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"THE EFFECTS OF KISSPEPTIN IN THE BIOLOGY OF BREAST CANCER"

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ABBREVIATIONS

AXL - AXL receptor tyrosine kinase

BCRP - breast cancer resistance protein

CEA - carcinoembryonic antigen

CRAd - conditionally replicative adenovirus

CRC - colorectal cancer

CREB - cAMP response element-binding protein

EGFRs/HERs - epidermal growth factor receptors

EMT - epithelial to mesenchymal transition

ERα - estrogen receptor alpha

ERBB2/ HER2/ *HER2/neu* - epidermal growth factor 2

EREs - estrogen response elements

ERK - extracellular signal-regulated kinase

EVs - extracellular vesicles

GnRHR - gonadotropin-releasing hormone receptor

GPCR - G protein-coupled receptor

GSIS - glucose-stimulated insulin secretion

HPG - hypothalamus-pituitary-gonadal

HRT - hormone replacement therapy

LCIS - lobular carcinoma in situ

lncRNAs - long non-coding RNAs

MEFs - mouse embryonic fibroblasts

MMP - matrix metalloproteinase

NSCLC - non-small cell lung cancer

PR - progesterone receptor

PI3K - phosphatidylinositol 4,5-bisphosphate 3-kinase

RT-PCR - reverse transcription polymerase chain reaction

SMURF1 - SMAD ubiquitin regulatory factor-1

TNBC - triple negative breast cancer

UHRF1 - Ubiquitin-like with PHD and RING finger domains 1

CHAPTER 1: BREAST CANCER

1.1 History

The Greek physician Hippocrates (460–370 BCE) assumed that the human body consisted of a balance of four “humors” (blood, phlegm, yellow bile and black bile), which corresponded to the building blocks of nature (air, water, fire and earth respectively). Hippocrates believed that diseases result from an imbalance in the system of humors and that the cause of breast tumors is the excess of black bile. He first used the term “karkinos” (crab in Greek), to describe the crab-like shape of tumors. He also believed that cancer cases were incurable and that surgical treatment put patients’ life in danger. Hippocrates reported only one case of breast cancer, an Abdera case, in which there was bloody fluid excretion from the nipple. Hippocratic physicians used the term “karkinoma” or “carcinoma” for the first time in the 4th century BC to describe malignant or non-healing swellings or ulcers (Lukong, 2017).

Galen (131–203 CE), a Greek physician, described in detail an atypical swelling in the breast in 200 CE. He mentioned that breast cancer was more usual in women who were not menstruating or had abnormal menstrual cycles. Galen also proposed that black bile release in ulcerated tumors resulted in illness caused by breast cancer. He also suggested a type of lumpectomy, as well as the use of medications like opium, castor oil, licorice and sulfur, as possible treatments (Lukong, 2017).

In the Middle Ages vague tumor/breast ablation methods, without anesthesia, were utilized using instruments such as Gerard Tabor's mastectomy instrument. René Descartes (1596–1650) formulated the lymphatic theory for the origin of breast cancer, which contradicted the prevailing humoral explanation. John Hunter (1728–1793), the father of scientific surgery, introduced the excision of the cancer along with the lymphatic spread. Bernardino Ramazzini (1633–1714), who established the field of occupational medicine, reported higher incidence of breast cancer in nuns in Italy than in married women. Nowadays, it is known that there is lower incidence of

premenopausal breast cancer in women who nurse their children. The cause of breast cancer was also blamed on pus-filled inflammations in the breast (Johanes de Gorter, 1689–1762), depressive mental disorders (Claude-Nicolas Le Cat, 1700–1768) and childlessness (Lorenz Heister, 1683–1758). Henri Le Dran (1685–1770), a French physician, described cancer as a disease that proceeds in stages and proposed the surgical removal of the breast tumor in order to avoid its spread to the lymph nodes of the armpits. This practice continued also in 20th century and finally resulted in the application of radical mastectomy or extensive removal of the breast (Lukong, 2017).

Rudolph Virchow (1821–1902) showed that tumors were composed of cells. William Halsted (1852–1922), a professor of surgery at Johns Hopkins Hospital (Baltimore, USA) introduced radical mastectomy for breast cancer in 1882. Halsted's removed the whole breast and the underlying chest muscle and the axillary contents. Later he extended his operation by removing the supraclavicular lymph nodes after dividing the clavicle. The main achievement of Halsted's operation was the reduction of local recurrence rates compared with other procedures, although the operation did not improve overall survival. Importantly, in the late 19th century radical mastectomy became the standard operation for breast cancer worldwide and remained the gold standard throughout most of the 20th century (Lukong, 2017).

Additionally, in the 19th century it was discovered that some breast cancers were hormone-dependent. There had been observations that the growth of breast cancer in patients fluctuated with the menstrual cycle and it was slower in postmenopausal women. Thomas Beatson (1848–1933), the father of anti-hormonal treatment of breast cancer, in 1896, reported the observation of temporary regression of metastatic breast cancer in two patients treated by surgical oophorectomy and hypothesized “that internal secretion of the ovaries in some cases favors the growth of the cancer” (Lukong, 2017). In the 20th century breast cancer research led to the molecular and pathological classification of breast cancer. Breast cancer was recognized as a major public health issue in the Western world.

1.2 Ontology

Breast cancer is a complicated disease that consists of a compilation of breast diseases characterized by diverse histopathology, genetic and genomic variations and clinical outcomes (Vargo-Gogola, 2007). The primary abnormality resulting in the development of breast cancer is the uncontrolled proliferation of cells within any of the breast components (lobules, ducts, connective tissue, fatty tissue, lymphatic tissue) (Winters, 2017). Initially breast cancer undergoes an in situ or invasive phase. At this stage it may be detected during routine self-breast exams, mammographic screening, or once it shows signs or symptoms. The first symptom is a palpable or visible, painless breast lump. Swollen and enlarged lymph nodes appear during the early stages of metastasis. Evidence of bloody nipple discharge, heaviness, redness, swelling, breast deformity, or retractions are signs of breast malignancy related to advanced stages of disease (Winters, 2017).

Metastatic cells are thought to be epithelial stem cells or differentiated epithelial cells that accumulate gene mutations and get transformed into mesenchymal tumor cells (Seyfried & Leanne, 2013). Several investigators support that metastatic cancer cells come from populations of undifferentiated or semi-differentiated tissue stem cells that physiologically replace the dead or damaged cells. This notion is supported by the fact that stem cells and cancer cells present similar gene expression profiles and also have the ability to proliferate and migrate during tissue morphogenesis and differentiation (Seyfried & Leanne, 2013). The spreading of cancer cells from the primary tumor involves a procedure known as epithelial-mesenchymal transition (EMT). During EMT the cancer cells lose the epithelial cell-to-cell adhesion and gain a spindle-shaped morphology. Additionally, the expression of epithelial markers is lost and the expression of mesenchymal markers is acquired, so the cells can migrate and invade through the basement membrane into the surrounding extracellular matrix (Guzman, 2019). Importantly, disseminated cancer cells must have the capability to survive at the sites of spread, which greatly depends on their crosstalk with the new microenvironment (Ulasov, 2019).

1.3 Risk factors

1.3.1 Genetic predispositions

About 5-10% of breast cancers are linked to inherited gene mutations. The most common mutations are in the tumor suppressor genes BRCA1 or BRCA2, that can be responsible also for cancer in other organs, such as the ovaries, pancreas, and prostate (Winters, 2007). Mutations in BRCA1 and BRCA2 genes are the cause of approximately 3% of breast cancers and 10% of ovarian cancers (Winters, 2017). A BRCA1 mutation is linked to 55-65% lifetime risk of breast cancer development, while a BRCA2 mutation is linked to a 45% lifetime risk. A woman with a BRCA1 or BRCA2 gene mutation has about 70% chance of developing breast cancer by age 80. Having close relatives with triple negative breast cancer (TNBC), premenopausal breast cancer, ovarian cancer, bilateral breast cancer or breast cancer in a male relative or multiple relatives with breast cancer is associated with increased possibility of having a gene mutation in BRCA1 and BRCA2 (Winters, 2017). BRCA1 mutations are less frequent in breast cancers in men and BRCA2 mutations are linked to a lifetime risk of about 6.8%. Totally, more than 1800 inheritable mutations in BRCA1 gene have been linked to increased breast cancer development in both men and women (Winters, 2017). There are also inherited mutations in other genes, that are not so frequent, and can also lead to breast cancer development. Some of these mutated genes are ATM, TP53, CHEK2, PTEN, CDH1, STK11, and PALB2 (Veronesi, 2005; Collaborative Group on Hormonal Factors in Breast C., 2001; Hulka, 1996; Colditz, 2012; Polyak, 2007; Allison, 2012).

1.3.2 “Non-genetic” risk factors

- Race and ethnicity

  Caucasian women are slightly more likely to develop breast cancer than African American women. Other races, such as Asian, Hispanic and Native American women have a lower risk of developing and dying from breast cancer (Feng, 2018).

- Residential segregation by race

  Residential segregation is linked to several factors that affect health, such as access to medical care, availability of nutritious foods and safe places to exercise (Coughlin, 2019). Warner and Gomez (2010) studied the association between racial residential segregation and breast cancer stage at diagnosis and breast cancer-specific mortality of black and white women diagnosed with breast cancer in California from 1996 to 2004. Black women who lived in neighbourhoods with more Blacks showed lower mortality levels than white women who lived in the same neighbourhoods. Pruitt *et al.* (2009) studied the relationship between residential racial segregation and mortality among black, white and Hispanic urban breast cancer patients in Texas. The greater segregation of black and Hispanic women was adversely linked to mortality by breast cancer. Additionally, it was demonstrated that women who lived in higher Hispanic density neighbourhoods showed generally augmented mortality; the birthplace and neighbourhood poverty had minor effect. Hossain *et al.* (2019) demonstrated that neighbourhood disadvantage was linked to diagnosis at more advanced stages and poorer stage-specific survival among patients with TNBC.

- Immigration status

  Studies of Ziegler (1993), Keegan (2010) and Schuldiner (2018) in the United States and Canada demonstrated that immigrants show lower breast cancer risk compared to women who were born in the United States. Additionally, breast cancer risk among immigrant women seems to increase over several generations. These findings may be owed to lower breast cancer rates in the country of origin, different life style, reproductive factors or lower frequency of screening mammography among immigrants (Feng, 2018).

- Certain breast conditions

  Women with dense breasts on mammogram have 1.5-2 times higher risk of breast cancer; however, breast density depends on a variety of factors, such as age, menopausal status, use of certain drugs and pregnancy (Feng, 2018).

- Lobular carcinoma in situ (LCIS) or lobular neoplasia

  LCIS cells are cancer-like, grow in the lobules of the milk-producing glands of the breast and are limited within the walls of the lobules. LCIS is categorized as a non-invasive breast cancer, however, if no treatment is applied it may become an invasive cancer (Feng, 2018).

- Chest radiation therapy

  Radiation therapy to the chest at a younger age increases the risk for breast cancer development. The risk is higher if the individual had radiation when the breasts were still at a developing stage; after age 40 radiation does not increase breast cancer risk (Feng, 2018).

- Exposure to diethylstilbestrol

  Diethylstilbestrol treatment that some pregnant women were given from 1940 to early 1970s in order to diminish the incidence of miscarriage, slightly increased risk of breast cancer. Also women whose mothers were treated with DES may also have a slightly higher risk of breast cancer (Feng, 2018).

- Lifestyle and Personal Behaviour-Related Risk Factors of Breast Cancer

  Approximately 85% of breast cancers occur in women without family history of breast cancer. These cancers may be caused by genetic mutations that occur during aging process and by lifestyle-associated risk factors (Feng, 2018).

- Birth control and contraceptives

  Use of hormones and oral contraceptives may slightly increase breast cancer risk. Depo-Provera increases breast cancer risk five years after the end of the treatment. Birth control implants, intrauterine devices, skin patches and vaginal rings that usually use hormones may also increase breast cancer risk (Feng, 2018).

- Hormone replacement therapy (HRT) after menopause

  Postmenopausal combined hormone therapy (estrogen and progesterone) increases the risk of developing breast cancer and dying from breast cancer. This increased risk lasts five years after stopping HRT. Short term use of estrogen alone after menopause is not linked much to increased risk of breast cancer, in contrast to long-term use, e.g. >15 years (Feng, 2018).

- Excessive alcohol consumption

  Alcohol consumption is related to an elevated risk of breast cancer; the risk is proportional to the amount of alcohol consumed (Feng, 2018). In the study of Chen *et al.* (2011), during 1980–2008, it was demonstrated that low levels of alcohol consumption (5–9.9 g/day, or 3–6 glasses of wine/week) is associated to increased breast cancer risk. Additionally, women who consumed at least two alcoholic drinks per day (more than 30 g/day) had an even greater risk of developing breast cancer (Chen, 2011).

- Significant overweight or obese

  Before menopause, body's estrogen is made mainly by women's ovaries, but also by fat tissue; after menopause, most of it is produced by fat tissue. So, increased fat tissue after menopause results in elevated estrogen levels that cause increased breast cancer risk. Additionally, higher blood insulin levels in overweight persons are also linked to breast cancer (Feng, 2018). Women who lost and maintained more than 10 kg

of weight are linked to more than 50% reduced breast cancer risk (Winters, 2017).

- Not having children or not breastfeeding

  Women without children or who have given birth after age 30, have a slightly higher risk for developing breast cancer. However, pregnancy seems also to augment TNBC risk. Breastfeeding may reduce breast cancer risk; maybe because it reduces the number of women's menstrual cycles (Feng, 2018).

