.

(242) Kim, K.; Shin, D. G.; Park, M. K.; Baik, S. H.; Kim, T. H.; Kim, S.; Lee, S. Circulating Cell-Free DNA as a Promising Biomarker in Patients with Gastric Cancer: Diagnostic Validity and Significant Reduction of CfDNA after Surgical Resection. *Ann. Surg. Treat. Res.* **2014**, *86* (3), 136.

(243) Shao, X.; He, Y.; Ji, M.; Chen, X.; Qi, J.; Shi, W.; Hao, T.; Ju, S. Quantitative Analysis of Cell-Free DNA in Ovarian Cancer. *Oncol. Lett.* **2015**, *10* (6), 3478–3482.

(244) Lin, Z.; Neiswender, J.; Fang, B.; Ma, X.; Zhang, J.; Hu, X. Value of Circulating Cell-Free DNA Analysis as a Diagnostic Tool for Breast Cancer: A Meta-Analysis. *Oncotarget* **2017**.

(245) Wang, H.; Liu, Z.; Xie, J.; Wang, Z.; Zhou, X.; Fang, Y.; Pan, H.; Han, W. Quantitation of Cell-Free DNA in Blood Is a Potential Screening and Diagnostic Maker of Breast Cancer: A Meta-Analysis. *Oncotarget* **2017**, *8* (60).

Appendices

Appendix A – Total cfDNA levels

Appendix Table 1: Total cfDNA levels in BC patients.

Patients	Total cfDNA levels (ng)	Patients	Total cfDNA levels (ng)
P#004	2,520	P#042	19
P#005	283	P#043	424
P#006	2,784	P#044	101
P#007	680	P#045	84
P#009	394	P#046	399
P#010	238	P#047	44
P#011	255	P#048	66
P#012	440	P#049	38
P#013	134	P#050	182
P#014	712	P#051	39
P#015	289	P#052	54
P#016	556	P#053	70
P#017	1,172	P#054	166
P#018	282	P#056	110
P#019	132	P#057	314
P#020	93	P#058	49
P#021	153	P#059	50
P#022	247	P#060	5
P#023	266	P#061	544
P#024	28	P#062	30
P#025	10	P#064	64
P#026	40	P#065	22
P#027	20	P#066	38
P#028	143	P#067	8
P#029	52	P#068	138
P#030	97	P#069	48
P#031	174	P#070	21
P#032	1,204	P#071	27
P#033	496	P#072	34
P#036	37	P#073	46
P#037	342	P#074	46
P#038	184	P#075	92
P#040	25	P#076	322
P#041	564		

Appendix Table 2: Total cfDNA levels in healthy donors.

Donors	Total cfDNA levels (ng)	Donors	Total cfDNA levels (ng)
N#001	124.4	N#071	600.0
N#002	228.8	N#072	110.4
N#003	18.8	N#073	177.6
N#004	100.0	N#074	124.4
N#005	76.8	N#075	137.6
N#006	124.8	N#076	70.8
N#007	77.2	N#077	91.2
N#008	88.0	N#078	81.2
N#009	39.6	N#079	208.0
N#010	69.2	N#080	131.2
N#011	38.3	N#081	235.2
N#012	8.0	N#082	57.2
N#013	19.3	N#083	47.2
N#014	11.0	N#084	76.4
N#016	60.8	N#085	60.8
N#017	75.2	N#086	30.0
N#019	61.2	N#087	7.2
N#020	43.6	N#088	86.0
N#021	37.3	N#089	44.4
N#022	88.0	N#090	72.8
N#023	648.0	N#091	125.2
N#024	86.8	N#092	100.8
N#025	4.8	N#093	73.6
N#026	41.2	N#094	43.2
N#027	89.6	N#095	44.8
N#028	51.2	N#096	187.2
N#029	230.8	N#097	55.6
N#030	64.0	N#098	15.8
N#031	58.0	N#099	112.8
N#032	80.4	N#100	41.6
N#033	36.8	N#101	145.2
N#034	19.5	N#102	29.6
N#035	16.6	N#103	10.3
N#036	45.2	N#104	10.3
N#037	45.2	N#105	209.2
N#038	456.0	N#106	71.2
N#039	58.0	N#107	148.8
N#040	20.8	N#108	34.3
N#041	161.6	N#109	55.6
N#042	42.0	N#110	276.0
N#043	88.0	N#111	138.4
N#044	58.4	N#112	166.8

Appendix Table 2: Total cfDNA levels in healthy donors.