- Starting menstruation early or stopping menopause after age 55

  Women that start menstruating early are exposed to estrogen and progesterone for a longer period of time, so breast cancer risk increases. Women that go through menopause after age 55, are also exposed to estrogen and progesterone for longer time and have higher risk of breast cancer (Feng, 2018).

- Lack of physical activity

  Regular physical activity, particularly past menopause, may diminish breast cancer risk, maybe because body weight, inflammation, hormones and energy balance are affected by physical activity (Feng, 2018). Seven hours walking per week reduced 14% the risk of postmenopausal breast cancer compared to less than three hours per week (Winters, 2017).

1.4 Treatment

Primary surgery, chemotherapy, radiation therapy and hormone therapy and removal of the tumour or primary systemic therapy are chosen depending on the histological and molecular tumour subtype (Harbeck, 2017; Winters, 2017). Patient survival is mainly affected by the stage of diagnosis; early stages of diagnosis are linked to higher survival rates (99% 5-year survival in the USA) compared to late stages (26% 5-year survival in the USA).

For non-metastatic breast cancer patients, the goal of therapy is to extinguish the tumor from the breast and regional lymph nodes, in order to avoid the metastasis. Local therapy includes the surgical removal and sampling or the removal of axillary lymph nodes and postoperative radiation. Systemic therapy can be preoperative (neoadjuvant), postoperative (adjuvant), or both. The standard systemic therapy administered depends on the breast cancer subtype; endocrine therapy for all HR+ tumors (some patients may need chemotherapy as well), trastuzumab-based ERBB2-directed antibody therapy plus chemotherapy for all *ERBB2*+ tumors (with endocrine therapy given in addition, if concurrent HR positivity) and chemotherapy for TNBC (Waks, 2019).

For metastatic breast cancer patients, therapy is used to prolong the duration and quality of life since there is no effective therapy for these patients yet. The types of systemic therapy used are the same as for non-metastatic breast cancer. Surgery and radiation are used to relief disease symptoms (Waks, 2019).

1.5 Biology

1.5.1. Molecular subtypes of breast cancer

According to the work of Perou, Sørlie, and colleagues on 2000 and 2001, there are at least four different clinically relevant molecular subtypes of breast cancer: luminal A, luminal B, HER2-enriched and basal like. The four

subtypes of a paraffin-embedded tumour sample can be distinguished by a multigene assay (Prosigna; NanoString Technologies or Blueprint; Agendia) or by immunohistochemistry. Depending on the expression of steroid hormone receptor (ERα, PR and ERBB2 status), as well as tumour proliferation measured by Ki67, the following subtypes are distinguished: luminal A-like subtype (ER or PR positive, or both, ERBB2 negative, low proliferation); luminal B-like subtype (ER or PR positive, or both, ERBB2 negative, high proliferation); ERBB2 subtype, non-luminal (ERBB2 positive and ER and PR negative) or luminal (ERBB2 positive and ER or PR positive, or both); basal-like subtype (ERBB2 negative and ER and PR negative; triple negative breast cancer) (Harbeck, 2017). Considering the values of the proliferation marker Ki67, values ≤ 10% are generally considered as low risk, while values 20 - 29% are considered as a minimum criterion for high proliferation. However, international standardisation for Ki67 is still missing (Harbeck, 2017).

In the study of Heng *et al.* (2016) genomic, transcriptomic and protein data were combined to create a molecular profiling of breast cancer morphological features. Their findings revealed that often in breast tumors co-exist inflammation, necrosis, medium/high mitotic count and marked nuclear pleomorphism. All these features have similar molecular basis and are linked to basal-like subtypes. Lobular carcinoma is characterized by a distinct molecular profile that may be linked to mitochondria dysfunction. In ductal carcinoma *in situ,* myoepithelial cells presented altered gene expression profile.

1.5.2 Extracellular vesicles in breast cancer development and evolution

1.5.2.1. Characteristics of extracellular vesicles

Cells are known to communicate through the secretion of soluble factors or by direct interaction. Nearly 30 years ago, some membrane-derived vesicles were described for the first time in the extracellular space (Pan, 1983; Harding, 1984). Nowadays, extracellular vesicles (EVs) have been isolated not only from almost all mammalian cell types, but also from lower eukaryotes and

prokaryotes (Andaloussi, 2013). Extracellular vesicles can be categorized into exosomes, microvesicles and apoptotic bodies, based on their cellular origin, biological function and biogenesis. All EVs are cell-derived, enclosed by a lipid bilayer (30 - 2,000 nm in diameter) and contain cytoplasmic proteins, lipid raft-interacting proteins and RNAs. Exosomes are generated from the endolysosomal pathway and typically contain some additional components compared to microvesicles. Microvesicles are generated by plasma membrane budding (Andaloussi, 2013) and they interact with target cells *via* receptors, endocytosis, fusion with plasma membrane or the release of their content (Ogorevc, 2013; Lee, 2014). EVs have been shown to be involved not only in the regulation of normal physiological processes, but also in the pathology of several diseases (Andaloussi, 2013). Additionally, EVs have been tightly linked to tumorigenesis (Rak, 2012) and they seem to represent a novel and missing class of therapeutic targets in cancer, including breast cancer. More specifically, malignant cells have been reported to use exosomes to transfer proteins and nucleic acids to other cancer cells in the tumor microenvironment, but also to normal cells (Jia, 2017). In this way exosomes play a role in tumorigenesis and cancer progression, including immune suppression, angiogenesis, cell migration and invasion (Jia, 2017).

1.5.2.2. Exosomes' function in breast cancer

In breast cancer, exosomes are implicated in the stimulation of tumor angiogenesis, stroma reorganization to establish the tumor microenvironment, as well as in tumor growth promotion and drug resistance. Exosomes secreted by malignant cells usually transfer disease-related molecules, such as mutant oncoproteins, oncogenic transcripts, microRNA and DNA sequences. After taken up by normal cells, exosomes from malignant cells alter the transcriptomes of target cells contributing to their oncogenic transformation and tumor formation. Additionally, exosomes can deliver signaling molecules to cancer cells within the tumor microenvironment and help tumor cells evade immune response, promote tumor invasion and metastasis, remodel the tumor microenvironment and stimulate angiogenesis (Jia, 2017). Harris *et al.* (2015)

showed that breast cancer cells-derived exosomes can stimulate cell migration proportionally to the metastatic potential of the cell that secretes them. Also, several studies have shown that miRNAs secreted from exosomes enhance the invasive and migration capacity of several breast cancer cell lines. For example, exosome-derived miR-10b inhibited the expression of its target genes, such as HOXD 10 and KLF4, and enhanced the invasion ability of breast cancer cells. Additionally, tumor-secreted exosomal miR-9, overexpressed in several breast cancer cell lines, can transform normal fibroblasts into cancer-associated fibroblasts, enhancing tumor growth. Dhani *et al.* (2015) also demonstrated that exosomes enhance the ability of breast cancer cells to survive and grow under hypoxic conditions. Furthermore, it has been reported that hypoxic conditions stimulate exosomes secretion (Wang, 2014; King, 2012) and that hypoxia-induced exosomes from cancer cells result into neoplastic transformation, malignant cell growth and invasion (Kucharzewska, 2013; Zhang, 2012). Tumor cell-derived exosomes also participate in the establishment of premetastatic niche that facilitates cancer invasion and migration, transferring miRNAs and proteins between epithelial cells, stromal cells, macrophages and fibroblasts and tumor cells (Vlassov, 2012; Dutta, 2014).

Exosomes have been also demonstrated to play a role in breast cancer radiotherapy resistance and immunotherapy. More specifically, Chen *et al.* (2004) showed that drug-resistant breast cancer cells may transmit their resistance capacity and alter chemosusceptibility in the recipient sensitive cells *via* exosomal miRNAs (such as miR-100, miR-222, and miR-30a). Exosomes were shown to mediate drug resistance *via* the intercellular transport of P-glycoprotein from docetaxel-resistant cells to sensitive ones (Lv, 2014). Also, exosomes derived from doxorubicin-resistant breast cancer cells transmitted resistance capacity in recipient cells by delivering miR-100, miR-222 and miR-30a. Considering the field of cancer therapy, exosomes derived from tamoxifen resistant cells (MCF-7TamR) in ER-positive breast cancer, favoured the proliferation and colony-forming ability of tamoxifen sensitive cells (MCF-7wt) in the presence of tamoxifen; the exosome-induced result was partly mediated by miR-221/222 (Wei, 2014). Anti-HER2 therapy, such as trastuzumab and

lapatinib, are really promising for the treatment of HER2 positive breast cancer, however some patients acquire resistance to it. HER2+ tumor exosomes carrying antigens that are targets for anti-tumor antibodies, competitively inhibit the drug activity contributing to treatment failure. Studies showed that exosomes secreted from BT-474 and SK-BR-3 cells prevented the interaction of HER2-targeted therapeutic drugs with cancer cells by binding to trastuzumab (Ciravolo, 2012). Moreover, HER2+ exosomes can be indicative of the tumor stage and aggressiveness. It's noteworthy that exosomes isolated from the serum of late stage HER2-positive breast cancer patients bind with higher affinity to trastuzumab compared to those isolated from early stage patients (Chen, 2014).

Table 1. Major miRNAs implicated in breast tumor progress (Jia, 2017)

miRNA/Protein	**target genes**	**physiological function**
miR-130a	TGB-β/Smad signaling	tumorigenesis
miR-328	CD44	reduce cell adhesion and enhance cell migration
miR-301a		a negative prognostic maker
miR-34a	p53	
miR-106b	BRMS1; RB	an early process of tumor metastasis
miR-10b	HOXD10; KLF4	transfer to non-maligant HMLE cells and promote cell invasion
miR-105	ZO-1	cell migration and metastasis
miR-127, -197, -222, and -223	CXCL12	suppress cell proliferation and elicit cell cycle quiescence
miR-23b		promote cell dormancy in a metastatic niche
miR-373		downregulate ER expression and inhibition of apoptosis
miR-122		inhibit glucose uptake in a premetastatic niche and promote metastasis
miR-9		transfer cancer-associated fibroblasts phenotype to normal fibroblasts
miR-223	Mef2c–β-catenin pathway	promote invasiveness of breast cancer
miR-16	VEGF	suppress angiogenesis
miR-210		key factors for the tumor angiogenesis and brain metastasis
miR-451, miR-326, miR-100, miR-222, and miR-30a		drug resistance
miR-221/222		enhance tamoxifen resistance
miR-155, miR-21 and miR-1246,	the shelterin component TERF1	cancer diagnose and predict a poor prognosis
miR-215, miR-299, and miR-411		lower expression in untreated patients with metastatic breast cancer
miR-155, miR-19a, miR-181b, and miR-24		an early marker for breast cancer risk
miR-101, miR-939, miR-373		breast tumor subtype and stage

1.5.2.3. Exosomes' potential role in breast cancer diagnosis and prognosis

Exosomes secreted by cancer cells can be isolated from serum, pleural effusions, urine and ascites fluids of cancer patients. So the expression changes of exosomal biomarkers can be studied and used as an early marker for diagnosis and prognosis monitoring. For example, the expression levels of CEA and CA153 in exosomes of breast cancer patients are related to cancer progression (Andre, 2002). Moreover, microRNAs contained in exosomes can serve as an early biomarker for breast cancer, as well as, as an indicator for tumor malignancy degree and prognosis (Joyce, 2016). Several miRNAs have been shown to be related with breast tumor subtype and stage. More specifically, elevated levels of exosomal miR-939 in the basal-like tumor subtypes were linked to worse prognosis in triple-negative breast cancers (Di Modica, 2017). Takahashi *et al.* (2015) demonstrated that decreased levels of exosomal miR-101 concentrations are associated with lymph-node positive cancer and that miR-373 levels were significantly upregulated in TNBC and ER and PR negative breast cancer patients. These results indicate that evaluating and comparing the expression levels of tumor derived exosomes during treatment can help to draw conclusions of the therapeutic response and patient prognosis.

Table 2. Characteristics of exosome biomarkers studies for breast cancer diagnosis (Wang, 2017)

First author	Total patient numbers	Groups (number)	Exosome biomarkers	Detection methods	*P* value
Fang [13]	NR	BC (19) Healthy donors (NR)	HER2	Microfluidic chip	< 0.0500
Domenyuk [14]	500	BC (206) Benign breast tumor (177) Healthy donors (117)	ssODNs	ADAPT	< 0.0500
Kibria [16]	90	BC (50) Healthy donors (40)	CD47	ELISA	< 0.0100
Hannafon [3]	NR	BC (16) Healthy donors (NR)	miR-1246 miR-21	qRT-PCR	< 0.0500
Lee [15]	115	Early BC	Del-1	ELISA	< 0.0500

NR not reported, *BC* breast cancer, *ssODNs* single-stranded oligodeoxynucleotides, *ADAPT* adaptive dynamic artificial poly-ligand targeting, *ELISA* enzyme-linked immunosorbent assay, *qRT-PCR* quantitative reverse transcription polymerase chain reaction

1.5.2.4. Exosomes' potential role in breast cancer therapy

Exosomes as natural carriers of nucleic acids and proteins could be utilized as a drug delivery system for cancer therapy. Tian *et al.* (2014) used exosomes to deliver chemotherapeutics to tumor and achieved cancer growth inhibition. Ohno *et al.* utilized modified exosomes to deliver nucleic acid drugs (such as let-7a) to EGFR-positive breast cancer cells. In mouse models of breast and ovarian cancer, exosomes' administration that delivered doxorubicin significantly increased the therapeutic index, limited the crossing of doxorubicin through the myocardial endothelial cells and avoided accumulation of drug in the heart without affecting other organs (Hadla, 2016). However, several technical problems concerning the efficient transfection of exosomes with nucleic acid drugs and the selection of the suitable host cells for exosome injection have to be solved (Jia, 2017). Additionally, tumor derived-exosomes can be utilized as a cancer vaccine since they carry several tumor antigens (Tan, 2010).

1.6 The implicated signaling pathways

1.6.1 Estrogen receptor (ER) signaling

ER signaling consists of membrane (mostly G protein-coupled receptors) and nuclear ERa and ERb (Renoir, 2013; Cheskis, 2007). Both ERa and ERb regulate the transcription of target genes upon ligand binding. They contain six functional domains with various degrees of similarity and have the ability of forming heterodimers (Kumar, 1987). Using their DNA-binding domain region ER dimers interact with estrogen response elements (EREs) of target genes, however ERs can also regulate the transcription of genes in the absence of EREs (Bjornstrom, 2005). Several co-activators and co-repressors, such as BRCA1, are involved in ER-mediated transcription (Fan, 1999).

The ERα is a steroid hormone receptor and a transcription factor that is activated by estrogen and consequently induces oncogenic growth pathways in breast cancer cells; it is present in approximately 70% of invasive breast cancers (Walks, 2019). Not only the full-size ERa, but also many of its isoforms, are involved in breast cancer biology and therapeutics (Fuqua, 2004). For example, elevated expression of a truncated isoform of ERa, ERa36, was found to be associated with metastatic phenotype and poor prognosis in breast cancer patients (Wang, 2018). The tissue expression pattern of ERb is partially overlapped with that of ERa. The role of ERb signalling in breast cancer has not been studied in detail yet (Bado, 2017). ERb is often expressed in normal breast tissue and its expression levels decrease as the breast tumors grow. Several studies have demonstrated that ERb has a breast cancer suppressor role both *in vitro* and *in vivo* (Paruthiyil, 2004; Lin, 2007).

1.6.2 HER2 signaling

The ERBB2 (formerly HER2 or *HER2/neu*) is a transmembrane receptor tyrosine kinase in the epidermal growth factor receptor family that has been related to poor prognosis in the absence of systemic therapy; it is overexpressed in approximately 20% of breast cancers. The indicated treatment for patients with *ERBB2*-amplified or -overexpressing breast cancer is anti-*ERBB2* antibodies (trastuzumab and pertuzumab) and small-molecule tyrosine kinase inhibitors (lapatinib and neratinib).

Human epidermal growth factor receptors (EGFRs, or HERs) 1 to 4 belong to a family of tyrosine kinase receptors that is expressed in normal tissues and in malignancies (Sergina, 2007). HER2 is a tyrosine kinase receptor that consists of an extracellular ligand-binding domain, a transmembrane domain and an intracellular domain (Wee, 2017). Constitutively active HER2 forms dimers with other molecules affecting many cellular functions through several pathways (Graus-Porta, 1997). Upon ligand binding, HER2 is dimerized and the tyrosine residues in the intracellular domain of HER2 are phosphorylated; this leads to the activation of multiple downstream signalling

pathways, such as the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) pathways (Burgess, 2008), which are involved in breast tumorigenesis (Elizalde, 2016). HER2 is also overexpressed in various human breast cancer cell lines. HER2 protein overexpression is associated with tumor cell proliferation and cancer progression (Elizalde, 2016). HER2 was also found to be linked to inflammation and the expansion of cancer stem-like cells in breast cancer (Liu, 2018). Additionally, breast cancer cells that express HER2 are more likely to progress to metastasis (Robert, 2017).

1.6.3 Canonical Wnt/b-catenin signaling in breast cancer

Wnt proteins are members of a family of highly glycosylated, secreted proteins with important roles in embryonic induction, generation of cell polarity, cell fate specification and adult tissue homeostasis maintenance (Logan, 2004). The secreted Wnt proteins bind to both co-receptors Frizzled and low-density lipoprotein receptor-related protein 5 and 6. This interaction is followed by the recruitment of Axin and Dishelved proteins to the cell membrane and the inhibition of glycogen synthase kinase -3b protein (Yang, 2016; Mohammed, 2016; Nusse 2017) together with CREB binding protein and T-cell factor/lymphoid enhancing factor. Mammary gland-specific expression of stabilized b-catenin has been shown to result in aggressive adenocarcinomas, consisting predominantly of glandular and undifferentiated cells (Imbert, 2001).

In breast cancer, Wnt signaling may be constitutively activated through an autocrine mechanism. Mutations in Wnt signaling in breast cancer is not a usual phenomenon, however, high levels of stabilized b-catenin are detected in approximately 50% of clinical breast cancer cases (Brennan, 2004; Bafico, 2004). Additionally, activated b-catenin has been demonstrated to promote TNBC. Wnt signalling has also been shown to be implicated in resistance to current anticancer drugs, possibly by regulating stem and progenitor cell populations (Chen, 2007; Woodward, 2007).

Kakarala *et al.* (2008) demonstrated that a small population of either stem cells, or cells with stem-like properties within the tumor, regulate tumor generation and progression. Ayyanan *et al.* (2006) demonstrated that Wnt1 in human mammary epithelial cells enhanced stem cell self-renewal, resistance to apoptosis and failure to senescence. Furthermore, Wnt-1 transgenic mice had expanded stem/progenitor cell populations in pre-neoplastic lesions and tumors (Li, 2003). Inhibition of WNT1 function was shown to diminish tumor formation and cellular migration (Jang, 2015). Similarly, the use of inhibitor of GSK3/b catenin signaling kinase D1 resulted in reduction of the stem cell features of breast cancer cells (Kim, 2016). So, Wnt signaling seems to have an intrinsic role in maintaining mammary cancer stem cell properties.

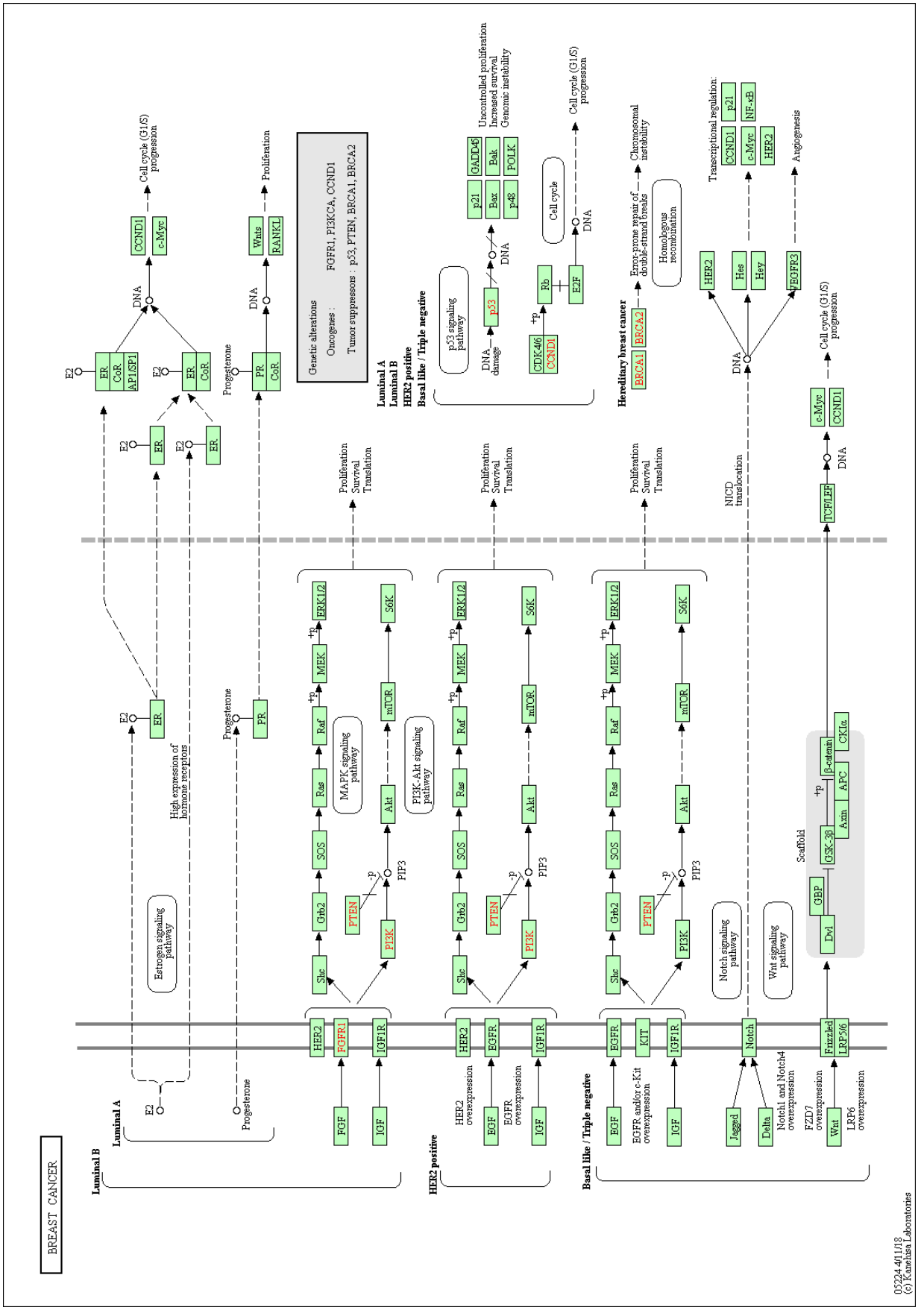
1.6.4 Other pathways implicated in breast cancer

The VEGF family was first studied by cancer biologists in the early 1980s. Since then, many genes encoding members of the VEGF family have been isolated and their protein products have been characterized. The VEGF family signals predominantly through the receptor tyrosine kinases VEGF receptor VEGFR-1, VEGFR-2, and VEGFR-3, in combination with the co-receptors neuropilin NRP1 and NRP2, and, in some cases, through other receptors, such as integrins (Stacker, 2013).

Because of the fact that blood supply is essential for malignancies to maintain cell viability, it was suggested that the generation of a humanized monoclonal antibody (mAb) that inhibits the binding of VEGF-A to its receptors would reduce tumor growth by limiting the generation of new blood vessels (Nahleh, 2019). Bevacizumab (Bev) was the first vascular endothelial growth factor (VEGF)-A inhibitor to be used in several solid cancers, including breast cancer and has been studied in combination with a variety of cytotoxic chemotherapeutic agents. The specific mechanism of action of Bev has not yet been clear; it is thought that it exerts an anti-tumor effect partly by interfering with VEGF binding to its receptor on vascular endothelium, inhibiting the usual mechanisms of endothelial proliferation and survival (Varella, 2017). Although

Bev has been shown to be clearly beneficial in a subset of cancer patients, some tumours develop resistance, while some others are not susceptible to it (Stacker, 2013). Currently, Bev is approved by the USA Food and Drug Administration (FDA) for use in several cancer types, but is only approved for the treatment of breast cancer outside the USA (Nahleh, 2019).

Additional pathways that are involved in breast cancer development in case of dysregulation include: CDKs, Notch, SHH, PI3K/Akt/mTOR, and others.


BREAST CANCER
Luminal A
Luminal B
HER2 positive
Basal like / Triple negative
Genetic alterations
Oncogenes : FGFR1, PI3KCA, CCND1
Tumor suppressors : p53, PTEN, BRCA1, BRCA2
Estrogen signaling pathway
High expression of hormone receptors
MAPK signaling pathway
PI3K-Akt signaling pathway
p53 signaling pathway
Hereditary breast cancer
Homologous recombination
Notch signaling pathway
Wnt signaling pathway
Scaffold
Cell cycle (G1/S) progression
Proliferation
Survival
Translation
Uncontrolled proliferation
Increased survival
Genomic instability
Chromosomal instability
Error-prone repair of double-strand breaks
Transcriptional regulation:
Angiogenesis
NICD translocation
HER2 overexpression
EGFR overexpression
EGFR and/or c-Kit overexpression
Notch1 and Notch4 overexpression
FZD7 overexpression
LRP6 overexpression
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CHAPTER 2: KiSS-1 AND KISS-1R GENES

2.1 KiSS-1 gene discovery and characterization

KiSS-1 (KI in reference to the place of discovery, Hershey Pennsylvania, home of the Hershey Chocolate Kisses and SS as suppressor sequence) gene was first described in 1996, in non-metastatic melanoma cells, by Lee and colleagues (Lee, 1996). It has been demonstrated that ectopic expression of *KiSS-1* in C8161 melanoma cells reduced their metastatic potential in an expression-dependent manner (Guzman, 2018). Furthermore, administration of *KiSS-1*-derived polypeptides eliminated metastasis occurrence in mice injected with melanoma cells that overexpressed KISS-1R (Ciamarella, 2018). More precisely, the kisspeptins/KISS-1R system has been shown to have an impact on many different cancer cell functions, such as suppression of motility, repair after scratch wound assay, proliferation, metastasis and invasion of human cells *in vitro* (Chianese, 2019). Several intracellular signaling pathways activated by kisspeptins/ KISS-1R system have already been identified (Chianese, 2019).

The *KiSS-1* gene, located on human chromosome 1q32 (Fratangelo, 2018), encodes a 54-amino acid peptide kisspeptin-54 (Kp-54; metastin), which is further cleaved in the blood by furin and matrix metalloproteinases (MMPs), such as MMP-9 and the membrane type I matrix metalloprotease (MT1-MMP), into shorter peptides 10, 13, 14, or 54 amino acids in length (Guzman, 2019). In mouse, the *KiSS-1* gene is located on chromosome 1 and in rat on chromosome 13 (Wolfe & Hussain, 2018). Interestingly, the rodent *KiSS-1* gene gives rise to a number of splice variants that are translated into the same protein (Wolfe & Hussain, 2018). All kisspeptins share a common arginine–phenylalanine residue at the amino terminal which is required for binding and activating the KISS-1R (Clarke & Dhillo, 2016). All kisspeptins have similar affinity for KISS-1R (Guzman, 2019). Although *KiSS-1* gene was initially identified in cancer cells, it has been found to be expressed in several tissues under physiological conditions, such as brain, pituitary gland, placenta, gonads,

gastrointestinal tract and liver, as well as the vascular system (Clarke & Dhillo, 2016).