Donors	Total cfDNA levels (ng)	Donors	Total cfDNA levels (ng)
N#045	46.0	N#113	492.0
N#047	101.2	N#115	206.8
N#048	47.2	N#116	25.9
N#049	57.2	N#117	5.6
N#050	87.6	N#118	27.0
N#051	32.6	N#119	46.0
N#052	38.3	N#120	19.2
N#053	40.8	N#121	29.3
N#054	20.1	N#122	13.6
N#055	205.2	N#123	5.2
N#056	37.5	N#124	17.3
N#057	107.2	N#125	7.2
N#058	49.6	N#126	16.6
N#059	70.4	N#127	19.7
N#060	56.4	N#128	39.6
N#061	331.2	N#129	22.4
N#062	50.0	N#130	27.3
N#063	315.6	N#131	72.0
N#064	17.6	N#132	28.1
N#065	49.2	N#133	6.8
N#066	43.6	N#134	10.1
N#067	75.6	N#135	38.6
N#068	39.5	N#136	10.8
N#069	178.8	N#137	17.2
N#070	179.2		

Appendix B – Copyright Permissions

Copyright permission for Figure 1.2, taken from Atoum Manar *et al.*, 2010[23].

WOLTERS KLUWER MEDKNOW PUBLICATIONS LICENSE TERMS AND CONDITIONS

Jul 29, 2018

This Agreement between Ms. Ayse Sedef Koseer ("You") and Wolters Kluwer Medknow Publications ("Wolters Kluwer Medknow Publications") consists of your license details and the terms and conditions provided by Wolters Kluwer Medknow Publications and Copyright Clearance Center.

The publisher has provided special terms related to this request that can be found at the end of the Publisher's Terms and Conditions.

License Number	4394880475368
License date	Jul 23, 2018
Licensed Content Publisher	Wolters Kluwer Medknow Publications
Licensed Content Publication	Indian Journal of Cancer
Licensed Content Title	TNM staging and classification (familial and nonfamilial) of breast cancer in Jordanian females
Licensed Content Author	Atoum M F, Hourani H M, Shoter A, Al-Raheem S N, Al Muhrib T K
Licensed Content Date	Apr 1, 2010
Type of Use	Thesis/Dissertation
Requestor type	Student
Format	Print, Electronic
Portion	image/photo
Number of images/photos requested	1
Rights for	Main product
Creation of copies for the disabled	no
In the following language(s)	Original language of publication
With incidental promotional use	no
The lifetime unit quantity of new product	0 to 499
The requesting person/organization is:	Ayşe Sedef Köseer
Order reference number	
Title of your thesis / dissertation	Evaluation of TAGLN as a diagnostic marker in breast cancer.
Expected completion date	Jul 2018
Total	0.00 USD

Copyright permission for Figure 1.3, taken from Estibaliz Alegre *et al*., 2015[116].

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jul 29, 2018

This Agreement between Ms. Ayse Sedef Koseer ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4398440344157
License date	Jul 29, 2018
Licensed Content Publisher	Elsevier
Licensed Content Publication	Elsevier Books
Licensed Content Title	Advances in Clinical Chemistry
Licensed Content Author	Estibaliz Alegre,Miguel Sammamed,Sara Fernández-Landázuri,Leyre Zubiri,Álvaro González
Licensed Content Date	Jan 1, 2015
Licensed Content Pages	43
Start Page	47
End Page	89
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier chapter?	No
Will you be translating?	No
Original figure numbers	Figures 5
Title of your thesis/dissertation	Evaluation of TAGLN as a diagnostic marker in breast cancer.
Expected completion date	Aug 2018
Estimated size (number of pages)	105
Requestor Location	Ms. Ayse Sedef Koseer Bilkent Universitesi Merkez Kampus Fen Fakultesi SB Binasi Molekuler Biyoloji ve Genetik Bolumu Ankara, 06800 Turkey Attn: Ms. Ayse Sedef Koseer
Publisher Tax ID	GB 494 6272 12
Total	0.00 USD