2.2 Kisspeptin-1 Receptor gene (KiSS-1r) and protein

The *KISS-1R* gene (*KiSS-1r,* also called *Gpr54*, *Axor12,* and hOT7T175) encodes of a class A (rhodopsin-like) G protein-coupled receptor (GPCR) that has homologous domains to galanin and the somatostatin receptor family. *KiSS-1r* gene is located on chromosome 19 in human and on chromosome 10 in mouse; it consists of five exons and is translated into a 398 amino acid peptide in humans and a 395 amino acid peptide in the mouse (Wolfe & Hussain, 2018). *KISS-1R* signals through a Gq/11-mediated pathway, inducing the increase of intracellular Ca^{2+}, the activation of the extracellular signal-regulated kinase (ERK)-signaling pathway and the subsequent stimulation of GnRH secretion. Recently, *KISS-1R* was also reported to signal via a Gq/11-independent, but β-arrestin-dependent pathway, inducing ERK activation (Wolfe & Hussain, 2018). The kisspeptin receptor has been found to be expressed in the human placenta and brain, being abundant in cerebellum, cerebral cortex and brain stem (Clarke & Dhillo, 2016), while lower levels of KISS-1R were detected in lymph nodes, peripheral blood lymphocytes, adipose tissue and spleen (Clarke & Dhillo, 2016; Fratangelo, 2018). These findings suggest that kisspeptin may possess multiple roles in different physiological systems. Additionally, kisspeptin-independent activation of the KISS-1R has been demonstrated, while kisspeptin can also signal independently of the KISS-1R (Wolfe & Hussain, 2018).

KISS-1R has an analogous tissue-specific expression pattern in mammalian and non-mammalian vertebrates, so kisspeptins were considered to be evolutionarily conserved molecules (Chianese, 2018). Deletion or inactivation of the *KiSS-1/KISS-1R* genes causes lack of sexual maturation and hypogonadotropic hypogonadism in rodents and humans, while gain-of-function mutations in these genes cause early puberty (Chianese, 2018).

2.3 The KiSS-1/KISS-1R system in reproduction control

Kisspeptin-expressing neurons are located in the hypothalamus and in the pre-optic region and infundibular nucleus in humans (Clarke & Dhillo, 2016). These neurons have an intrinsic role in normal physiological development at puberty and they also monitor the reproductive function during human lifetime. Interestingly, the presence of kisspeptin-producing neurons has been identified *in utero* and in the early postnatal periods (Wolfe & Hussain, 2018). Messager *et al.* (2005) showed first that kisspeptin directly regulates GnRH release. Intracerebroventricular administration of kisspeptin in sheep resulted in augmented GnRH levels. Importantly, high KISS-1R expression levels have been detected on GnRH neurons, which were shown to be stimulated by kisspeptin to release GnRH (Franssen, 2018). So, there is the notion that kisspeptin directly induces GnRH secretion via the kisspeptin/KISS-1R pathway at GnRH neurons and then GnRH induces gonadotropins secretion and puberty onset (Ke, 2018). Concerning the mechanism by which kisspeptin regulates gonadotropins, some studies support that kisspeptin expressed in pituitary cells acts on the pituitary instead of GnRH neurons, while some others propose that serum/plasma levels of kisspeptin regulate pubertal development (Ke, 2018).

Additionally, it has been shown that central kisspeptin expression is altered not only in response to food restriction (negative energy balance), but also in models of obesity (positive energy balance). Importantly, in most studies that used fasted rodents, a reduction in *KiSS-1* expression was reported in both hypothalamic kisspeptin neuron populations. More precisely, fasting in male mice downregulated *KiSS-1* mRNA levels in hypothalamus compared to fed controls (Luque, 2007), while the same result was reported in both male and female rats (Castellano, 2005). However, the study of Kalamatianos *et al.* (2008) reported no differences in ARC *KiSS-1* expression in response to a 48-h fast and a reduction in AVPV *KiSS-1* mRNA only in ovariectomized/estrogen-replaced female rats. Studies in the leptin-deficient Ob/Ob mouse, a genetic model of obesity, showed reduced (Smith, 2006; Quennell, 2011; Donato, 2011) or unchanged (Luque, 2007) mRNA levels of ARC *KiSS-1,* compared to control

mice. Studies by Smith *et al.* (2006) indicated that leptin treatment of Ob/Ob mice partially restored the decreased *KiSS-1* expression in these mice.

2.3.1 Kisspeptin receptor in metabolism regulation

KISS-1R deficiency (*KiSS-1r* KO mice) caused significant changes in body weight and glucose metabolism in female mice and changes in body composition and circulating leptin levels in male and female mice (Tolson, 2014). Tolson *et al.* showed that the loss of kisspeptin signaling contributed to the obesity observed in female mice. On the other hand, *KiSS-1r* KO males exhibited increased adiposity and circulating leptin levels, but no KISS-1R-mediated weight difference compared to controls. Studies by the Kauffman laboratory have tried to shed light on the tissue-specific mechanisms that underlie the regulation of glucose and lipid metabolism, food intake and body weight by kisspeptin. Kauffman *et al.* (2007) mentioned the presence of changes in the body composition, leptin levels and RER in 6-week-old female KO mice before the body weight increase. The body weight phenotype in *KiSS-1r* KO mice may be partly the result of an altered hypothalamic control of food intake or energy consumption. Kisspeptin treatment has been shown to cause alterations in neuropeptide Y and pro-opiomelanocortin neuronal activity in mice (Fu, 2010). De Bond *et al.* (2016) found no differences in the gene expression pattern of key genes of the hypothalamic appetite-regulating system (*Pomc* and *Npy*), as well as of the genes that encode for the receptors for leptin, ghrelin and the melanocortins between ovariectomized *KiSS-1r* KO and ovariectomized control mice. This result implies that kisspeptin acts peripherally contributing to altered metabolism in the *KiSS-1r* KO mouse (De Bond, 2016).

2.3.2 Liver-derived kisspeptin in islet hormone cross-talk

Glucagon is secreted during fasting to participate in adaptive energy mobilization in the liver and fat. It was demonstrated that *KiSS-1* expression augmented in overnight fasted WT mice, but not in mice with a liver-specific deletion of the glucagon receptor gene, suggesting that activation of liver

glucagon receptor can both induce insulin secretion and inhibit insulin secretion by stimulating kisspeptin production. When mice with selective ablation of the pancreatic *KiSS-1r* gene (Panc-KISS-1R mouse) (Novaira, 2014) were treated with kisspeptin before glucose injection presented glucose tolerance and insulin secretion similar to vehicle-injected mice, compared to control mice that presented impaired glucose tolerance and attenuated insulin secretion (Song, 2014). Opposite results have been reported about kisspeptin role in insulin secretion; some groups support that KiSS-1 stimulates glucose-stimulated insulin secretion (GSIS) (Hauge-Evans, 2006; Schwetz, 2014), while others support the opposite (Silvestre, 2008; Vikman, 2009). Subsequent studies showed that the different kisspeptin concentrations used was the cause of these controversial results. Nanomolar concentrations of kisspeptin mediated suppression of GSIS through KISS-1R, whereas supraphysiological levels of kisspeptin stimulated GSIS in a non-KISS-1R-mediated manner. Additionally, Song *et al.* (2014) demonstrated the existence of a hepatopancreatic circuit in which pancreas-produced glucagon induces hepatic expression of the genes regulating gluconeogenesis and kisspeptin. Overexpression of *Pepck* and *G6pase* increases hepatic glucose output and blood glucose levels and stimulates insulin secretion, whereas increased kisspeptin secretion inhibits insulin secretion. These results support the use of kisspeptin in the treatment of metabolic diseases.

2.3.3 Placenta-produced circulating kisspeptin

The circulating levels of kisspeptin increase approximately 10,000-fold by the placenta at the end of pregnancy (Horikoshi, 2003). These levels diminish rapidly after delivery, implying that they are produced by the placenta (Horikoshi, 2003). Horikoshi *et al.* (2003) supported also that the increased kisspeptin production during pregnancy might negatively regulate the trophoblast invasion due to the similarities between invasive placental cells and invasive cancer cells.

Placenta-produced kisspeptin could also have a metabolic role. During normal human pregnancy there is a slight excess of energy substrates in the blood for use by the developing fetus (Ryan, 2003). Due to this fact, insulin resistance is developed by several hormones and peaks during late pregnancy (34–36 weeks of gestation). Two possible explanations have been proposed for the high levels of kisspeptin late in pregnancy: kisspeptin acting through kisspeptin receptor may reduce increased insulin secretion to maintain a modest excess levels of energy substrates (glucose, free fatty acids) in the blood, or kisspeptin acting *via* a kisspeptin receptor independent mechanism may augment insulin secretion to counterbalance for the increased insulin resistance (Wolfe, 2018).

2.3.4 Fat as a source of circulating kisspeptin

Although it is still unclear, adipocytes could also be a source of circulating kisspeptin. *KiSS-1* mRNA has been detected in rat adipose tissue (Brown, 2008; Cockwell, 2013). Food restriction increased *KiSS-1* mRNA in the fat of both male and female rats (Brown, 2008). On the contrary, *KiSS-1* mRNA was reduced in obese HFD fed and obese Zucker rats (Brown, 2008). The Wilkerson group also demonstrated that kisspeptin expression was induced by estradiol in female adipocytes and by testosterone in male adipocytes (Brown, 2008). The adipocytes also express the KISS-1R (Pruszynska-Oszmalek, 2017). Human fat has also been reported to express *KiSS-1* (Cockwell, 2013). In women older than 19 years' old who were not post-menopausal, a positive correlation was observed between *KiSS-1* mRNA in visceral adipose tissue and body mass index. Similarly, Song *et al.* (2014) showed that circulating kisspeptin levels increased 2- to 4-fold in high-fat diet fed and db/db obese mice and nearly 10-fold in humans with type 2 diabetes mellitus, compared to lean mice and non-diabetic humans, respectively.

2.3.5 Other potential effects of kisspeptin on Peripheral Metabolic Function

Kisspeptin has been demonstrated to regulate smooth and cardiac muscles. Kisspeptin receptor is expressed in cardiomyocytes, as well as in the cells of the smooth muscle of the intramyocardial blood vessels (Maguire, 2011; Zhang, 2017). Although kisspeptin has been shown to induce inotropic actions on cardiac function through KISS-1R, cardiac dysfunction is not presented by humans or mice lacking KISS-1R (Maguire, 2011). Kisspeptin immunoreactivity was detected in human, mouse, and rat vascular and endocardial endothelial cells and in human cardiomyocytes (Maguire, 2011). Moreover, a recent study demonstrates that kisspeptin can stimulate gastrointestinal motility by central and peripheral mechanisms (Jiang, 2017).

CHAPTER 3: KISSPEPTINS AND KISS-1R IN CANCER AND METASTASIS

3.1 KISSPEPTINS-KISS-1R signaling pathways

The specific mechanism that underlies the antimetastatic potential of kisspeptins has not been elucidated yet, however several pathways induced by kisspeptin that might be implicated have been described (Ciaramella, 2018).

KiSS-1/KISS-1R system couples to Gq/11 inducing a) the activation of the phospholipase C signaling pathway and the subsequent Ca^{2+} recruitment, b) the phosphorylation of focal adhesion kinase inducing the formation of excessive focal adhesions and stress fibers (chemotaxis inhibition) and c) calcineurin activity reduction (metastasis suppression) (Ciaramella, 2018). G-protein-coupled signaling is regulated by β-arrestins, which can desensitize it and also act as molecular scaffolds that activate a series of signaling pathways including ERK 1/2, p38, PI3K/Akt, and cJun N-terminal kinase 3 (Fratangelo, 2018).

The antimetastatic activity of kisspeptins has been demonstrated in several types of cancer, such as melanoma, thyroid, ovary, bladder, gastric, pancreas, and lung cancers (Ciaramella, 2018). In some of these cases there is a common antimetastatic mechanism involving the inhibition of tumor invasion via a suppression of the MMP-9 expression and activity, and by inhibiting MAPK (Guzman, 2019). Thus, *KiSS-1* gene acts as a cancer suppressor gene and activation of kisspeptin signaling inhibits cancer cell invasion, metastasis, and tumor recurrence. Contradictory data on the role of KiSS-1 and KISS-1R in carcinogenesis may result from the presence of alternative forms of these genes or their different epigenetic regulation.

3.2 The role of KiSS-1 and KISS-1R complex in human cancers

Metastasis is a complex procedure, responsible for about 90% of cancer deaths (Seyfried & Huysentruyt, 2013). This process is known as the invasion-metastasis cascade and is multi-step. Initially, cancer cells disseminate from primary tumors and invade locally into surrounding tissues. Then, these cells intravasate into the circulatory system, arrest and extravasate into the parenchyma of distant tissues formatting micrometastatic colonies (Lambert, 2017).

Kisspeptin has been described to suppress the metastatic potential of cancer cells, to sensitize them to conventional chemotherapy and to induce a dormancy state to tumour disseminated cells (Beck and Welch, 2010; Jiffar et al., 2011).

In several clinical studies downregulation of *KiSS-1* and/or *KISS-1R* expression is linked to poorer prognosis in patients, indicating that *KiSS-1* and/or *KISS-1R* might be used as predictive biomarkers. More precisely, in gastric cancer, reduced expression of *KiSS-1* was associated with recurrent cancer invasion and shorter survival. In ovarian cancer, lower *KiSS-1* gene expression was linked to more resistant cancer, cell invasion and worse patient's prognosis (Hata, 2007), while in urinary bladder cancer, loss of *KiSS-1* expression was a common feature of all invasive cancers. Similarly, in esophageal squamous cell carcinoma, *KiSS-1* was downregulated in ≥ 85% of tumors with lymph nodes metastases, indicating a role of kisspeptin signaling in esophageal carcinoma metastasis (Ikeguchi, 2004).

Studies on the role of kisspeptins in breast cancer have resulted into contradictory data. Blake *et al.* (2017) demonstrated that KISS-1R signaling enhances drug resistance in ERα-negative breast cell lines and in TNBC cells by increasing the expression of the efflux drug transporter breast cancer-resistance protein and also by enhancing tyrosine kinase expression and activity. The researchers used a KISS-1R antagonist that was able to restore cell sensitivity to doxorubicin. Their findings support the notion that KISS-1R

can serve as a novel therapeutic target to restore drug sensitivity in patients affected by TNBC.

An additional role that has been assigned to KiSS-1 is the maintenance of the disseminated melanoma cells in a state of dormancy, inhibiting metastasis to multiple organs (Nash, 2007). Importantly, this ability of KiSS-1 was reported to be KISS-1R independent. These findings support the potential use of exogenous KiSS-1 as a treatment of tumor patients.

3.2.1 Colorectal Cancer

Colorectal cancer (CRC) represents the second most common cancer type among women and the third most common among men (Fratangelo, 2018). It is one of the deadliest types of cancer due to the disease progression *via* liver metastases; 30% of patients already have metastasis when diagnosed (Siegel, 2017). Current treatments have significantly improved the survival of patients with metastatic CRC, however, many patients become resistant to therapies.

The study of Okugawa *et al.* (2013) indicated that patients with tumors with low *KiSS-1* mRNA expression had poorer prognosis and exhibited lymph node metastasis, in comparison to patients with high *KiSS-1* levels. Immunohistochemistry experiments for KiSS-1 showed high expression levels in primary CRC and in early stages of the disease and lower expression levels in advanced stage-tumors. Canbay *et al.* (2012) measured plasma KiSS-1 levels in blood samples from CRC patients and age-matched healthy controls. KiSS-1 plasma levels were significantly higher in CRC patients (86.2 ± 20.5 ng/ml) compared to controls (49 ± 12.7 ng/ml). It was also found that KiSS-1 levels were significantly correlated with nodal metastases of CRC. Ji *et al.* (2014) examined KiSS-1 and KISS-1R mRNA levels in samples from colorectal cancer tissues and normal tissues. Their results showed that the levels of KiSS-1 mRNA had a negative correlation with Duke's staging, TMN staging, tumor size and lymph node metastasis.

Additionally, reduced KISS-1R expression was linked to poor prognosis in patients. Importantly, patients who were undergoing chemo/radiotherapy showed a higher expression of KISS-1R compared to those without. Chen *et al.* (2014) also showed that a decrease in *KiSS-1* gene and protein expression was associated with an increased invasion and lymph node metastasis in CRC patients compared to normal controls. The researchers also discovered that low *KiSS-1* expression levels were caused due to inactivation by epigenetic mechanisms (Chen, 2014). In 88.33% of *KiSS-1* of CRC samples there was hypermethylation; this was significantly higher compared to the rate observed in normal colorectal tissues (Chen, 2014). The DNA methyltransferase inhibitor azacitinde (5-Aza-2-deoxycytidine) was able to restore *KiSS-1* expression and the corresponding reduction of CRC cell invasion (Chen, 2014). Chen *et al.* (2016) showed that *KiSS-1* gene inhibits the metastatic potential of CRC cells by suppressing the expression of MMP-9. High levels of *KiSS-1* expression inhibited the proliferation and the invasiveness of HCT-119 CRC cells and boosted their apoptosis by MMP-9 downregulation through blocking of PI3K/Akt/NF-κB pathway.

Shen *et al.* (2016) compared the miRNA expression profiles in CRC tissues and hepatic metastasis; their results showed that downregulation of miR-199b was linked to distant metastasis in CRC and a longer survival. Additionally, using a human tumor metastasis PCR array, the researchers found that *KiSS-1* is one of the downstream targets of SIRT1. Silencing of SIRT1 enhances the acetylation of the transcription factor CREB which binds to the promoter of *KiSS-1* upregulating its expression. Thus, Shen *et al.* identified miR-199b as a regulator of SIRT1/CREB/KiSS-1 signaling pathway and proposed that it might be used as a prognosis marker for patients with CRC (Shen, 2016).

3.2.2 Bladder Cancer

Urinary bladder cancer is the ninth most common malignant disease (Guzman, 2018). In bladder cancer, KiSS-1 expression was significantly

decreased or lost in invasive bladder tumors compared to their respective normal urothelium (Sanchez-Carbayo, 2003). Patients with lower KiSS-1 expression in bladder tumors had worse survival. Also, KiSS-1 expression levels were significantly decreased in bladder tumors with vascular invasion compared with normal urothelium. Moreover, all bladder tumors that developed distant metastases showed loss of KiSS-1 expression. As observed in CRC, *KiSS-1* was found to be hypermethylated in over 83% of 804 primary bladder tumors (Cebrian, 2011). Epigenetic loss of KiSS-1 expression was associated with poor survival, indicating that KiSS-1 levels can be used as a predictive value for patients' disease outcome.

Takeda *et al.* (2012) examined KiSS-1 and KISS-1R expression in bladder cancer patients to evaluate their prognostic significance. Their findings showed that KiSS-1 immunoreactivity was significantly reduced in advanced stages of the disease and inversely associated with tumor grade and stage. However, KISS-1R expression levels were not associated with disease progression. Interestingly, this study demonstrated that KiSS-1 administration significantly reduced the invasiveness of bladder cancer cells and lung metastasis; the underlying mechanism involved the inhibition of MMP-9 expression and activity via blockage of NF-kB nuclear translocation. Moreover, multivariate analysis demonstrated that KiSS-1 expression levels were an independent predictor of bladder cancer metastasis, as well as of patient survival.

Sanchez-Carbayo *et al.* (2003) detected loss of *KiSS-1* expression in bladder cancer tissues that was associated with bladder cancer progression and clinical outcome. Cebrian *et al.* (2011) demonstrated that the lower levels of *KiSS-1* transcripts in invasive bladder cancer compared to superficial tumors, were the result of the epigenetic silencing of *KiSS-1* gene due to a CpG island hypermethylation near to *KiSS-1* promoter. Similarly, Zhang *et al*. (2014) showed that ubiquitin-like with PHD and RING finger domains 1 increased the methylation of CpG islands of *KiSS-1*, reducing its expression.

3.2.3 Ovarian Cancer

Ovarian cancer is the deadliest cancer type of the female reproductive system and among the top five deadliest cancers in most countries (Ferlay, 2015). KiSS-1 expression levels have also been linked to survival in ovarian cancer patients. Hata *et al.* (2007) detected elevated levels of KiSS-1/KISS-1R expression by quantitative PCR, in ovarian cancer patients, which was linked to favourable prognosis. Cao *et al.* (2016) reported increased KiSS-1 expression in 40 pre-operative epithelial ovarian cancers primary tumors compared to normal tissue. Pre-operative KiSS-1 expression levels were negatively associated with metastasis development and tumor size. Additionally, patients with low KiSS-1 expression had worse clinical outcome than those with high expression.

In the largest study of ovarian biopsies done to date, Prentice *et al.* (2007) utilized a tissue microarray consisting of 518 ovarian carcinomas to perform immunohistochemical analysis of KiSS-1 and KISS-1R. The study results showed that intense KiSS-1 and KISS-1R immunostaining was associated to favourable prognosis and patients' survival. Jayasena *et al.* (2012) used an in-house radioimmunoassay to measure the concentration of plasma kisspeptin in 31 patients with ovarian carcinoma (Stages I to IV) and 31 healthy volunteers. Their results showed that stage I patients had increased KiSS-1 plasma levels compared to the control and stages II-IV. However, larger studies are required to further examine the relationship between plasma kisspeptins and ovarian cancer.

3.2.4 Prostate Cancer

Prostate cancer is the second leading cause of cancer mortality in men of 40 years of age and older (Siegel, 2016). Currently, chronically administered gonadotropin-releasing hormone receptor (GnRHR) agonists are used for treatment inducing androgen deprivation (Salciccia, 2016). Kisspeptin is known to stimulate GnRH secretion and to play a key role in the hypothalamus-pituitary-gonadal (HPG) axis. Several studies (Matsui, 2014; MacLean, 2014;

Millar, 2015) have shown that administration of kisspeptin agonists eliminated serum testosterone levels by suppressing the HPG axis in rats and humans. Wang *et al.* (2012) examined KiSS-1 protein expression in 253 prostate tissue samples (normal tissue and prostate cancer) in order to evaluate the direct anti-metastatic potential of KISS-1 in prostate cancer cells. Their results showed that there was a negative correlation between KiSS-1 expression levels and clinical staging (Wang, 2012). Additionally, they demonstrated that decreased mRNA expression of *KiSS-1* and KISS-1R correlated with increased metastatic ability of human prostate cancer cell lines. They also induced *KiSS-1* re-expression in PC3M cells that lack KISS-1 resulting in inhibition of cell migration and invasion and re-sensitization of cells to chemotherapeutics (Wang, 2012). Kim *et al.* (2017) showed that KISS-1R signaling induced the activation of eukaryotic translation initiation factor 2α kinase in prostate cancers that inhibited cell growth and metastasis. In conclusion, kisspeptin seems to act both indirectly via the HPG axis and directly on prostate cancer cells being a promising therapeutic agent.

3.2.5 Lung Cancer

Lung cancer is the most commonly diagnosed cancer and the leading cause of death in men (Wang, 2016). Zheng *et al.* (2010) showed an inverse correlation between KISS-1 expression and progression of non-small cell lung cancer (NSCLC), showing that KISS-1 functions as a metastasis suppressor. Furthermore, studies on cisplatin-resistant NSCLC cells demonstrated that overexpression of exogenous *KiSS-1* significantly decreased their capability to invade *in vitro* and *in vivo* (Zuco, 2010). Zheng *et al.* (2010) demonstrated that *KiSS-1* mRNA and protein levels were lower in the metastatic tissues compared to the primary tumors, indicating a metastasis suppressor role for *KiSS-1* in NSCLC associated with disease stage, metastasis, and survival of the patients.

Sun *et al.* (2013) examined the expression of *KiSS-1* and *KiSS-1R* genes in 56 NSCLC specimens divided into low stage (locally advanced) and metastatic (advanced) disease. Their results showed an inverse correlation

between *KiSS-1* and *KiSS-1R* expression and NSCLC progression. The expression levels of KiSS-1 and KiSS-1R were lower in cancer tissues compared to normal tissues, as well as in patients with advanced stage compared to patients with low stage of NSCLC. Moreover, low stage disease cells presented high apoptotic ratio and arrest in G1 phase, suggesting that KiSS-1/KISS-1R complex might have a role also in apoptosis and cell cycle (Sun, 2013). Karapanagiotou *et al.* (2011) measured the circulating levels of kisspeptin in 96 NSCLC patients (75 with metastatic disease and 21 with locally advanced disease) and healthy volunteers. Their results revealed no differences in plasma kisspeptin levels between NSCLC patients and healthy volunteers or between locally advanced and metastatic disease patients.

3.2.6 Gastric Cancer

KiSS-1 has been shown to inhibit the proliferation and invasion also of gastric carcinoma cells *in vitro* and *in vivo,* through the downregulation of MMP-9 (Li, 2012). Dhar *et al.* (2004) studied the *KiSS-1* mRNA expression levels into two groups of 40 gastric cancers and demonstrated that *KiSS-1* may represent an independent prognostic factor for gastric cancer patients. The decreased expression of *KiSS-1* in tumor tissues was associated with the ability of gastric cancers to invade, metastasize, and relapse, as well as to worse clinical outcome. Immunohistochemical analysis of tissue microarrays from 71 patients with gastric cancer revealed a statistically significant reduction of KiSS-1 protein expression in lymph node and liver metastases compared with primary tumors (Guan-Zhen, 2007).

3.2.7 Thyroid Cancer

Ringel *et al.* (2002) showed that KiSS-1 is expressed both in normal thyroid and in papillary thyroid carcinomas in contrast to KiSS-1R that is overexpressed in papillary thyroid cancer but not in normal thyroid and in follicular adenomas. The low expression levels of KiSS-1 and KISS-1R proteins

may be the reason for the increased ability of follicular carcinomas to metastasize. KISS-1R was also shown to activate MAPK, but not Akt, in thyroid cancer cells (Ringel, 2002). Additionally, KiSS-1 expression was significantly lower in early stages thyroid tumors compared to advanced tumors with extrathyroidal invasion (Ringel, 2002).

3.3 Kisspeptin-mediated signaling in cancer

3.3.1 KiSS-1 as a negative regulator

SMAD ubiquitin regulatory factor-1 (SMURF1) is a negative regulator of KiSS-1-mediated signaling (Yan, 2018). SMURF1 overexpression promoted BCPAP and K1 (papillary thyroid carcinoma-derived cell lines) cell viability, migratory, invasive and proliferation abilities. SMURF1 downregulates the protein levels of KiSS-1 in thyroid TPC-1 and SW579 cancer cells. When SMURF1 was overexpressed in these cancer cell lines, it enhanced the ubiquitination of KiSS-1 reducing its protein levels in a dose-dependent way. Additionally, high levels of KISS-1 blocked NF-κB signaling pathway in thyroid cancer TPC-1 and SW579 cells (Yan, 2018). Moreover, KiSS-1 was shown to suppress the metastatic abilities of cancer cells via an EIF2AK2-mediated pathway (Kim, 2017). KiSS-1 had also an anti-metastatic role in LoVo lung cancer cells in nude mice, through a EIF2AK2- mediated pathway (Kim, 2017).

3.3.2 KiSS-1 as a positive regulator

Overexpression of ABCG2/BCRP (breast cancer resistance protein) and receptor tyrosine kinase AXL promotes resistance against doxorubicin (Blake, 2017). Moreover, KISS-1R-overexpression significantly reduced doxorubicin accumulation in SKBR3 cancer cells. Use of inhibitors of drug efflux transporters or KISS-1R antagonists restored sensitivity to doxorubicin (Blake, 2017). KISS-1R signaling induced transcriptional upregulation of AXL via binding of a transcription factor, SP-1 (Blake, 2017). So, KISS-1R seems to

have an intrinsic role in chemoresistance development in breast cancer cells via AXL and BRCP/ABCG2 overexpression. KISS-1R signaling stimulated fibulin-3 in ERα- negative BCa cells. KP-10 was shown to stimulate MMP-9 activity in a fibulin-3 dependent way (Noonan, 2018). Fibulin-3 silencing in MDA-MB-231 cells induced reduced levels of lung metastasis in mice (Noonan, 2018).

3.4 The epigenetic modulation of KiSS-1 in cancer

Several studies have shown that epigenetic modifiers could be used as therapeutic agents for some cancers types (Mack, 2014; Rahimi, 2019; Vidoni, 2019). For this reason, epigenetic drugs characterized by increased specificity and efficiency have been developed for the treatment of human cancer (Mack, 2014; Rahimi, 2019; Vidoni, 2019). During tumor growth several epigenetic changes take place including: a) hypermethylation in promoter CpG islands, particularly in tumor-suppressor genes, b) histone modifications that result in gene expression alterations and c) deregulation of miRNA expression that is related to functional changes in target genes (Kanwal, 2012; Fraga, 2005; Hosseini, 2008; Esteller, 2011; Liz, 2015; Leenen, 2016; Sharma, 2010; Flavahan, 2017).

The epigenetic changes of *KiSS-1 gene* are of particular importance due to *KiSS-1* involvement in the development of metastasis. In cancer, the kisspeptin system might have an anti-metastatic role, affecting cellular migration and invasion, or might also be involved in other stages of tumor development like the dormancy state. KiSS-1 has been demonstrated to induce a dormancy state of the disseminated melanoma cells, inhibiting their metastatic colonization to multiple organs (Chianese, 2019).

CpG islands colocalize with the *KiSS-1* promoter and in cancer the hypermethylation of the *KiSS-1* promoter leads to reduced protein expression. In CRC, epigenetic modifications of the *KiSS-1* promoter are usual, in contrast with normal tissues and result in transcription and protein expression loss. More precisely, *KiSS-1* methylation was related to metastasis, predicted recurrence and disease-free and overall survival (Chen, 2014). So, *KiSS-1* may serve as a

possible target for the treatment of metastatic CRC. Additionally, the methylation values of *KiSS-1* in combination with the serum concentration of carcinoembryonic antigen (CEA) have been shown to have a greatest prognostic value compared to CEA alone (Moya, 2013). *KiSS-1* hypermethylation has also been detected in numerous cases of bladder tumors. Cebrian *et al.* (2011) used RT-PCR to show that the methylation of the *KiSS-1* promoter decreased *KiSS-1* expression. Low *KiSS-1* expression levels were also related to poor disease-specific survival. Moreover, Cebrian *et al.* (2011) demonstrated that *KiSS-1* promoter hypermethylation was frequently reported in bladder cancer cells and was related to a low *KiSS-1* gene expression, using methylation-specific PCR and bisulfite sequencing. As far as bladder cancer is concerned, it has been demonstrated that the epigenetic silencing of *KiSS-1* depends on the upregulation of Ubiquitin-like with PHD and RING finger domains 1 (UHRF1), which enhances the methylation of CpG islands. Additionally, UHRF1 was found to be overexpressed in most bladder cancer tissues compared to normal tissues and in metastatic tumors in comparison to non-metastatic tumors (Zhang, 2014).

Importantly, recent data show that miRNAs and long non-coding RNAs (lncRNAs) could affect kisspeptin-mediated signaling. Also, miRNAs regulate the expression of proteins that are involved in the modification and inhibition of *KiSS-1* expression (Farooqi, 2019). In fact, the cAMP response element-binding protein (CREB) induces the upregulation of *KiSS-1*. The NAD+-dependent deacetylase SIRT1 inhibits the CREB-mediated upregulation of *KiSS-1* through *miR-199b.* In contrast, in CRC, *miR-199b* upregulation inhibits SIRT1, thus enhancing the CREB-induced upregulation of *KiSS-1.* In this sense, *miR-199b* could represent a prognostic marker and/or therapeutic target for CRC patients (Shen, 2016). Additionally, TCF21 is a positive transcriptional regulator of KiSS-1 in C8161 cells, interacting with E12, a TCF3 isoform and TCF12 (Arab, 2011). TCF21 has been shown to be regulated by non-coding RNAs, TARID (for TCF21 antisense RNA inducing demethylation) (Arab, 2014) and LINC00163, another long non-coding RNA (Guo, 2018). TCF21 silencing reduced KiSS-1 expression in Caki-1 cells (Zhang, 2012). MiR-21 directly targeted TCF21 in

Caki-1 cells. So, it seems that miR-21, interacting with TCF21, inhibits *KiSS-1* expression in renal cell carcinoma cells.

Recent data also support an intrinsic role of lncRNAs in the modulation of *KiSS-1* expression in several cancer types. It has been shown that lncRNAs worked synergistically with EZH2 to regulate the transcriptional levels of their target genes. LncRNAs captured tumor suppressor miRNAs and removed oncogenic mRNAs. EZH2, a catalytic subunit of PRC2, is a transcriptional repressor of a plethora of target genes; it was demonstrated that it suppressed the transcription of KiSS-1 along with TP73-AS1, a long non-coding RNA (Liu, 2018). In pancreatic and ovarian cancer, the KiSS-1/KISS-1R system was demonstrated to have a tumor suppressor role and be overexpressed in the initial phases of cancer development. Importantly, in TNBC cells, *KiSS-1* was shown to induce tumor growth (Guzman, 2018). The mechanisms that underlie the function of non-coding RNAs have to be further investigated.

CHAPTER 4: KISSPEPTIN IN BREAST CANCER

4.1 Expression levels of KiSS-1 and KISS-1R in breast cancer

One year after the first paper on melanoma, Lee and colleagues published the results of another study on the effect of *KiSS-1* on the metastatic potential of the human breast carcinoma cell line MDA-MB-435 (Lee, 1997). Previous studies had already shown loss of heterozygosity for regions of chromosome lq in human breast cancers. The human breast carcinoma cell line MDA-MB-435 was transfected with the pcDNA3 constitutive expression vector that contained the full-length KiSS-1 cDNA. A vector without insert was used as a negative control. Transfected cells and controls were injected into the subaxillary mammary fat pads of 4-6-week-old female athymic nude mice. Tumors were measured weekly and one month later mice were sacrificed. Both tumor types, developed from MDA-MB-435 and KiSS-1 transfectant, exhibited characteristics of poorly differentiated invasive adenocarcinoma, without any differences in their histological properties. However, the number of macroscopic lung metastases in animals injected with the KiSS-1 transfectants was significantly decreased. In all mice inoculated with KiSS-1 transfectants, lung metastasis was eliminated by at least 50%. Similarly, regional lymph node metastasis was also reduced. The results of this study showed that *KiSS-1* expression in human MDA-MB-435 breast carcinoma cells significantly diminished their metastatic ability in athymic nude mice.

Song *et al.* (2013) investigated if KiSS-1 expression and methylation patterns were different in the breast cancer cell lines, MDA-MB-231 and MDA-MB-157. MDA-MB-231 cells showed relatively low KiSS-1 expression levels, whereas the MDA-MB-157 cell line expressed higher KiSS-1 protein levels. Treatment of hypermethylated (MDA-MB-231) and hypomethylated (MDA-MB-157) cell lines with a DNA demethylating drug (5-aza-2'-deoxycytidine - AZA) induced increase of KiSS-1 expression at the transcriptional level. Then, MDA-MB-231 cells were transfected with integrated or truncated KiSS-1 fragments, whereas MDA-MB-157 cells were transfected with KiSS-1 siRNA. MDA-MB-231

cells expressing intact or truncated KISS1 showed 4-5 times higher apoptotic ratio than the non-transfected ones. These cells also displayed decreased motility in wound-healing and transwell assays. The researchers also evaluated the inhibition potential of KiSS-1 protein on tumor growth in a breast cancer model. Tumors generated by intact or truncated KiSS-1-treated cells had significantly lower volume and weight compared to the other tumors. Additionally, the same tumors displayed significantly higher survival rate. Using immunohistochemical staining it was also shown that KiSS-1 induced apoptosis in breast cancer cells by suppressing the MEK/ERK signaling pathway. Overall, these results indicated that KiSS-1 protein exerted anti-tumor effects in breast cancer cells inducing increased apoptotic ratio, lower motility and lamellipodia formation *in vitro* via suppression of the Ras/Raf/MEK/ERK signaling pathway. *In vivo*, in a breast cancer model, KiSS-1 treatment induced decrease in tumor volumes and weights.

Kim *et al.* (2017) investigated the role of EIF2AK2 in the inhibitory effects of KiSS-1 on cancer metastasis. KiSS-1 activated EIF2AK2 in several highly metastatic types of cancer cells. Using a scratch wound assay and EIF2AK2-knockout MEFs, the researchers found that EIF2AK2 was implicated in kisspeptin-mediated inhibition of cellular migration and invasion. RhoA-mediated signaling was found to be involved in the mechanisms underlying the kisspeptin-induced phosphorylation of EIF2AK2. Using an *in vivo* experimental metastasis assay, the researchers showed that KiSS-1-induced EIF2AK2 activation reduced cancer cell migration and invasion, inhibiting distant metastasis to the lungs of nude mice. The results suggested that KiSS-1 protein exerts its anti-metastatic capabilities on cancer cells via EIF2AK2 phosphorylation.

Platonov *et al.* (2018) used an engineered conditionally replicative adenovirus (CRAd) with oncolytic capability to deliver and express the *KiSS-1 gene* in metastatic breast cancer cells. Two oncolytic vectors encoding either KiSS-1 (AdKISS1) under control of CMV promoter or no transgene were generated and tested for their ability to express KiSS-1 protein by measuring KiSS-1 protein levels in the infected A549 cells. The AdKISS1 was used to

induce oncolysis in human brain metastatic breast cancer cell lines (CN34Br and MDA231Br), human lung carcinoma cells (A549) and normal human lung fibroblasts (MRC5). Using an MTT assay the authors showed that AdKISS1 decreased cancer cell proliferation, especially in CN34Br and MDA231Br cells, whereas it had no effect on the proliferation of non-cancer fibroblasts (MRC5). The levels of apoptosis were measured following infection of cancer or normal human cells with Ad or AdKISS1 and the results revealed that the replicative AdKISS1 vector induced higher apoptotic levels to the cancer cells, compared to the control Ad vector.

The secretome of infected cells with AdKISS1 or Ad (control) viruses was analysed and the authors found that AdKISS1-infected cells displayed a significant upregulation of 94 cytokines and a downregulation of 90 different cytokines compared to Ad-infected cells. Five factors (GKN-1, OX40L, DKK3, IL-27, cystatin SN and NUDT-5) that are known to suppress cell proliferation were upregulated in AdKISS1-infected cells. Also, FGF9, FGF16, neugranin, desmin, galanin, TC-PTP and furin that possess a dual role in cancer were found to be induced by KiSS-1. However, KiSS-1 induced also a significant upregulation of genes, that are involved in MAPK, RAS and RAP1 signaling pathways and are implicated in cancer. On the contrary, KiSS-1-suppressed genes regulate lysosome activity, cell adhesion and cytokine-cytokine interactions, have a stimulatory effect on various cancer cells and affect their resistance to therapies, such as DSG1, NG2, SOX9, CPE, VEGF-C, and CD97. Additionally, using an EGFR protein array the researchers showed that KiSS-1 induced MAPK activation. Finally, Platonov *et al.* (2018) showed that *KiSS-1* knock-down with RNA silencing activates VEGF, MMP14, IL8, that are involved in tumor angiogenesis. Overall, ectopic expression of KiSS-1 in infected breast cancer cells suppressed cell proliferation, decreased cell viability and inhibited tumor angiogenesis, indicating that KiSS-1 can be administered as a new anti-cancer therapy.

Over the last decade, several studies have shown that the KiSS-1/KISS-1R system is related to breast cancer progression, contrasting the reports that support the antimetastatic potential of *KiSS-1* in breast cancer. Martin *et al.*

(2005) studied the expression and distribution of KiSS-1 and its receptor in human breast cancer tissues. They also tried to find a possible link between KiSS-1 expression levels and patient prognosis. The researchers used immunohistochemistry and an anti-human KiSS-1 antibody on breast cancer primary tumours (matched tumour 124 and background 33). Additionally, qRT-PCR was used to measure KiSS-1 mRNA levels. Their results showed higher KiSS-1 levels in tumour compared to background tissues and significantly increased KiSS-1 expression in node positive tumours compared to node negative. Increased KiSS-1 expression in patients was linked to death from breast cancer; patients who had remained healthy expressed lower levels. Moreover, transfection of the human breast cancer cell line MDA-MB-231 with the KiSS-1 gene increased cell motility and invasiveness and reduced cell adhesion to matrix. The results of this study showed that the levels of KiSS-1 expression were higher in metastatic disease patients compared to healthy individuals, and that this was associated with poor patient prognosis. Also, over-expression of KiSS-1 in the human breast cancer cell line MDA-MB-231, resulted in a more aggressive phenotype.

Blake *et al.* (2007) using immunohistochemistry, demonstrated that KiSS-1 and KISS-1R are expressed within the ductal carcinoma *in situ* and in invasive ductal carcinoma and that their expression was higher in tumors than in normal tissue. Tissue samples from ductal invasive breast carcinomas displayed significant downregulation of KiSS-1 mRNA in brain metastases compared to the primary tumors.

Similarly, Papaoiconomou *et al.* (2014) studied the protein expression levels of KiSS-1 and KISS-1R in breast cancer tissues compared to non-malignant mammary tissues. The study included 43 tissue specimens which consisted of 32 ductal carcinomas and 11 lobar carcinomas, as well as 11 non-malignant mammary gland tissues served as controls. Tissue staining with KiSS-1 and KISS-1R antibodies revealed significantly higher expression levels of KiSS-1 and KISS-1R in breast cancer tissues compared to controls. However, KiSS-1 and KISS-1R protein levels were not associated with the tumor grade, tumor size or lymph node metastasis. Additionally, there were no

differences in the expression levels of KiSS-1 and its receptor in lobar and ductal breast cancers and their expression was not related to the expression of ER, PR, cerb2, p53 or Ki67. This study resulted in KiSS-1/GPR54 expression significantly higher in breast cancer compared to non-malignant mammary tissues.

Ulasov *et al.* (2012) studied the relationship between KiSS-1 expression and breast cancer progression, as well as brain metastases development. The researchers utilized immunohistochemistry to examine KiSS-1 expression in primary invasive ductal carcinoma (IDC), ductal carcinoma in situ, brain metastasis from human breast (BMHB), metastatic lymph nodes and non-neo-plastic breast parenchyma from a total of 389 patients. Using a tissue microarray and a KiSS-1 biomarker, they compared KiSS-1 protein expression levels between the primary tumors and metastases. To study the relationship between mRNA and protein expression, they used qRT-PCR. Their results demonstrated a statistically significant downregulation of *KiSS-1* mRNA and protein in brain metastasis compared to primary tumors. The most significant difference was downregulation of KiSS-1 expression in BMHB compared with IDC.

In 2007, Marot *et al.* investigated whether KiSS-1 and/or KISS-1R expression is regulated by estrogen signaling in breast cancer cell lines and whether this regulation may have clinical relevance in the evaluation of the tumoral response to tamoxifen (TAM) treatment. Initially, the researchers evaluated KiSS-1 and KISS-1R mRNA levels in the following breast tumor cell lines: T47D, MCF7, ZR75-1, MDA-MB-231, MDA-MB-435 and L56Br. Then, in order to investigate the effects of E2 on KiSS-1 expression, MDA-MB-231 cells (ERa negative) were infected with recombinant adenoviruses encoding ERa. KiSS-1 mRNA levels were measured by real-time RT-PCR 24h later and the results showed that the expression of KiSS-1 and KISS-1R were regulated through estrogen signaling pathways in breast tumor cell lines. More precisely, E2 significantly decreased KiSS-1 mRNA levels in ERa- and ERb-infected ER-negative MDA-MB-231 cells compared to uninfected control cells. Interestingly, the contribution of ERb in ER-deficient breast tumor cells was as effective as

the reintroduction of ERa. Moreover, ER-expressing cell lines were treated with the synthetic anti-estrogen TAM for 24h to investigate if ER inhibition would result in an increase in KiSS-1 expression. TAM treatment resulted into a three- and two-fold increase in KiSS1 expression in MCF7 and T47D cells, respectively. The mRNA levels of KiSS-1R in TAM-treated MCF7, T47D and ZR75-1 cells increased 2.5-, 3.4- and 7.3-fold respectively.

To study the mRNA levels of KiSS-1 and KISS-1R during breast cancer progression, the researchers analysed 92 primary breast tumor samples from women that met the following criteria: primary unilateral non-metastatic postmenopausal invasive ductal carcinoma of the breast; ERa positivity; complete clinical, histological and biological information available; no radiotherapy or chemotherapy before surgery and full follow-up. Their analysis showed that patients with high KiSS-1 and KISS-1R expression in breast tumors had the poorest prognosis, while those with the best prognosis had low KiSS-1 mRNA levels and low KISS-1R mRNA levels. The groups that had low KiSS-1/high KISS-1R and high KiSS1/low KISS-1R presented intermediate outcomes.

To investigate the association between mRNA levels of KiSS-1 and KISS-1R and ERa expression status, 36 additional primary breast tumors were analysed, 12 ERa negative and 24 ERa positive. KiSS-1 mRNA levels were found to be significantly lower in the ERa positive breast tumors compared to the ERa negative breast tumors. Conversely, the mRNA levels of KISS-1R were slightly higher in the ERa positive breast tumors compared to the ERa negative.

To conclude, this study showed that high levels of *KiSS-1* were detected in breast tumors, but not in normal mammary tissues. *KiSS-1* level was high in the first stages of the disease and increased with tumor progression, whereas *GPR54* expression increased in invasive ductal tumors but not in benign tumors or ductal carcinoma *in situ*. The authors suggest that *KiSS1* may be used as a molecular marker for human breast tumors and *GPR54* as a marker of invasive-grade tumors.

In a similar study, Kostadima *et al.* (2007) evaluated *KiSS-1* mRNA levels in 272 women with dissected node-positive breast cancer and

investigated if *KiSS-1* mRNA levels are associated with tumour clinical and molecular characteristics, as well as with patients' relapse and survival. KiSS-1 mRNA levels were detected in only eight among 272 patients (3%), whereas they were undetectable in 264 women with breast adenocarcinomas. There was no association between *KiSS-1* mRNA levels and the number of involved axillary lymph nodes, histological grade, hormone receptor status or tumour size. The tumors that expressed KiSS-1 mRNA levels were hormone receptor-negative and of poor differentiation. The transcriptional levels of regulators of cellular proliferation, angiogenesis, DNA repair and apoptosis (HER2, VEGF, p53, BCL2, PAEP and BIRC5) were examined, however no relationship was found between the transcriptional levels of *KiSS-1* and those marker-genes.

The study by Cho *et al.* (2011) further supported the pro-metastatic role of KISS-1R in breast cancer. These researchers using mouse models of Kiss1r gene knockout and mouse mammary tumor virus-polyomavirus middle T antigen (MMTV-PyMT)-induced breast cancer showed that Kiss1r heterozygosity decelerated PyMT-induced breast cancer development and metastasis. Moreover, Kiss1r heterozygosity (Kiss1rþ/) in MMTV-PyMT/Kiss1r mouse models resulted in attenuated breast tumor initiation, tumor growth, latency, multiplicity and metastasis. Experiments of *KiSS-1* or *KiSS-1R* silencing showed that *KiSS-1* / *KiSS-1R* signaling was indispensable in pubertal breast epithelial cells for breast hyperplasia. Moreover, *KiSS-1R* heterozygous (PyMT/Kiss1rþ/) tumors exhibited a significant downregulation of MMP-9 mRNA levels compared to wild type (PyMT/Kiss1rþ/þ) tumors. Additionally, KISS-1R signalling activated RhoA, a key regulator of the cytoskeleton, inducing cell migration and invasion. This study indicated that KiSS-1R has an intrinsic role in breast tumor initiation, progression and metastasis through Gαq-p63RhoGEF-RhoA signaling pathway.

Cvetkovic *et al.* (2013) investigated the role of KISS-1R signaling in mammary epithelial cells. The researchers treated the non-malignant and non-invasive MCF10A cells, which endogenously express KISS-1R, with KP-10 or overexpressed KISS-1R in them. Using 3D Matrigel invasion assays they showed that KP-10 treatment or KISS-1R overexpression significantly

stimulated the invasiveness of MCF10A cells compared with control cells. Moreover, KP-10-treated cells displayed loss of membrane integrity and diffuse localization of the membrane protein laminin V. *In vivo,* the researchers investigated the ability of SKBR3 FLAG-KISS1R cells and vector controls to extravasate after intravenous administration into the CAM of chicken embryos. The number of SKBR3 FLAG-KISS1R cells that extravasated was significantly higher compared to vector control cells. KP-10 incubation of SKBR3 FLAG-KISS1R cells further increased the number of cells that extravasated; this phenomenon was inhibited upon treatment of cells with P-234, a KISS-1R antagonist. These data indicated that KISS-1R signaling plays a role in breast cancer cell invasion *in vitro* and extravasation *in vivo*.

Additionally, using the *in vitro* model of scratch wound assay the researchers investigated if KISS-1R activation by KP-10 was able to induce MCF10A cell migration. Their results showed that KISS-1R activation enhanced the motility of MCF10A and MCF10A FLAG-KISS1R cells. Importantly, treatment of MCF10A cells with KP-10 or stable expression of KISS-1R in MCF10A and SKBR3 cells induced EMT transition. Also, KP-10 treatment transactivated EGFR in the ER-negative non-malignant mammary MCF10A epithelial cells, in the MCF10A KISS1R cell lines, as well as in the ER-negative SKBR3 FLAG-KISS1R breast cancer cells. EGFR phosphorylation by KP-10 was conquered in IQGAP1-depleted cells, indicating a pivotal role of IQGAP1 in KISS-1R-induced EGFR activation. These data implied that the ability of KISS-1R signaling to induce cell migration, invasion and EGFR transactivation depends on the ER status of breast epithelia. KP-10 may influence metastatic potential of breast cancer cells that are deficient in the ER. KISS-1R was found to induce the extravasation of metastatic cells *in vivo* and the actin cytoskeletal protein IQGAP1 was required for KISS1R-induced transactivation of EGFR in breast cancer cells. The results of this study demonstrated that kisspeptin treatment or exogenous expression of KISS-1R in MCF10A cells induced EMT and stimulated cell invasiveness by inducing the expression of mesenchymal markers (N-cadherin, Snail/Slug) and loss of E-cadherin from cell-cell junctions.

4.2 Expression levels of KiSS-1 and KISS-1R in ERα positive and ERα negative breast tumors

Estrogen is a steroid hormone with a pivotal role in the development of the mammary epithelium during puberty. High lifetime exposure to estrogen increases breast cancer risk (Stingl, 2010). Breast tumors are divided into ERα positive and ERα negative. Mammary epithelial cells that express ERα in the normal adult mammary gland are typically not proliferating, although ERα+ cells often proliferate in tumors. Moreover, premenopausal exposure to estrogen decreases the rate of occurrence of ERα− tumors after menopause, while the rate of occurrence of ERα+ tumors steadily increases (Stingl, 2010). Patients with ERα+ tumors often become resistant to TAM, within 15 months of treatment and there are many possible reasons: a tamoxifen-resistant clone may exist within the tumor cell population, an ERα− subpopulation may be present within ERα+ cell lines, ER+ cancer (stem) cells may undergo a phenotypic shift from an ERα+ state to an ERα− state *in vitro* or ERα+ cancer stem cells may obtain intrinsic mutations that lead ligand-independent ERα activation (Stingl, 2010).

In their study, Marot *et al.* (2007) investigated E_2 effect on KiSS-1 expression levels. The breast tumor cell line MDA-MB-231 that is ERα negative, was infected with recombinant adenoviruses encoding ERα (AdERα) and ERβ (AdERβ). Twenty-four hours later, infected MDA-MB-231 cells expressed high levels of *KiSS-1* mRNA. *KiSS-1* mRNA levels were significantly reduced, 12h after addition of E_2 to the medium. These results were also confirmed by immunocytochemistry, using an antibody against amino acid residues 45–54 of human kisspeptin. Functional inhibition of ER by the synthetic anti-estrogen TAM upregulated *KiSS-1* gene expression levels. Moreover, *KiSS-1* expression was evaluated in different types of primary breast tumors. High levels of *KiSS-1* expression were detected in breast tumors, whereas in normal mammary tissues KiSS-1 expression was undetectable. Also, *KiSS1* expression levels were linked to tumor progression, whereas *KISS-1R* expression levels were associated with tumor metastatic capacity. Moreover, *KiSS-1* expression was observed to be negatively associated with ERα tumor status. In ER positive

tumors, high *KiSS-1* and *GPR54* mRNA levels were significantly associated with shorter relapse-free survival for postmenopausal women with invasive primary breast tumors. Patients with high *KiSS1* and *GPR54* levels had a worse clinical outcome than those with low KiSS-1 and GPR54 levels.

In summary, the results of this study correlate *KiSS-1* expression levels with breast tumor progression. High levels of *KiSS-1* were detected in breast tumors, but not in normal mammary tissues. *KiSS-1* might serve as a molecular marker for human breast tumors, whereas *GPR54* as a marker of invasive-grade tumors.

In the study of Cvetkovic *et al.* (2013), as mentioned above, ERα was demonstrated to negatively regulate KiSS-1 and KISS-1R expression, as well as KISS-1R-induced cell invasion. These researchers also reported that absence of ERα expression (e.g. in TNBC) leads to increased mRNA levels of KiSS-1/KISS-1R and receptor signalling, as well as to the acquisition of invasive characteristics and epithelial to mesenchymal transition.

4.3 Expression levels of KiSS-1 and KISS-1R in Triple Negative Breast Cancer (TNBC)

TNBC constitutes 15–20% of all breast cancer cases and usually appears in women under 50 years old, particularly those of African American and Hispanic descent, or in patients with BRCA1 / BRCA2 mutation (Blake, 2017; Guzman, 2019). TNBC is an aggressive subtype of breast cancer, which consists of a heterogeneous group of tumors. Histologically, TNBCs are frequently classified as high-grade, invasive, ductal carcinomas of no special type with basal-like features (Marotti, 2017). However, some TNBCs types have distinct morphology, such as secretory carcinoma, adenoid cystic carcinoma and adenosquamous carcinoma and a more favourable prognosis compared with basal-like TNBC (Marotti, 2017). TNBCs lack the expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2, which are used along with the histologic characteristics of the tumor, the tumor

size, the lymph node involvement and the tumor node metastases staging to define prognosis and therapeutic strategy. However, TNBCs express high-molecular-weight basal cytokeratins (cytokeratin 5/6, cytokeratin 14 and cytokeratin 17), epidermal growth factor receptor (EGFR), vimentin, p-cadherin, aB-crystallin, fascin and caveolins 1 and 2 (Marotti, 2017).

TNBC patients display high rates of distant metastasis, which are linked to morbidity (Noonan, 2018) and present a greater recurrence risk (Marotti, 2017). Hormone receptor or HER2-targeted therapies are not effective in patients with TNBC, making TNBC an orphan disease (Guzman, 2018). Treatment for TNBC patients is currently limited to standard cytotoxic anthracycline and taxane-based chemotherapy, however chemotherapy is not usually beneficial for these patients; they initially respond well, but often develop chemoresistance (Foulkes, 2010). The underlying mechanisms of TNBC drug resistance are poorly understood; increased survival factors, inactivation of cell death pathways and multidrug resistance proteins, such as ATP-binding cassette transporters, seem to be implicated (Blake, 2017).

Researchers have tried to better subclassify TNBCs using molecular approaches. Lehmann *et al.* (2011) identified six subtypes of TNBC: basal-like 1 and 2 (BL1 and BL2), immunomodulatory (IM), mesenchymal, mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). The BL1 subtype was associated with genes involved in the cell cycle and DNA damage repair, the BL2 subtype was related to higher expression of growth factor genes, the IM subtype was characterised by genes involved in immune cell processes, the mesenchymal and MSL subtypes were identified by cell motility and differentiation genes (EMT transition) and the LAR subtype was characterized by androgen receptor signaling. The different TNBC subtypes have displayed differing clinical outcomes and varying responses to therapy (Marotti, 2017).

TNBC cells express higher levels of KiSS-1 and KISS-1R in comparison with ERα positive breast cancer cells or nonmalignant breast MCF10A cells and secrete KP-10 (Dragan, 2020). It has not been clear yet if plasma kisspeptin levels are also increased in TNBC patients in order for kisspeptin to be used as a biomarker for metastasis and disease recurrence (Guzman, 2019).

Zajac *et al.* (2011) demonstrated that the Kp-10/GPR54 system stimulated the ability of human breast cancer cells to migrate and invade. In this process MMP-9 and b-arrestin 2 were also implicated. Additionally, the researchers showed that GPR54 directly interacted with EGFR and that upon stimulation of breast cancer cells, with either Kp-10 or EGF, the GPR54 and EGFR complex was endocytosed.

Cvetkovic *et al.* also demonstrated that KP-10/KISS-1R system stimulated the migration and invasion of the non-invasive MCF10A cell line, inducing an EMT transition.

Goertzen *et al.* (2016) showed that depletion of KISS-1R expression using shRNA in the highly invasive TNBC MDA-MB-231 and Hs578T cells that possess mutated Ras oncogene, resulted in loss of their mesenchymal cell morphology and downregulation of mesenchymal markers expression. The effect of KISS-1R depletion in the migration capacity of TNBC cells was further evaluated using scratch wound assays and transwell chamber migration assays. Loss of KISS-1R expression significantly reduced TNBC cells migration capacity under basal conditions, as well as in the presence of KP-10. Using Matrigel-transwell membrane invasion assays, the researchers also demonstrated that loss of KISS-1R expression reduced TNBC cell invasion. Furthermore, activation of KISS-1R signaling was found to augment the activity of cortactin, cofilin and MT1-MMP in TNBC cells inducing the formation of invadopodia and their invasion through ERK1/2, but independently of Src. Interestingly, KISS-1R was located to invadopodia only upon activation, where it interacted with invadopodia protein cortactin, as well as with β-arrestin-2. Using β-arrestin-2 knock-down and the highly selective ERK inhibitor U0126 the researchers demonstrated that β-arrestin-2 and ERK1/2 acted as downstream mediators of KP/KISS1R signaling in TNBC cell invadopodia formation.

Blake *et al.* (2017) using a TNBC cDNA array (Origene) demonstrated that the expression levels of *KiSS-1* and *KISS-1R* in 20 TNBC primary breast tumors were higher compared to 10 normal breast tissue biopsies. KISS-1R protein levels were also higher in TNBC primary tumors compared to normal breast, however, KiSS-1 protein levels were not detectable in these tissue

samples. Two ERα negative cell lines, normal (non-malignant) MCF10A and breast cancer SKBR3 cells that expressed very low levels of endogenous KISS-1R, were transfected with vectors containing FLAG-KISS1R to overexpress KISS-1R. The SKBR3FLAG-KISS-1R cells showed increased *KISS-1R* mRNA and protein levels, as well as higher levels of KiSS-1 mRNA and protein compared to pFLAG vector controls. KISS-1R overexpression in SKBR3 cells increased their motility compared to pFLAG vector controls, as was shown by a scratch wound healing assay.

Additionally, SKBR3 cells that overexpressed KISS-1R were found to express increased levels of the pro-survival molecules AXL, AKT, ERK, as well as the anti-apoptotic protein, survivin. TNBC tumors were also found to express increased *AXL* mRNA levels, compared to normal breast tissue. Furthermore, *AXL* expression was positively correlated with *KiSS-1* expression in the TNBC samples. Immunofluorescence analysis of TNBC patient tumors showed that *KiSS-1*, *KISS-1R* and *AXL* were aberrantly expressed in human TNBC tumors. Additionally, KISS-1R signaling was shown to be implicated in the acquisition of chemoresistance of TNBC cells through the expression of efflux drug transporter, breast cancer resistance protein (BCRP) and the activation of the tyrosine kinase, AXL (Blake, 2017).

Tian *et al.* (2018) investigated if TGFβ/p21 and KiSS-1 signaling pathways, that both promote metastasis in breast cancer, interfere. The researchers evaluated KiSS-1 gene expression in different breast cancer molecular subtypes. The results of their gene expression analysis showed that more aggressive HER2-enriched and basal-like breast cancer subtypes expressed higher KiSS-1 levels compared to other molecular subtypes. Higher levels of KiSS-1 expression were also detected in ER- breast cancer compared to less aggressive ER+ breast cancer. So, increased KiSS-1 expression was related to breast cancer invasiveness. Then, the researchers investigated if the TGFβ/p21 signaling axis could regulate KiSS-1 levels in breast cancer. TGFβ was found to strongly induce KiSS-1 mRNA and protein levels in various TNBC cell lines (MDA and SCP2), while it had no effect in the ER+ cell lines (BT474 and MCF7). In order to examine if KiSS-1 was implicated in TGFβ-induced

invasion, a specific siRNA was used to silence its expression. Using a Matrigel transwell invasion assay, it was demonstrated that blocking of KiSS-1 activity *in vitro* using a peptide antagonist, inhibited TGFβ-mediated MMP-9 induction and cell invasion. Kisspeptin-10 (KP-10) has also been shown to enhance cancer cell invasiveness through MAPK/Erk pathway.

Dragan *et al.* (2020) measured the plasma KiSS-1 levels among newly diagnosed, non-metastatic TNBC (early disease) and metastatic TNBC (advanced disease) patients; TNBC patients had significantly higher plasma KiSS-1 levels compared to healthy females. To examine if KISS-1R regulates tumor metabolism, mice were injected with SKBR3FLAG-KISS1R cells to induce tumor formation. The SKBR3FLAG-KISS1R primary breast tumors had significantly higher volume and expressed GLS (encodes glutaminase), in contrast to control tumors. A metabolomic analysis showed that KISS-1R overexpression resulted in significantly higher glutamate levels in the primary tumors, compared with control tumors, while there was no change in glutamine levels. Both serum glutamate and glutamine levels were significantly decreased in SKBR3FLAG-KISS1R xenografts compared with controls.

These data indicate that KISS-1R overexpression promotes glutamine metabolism in primary tumors by upregulating glutaminase expression to promote tumor growth. Metabolomic analysis of primary tumors revealed that several metabolites in the glutamine, nucleotide and lipid synthesis pathways and TCA cycle were significantly elevated in the SKBR3FLAG-KISS1R tumors compared with control tumors. These results indicate that KISS-1R overexpression promotes glutamine metabolism in primary tumors by upregulating glutaminase expression promoting tumor growth.

To investigate if human KISS-1R regulates metastasis, the researchers silenced KISS-1R expression in metastatic MDA-MB-231 cells and then injected these cells in a spontaneous metastasis xenograft model using NOD/SCID/IL2 receptor γnull mice. KISS-1R knockdown reduced primary tumor volume and eliminated the capacity of tumor cells to metastasize and colonize the lungs. Then, the researchers examined whether KISS-1R regulates lung colonization using an experimental metastasis xenograft model. Human ERα negative

luminal SKBR3 breast cancer cells (that express low levels of endogenous KISS1R) that stably overexpressed FLAG-KISS1R were intravenously injected into mice and induced a significantly higher number of lung metastases compared to control cells.

Additionally, it was shown that KISS-1R overexpression in ERα negative SKBR3 cells resulted in a significant increase in the expression of c-Myc, GLS1, GLUD1 and a decrease in the expression of glutamine synthetase (encoded by GLUL gene), that converts glutamate to glutamine. GLUD1 is essential for sustaining the TCA cycle in rapidly proliferating cells and is upregulated in breast cancer. These data suggest that KISS-1R overexpression may sustain the TCA cycle required for cell growth and proliferation. KISS-1R modulates the expression of key regulators of glutamine metabolism in ERα negative breast cancer cells. Overall, the results of this study suggest that TNBC patients had elevated plasma kisspeptin levels compared with healthy subjects. KISS1R-induced glutamine dependence of tumors and promoted glutaminolysis and nucleotide biosynthesis by increasing c-Myc and glutaminase levels.

Kim *et al.* (2017) aimed to investigate if melatonin regulates KiSS-1 expression in TNBC, as happens in the brain hypothalamus. Melatonin has been shown to suppress breast cancer growth and metastasis. Initially, TNBC cell lines, MDA-MB-231 and HCC-70, were treated with different concentrations of melatonin, for 48h, to examine the effect of melatonin on the ability of cancer cells to proliferate, migrate and invade. The study results showed that melatonin-treated TNBC showed unchanged proliferation rate, but inhibited metastatic abilities. The levels of KiSS-1 protein were evaluated in the conditioned medium from melatonin-treated MDA-MB-231 cells. It was revealed that melatonin induced GATA3-mediated KiSS-1 expression in TNBC (MDA-MB-231 and HCC-70) cells. When KiSS-1 was silenced in MDA-MB-231 cells using KiSS-1 siRNA, melatonin failed to inhibit the cell invasiveness, showing that melatonin suppressed cell invasiveness via KiSS-1 expression.

CONCLUSIONS

The KiSS-1/KISS-1R system was initially demonstrated to have a role in the reduction of metastatic potential of human melanoma cells by Lee *et. al* (1996). Since then, several studies have demonstrated that this system has also a physiological role in the onset of puberty, sexual maturity and pregnancy through direct regulation of the hormone releasing the gonadotropin produced by the hypothalamus.

In several types of cancer, such as pancreatic, ovarian, colorectal, lung, thyroid, bladder and gastric cancer, *KiSS-1* has been described as a gene suppressor of metastasis. On the contrary, Martin *et al.* (2005) firstly demonstrated that *KiSS-1* may have a detrimental role in breast cancer. *KiSS-1* expression was higher in metastatic disease patients compared to healthy individuals, and also was associated with poor patient prognosis. Moreover, KiSS-1 over-expression in human breast cancer cells resulted in a more aggressive phenotype. Since then, many other studies have associated the KISS1/KISS1R system with breast cancer initiation, progression, and lung metastasis, as well as with drug resistance of TNBC cells.

The reason for these contradictory data remains unknown. It is possible that kisspeptin has different effects on different cancer types. Moreover, alternative forms of KISS1/KISS1R genes or different epigenetic modifications could lead to controversial results. Importantly, the MDA-MB-435 cell line, that has been used in many studies that support the antimetastatic role of KiSS-1, was initially classified as a “breast cancer” line, however it has been found to express genes resembling melanoma cells; it is not considered to be a robust breast cancer cell model. As far as it concerns the studies that use cancer patients’ samples, many factors have to be taken under consideration, such as previous chemotherapy, the patients’ age, the genetic diversity of the population and the ER status of breast tumors.

TNBC is an aggressive subtype of breast cancer that is linked to high rates of distant metastasis. TNBC cells lack the expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2,

so researchers aim to identify other marker-proteins that these cells express in order to target them. KiSS-1/KISS-1R represents an attractive candidate target for TNBC therapy. Possible therapies could be exosomes effectively transfected with antibodies raised against human KISS-1, or with nucleic acids, such as miR-21, able to downregulate KISS-1 expression.

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