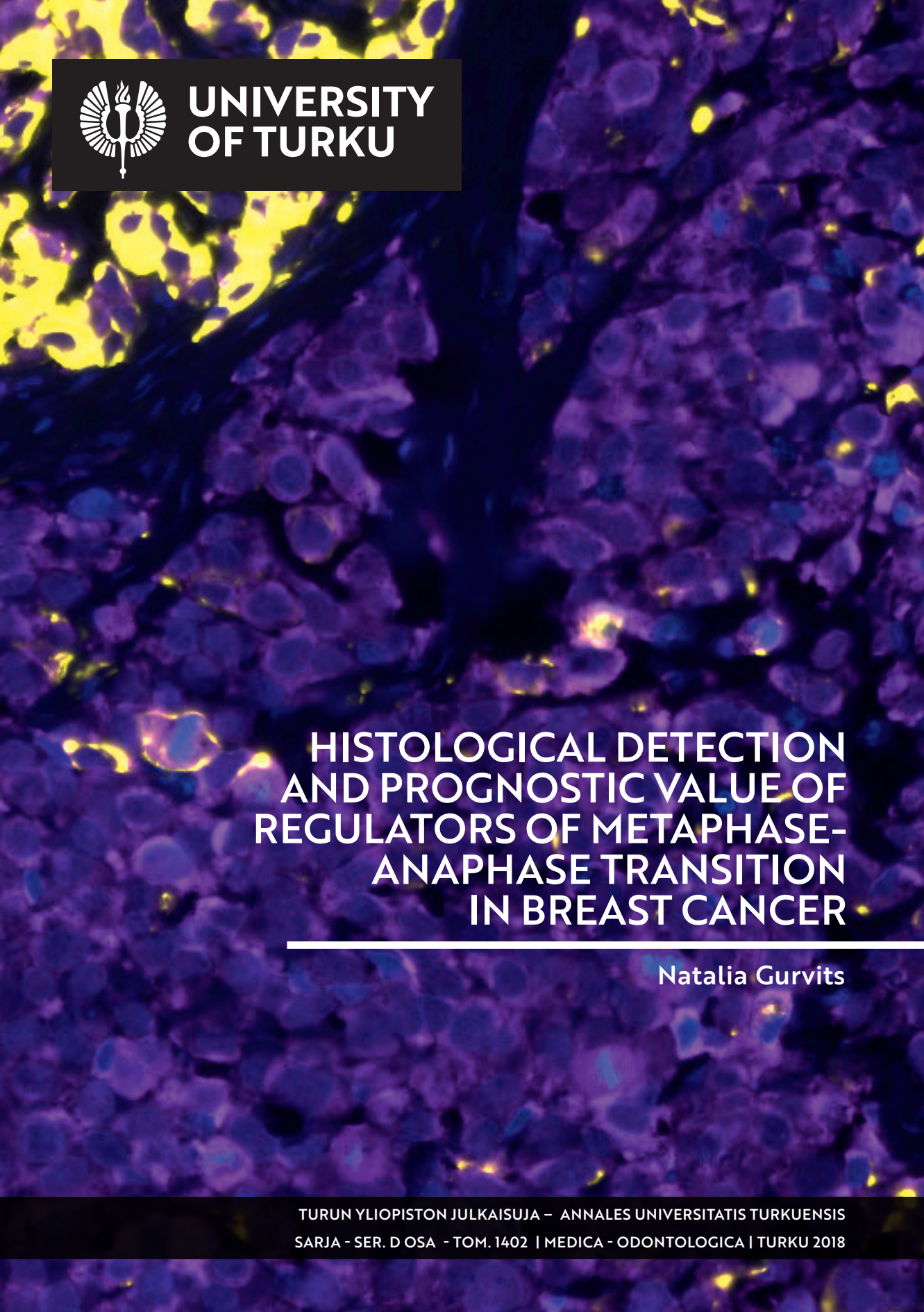

UNIVERSITY OF TURKU

HISTOLOGICAL DETECTION AND PROGNOSTIC VALUE OF REGULATORS OF METAPHASE-ANAPHASE TRANSITION IN BREAST CANCER

Natalia Gurvits

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To my family

ABSTRACT

Natalia Gurvits

Histological detection and prognostic value of regulators of metaphase-anaphase transition in breast cancer

University of Turku, Faculty of Medicine, Institute of Biomedicine, Pathology, Doctoral Programme in Clinical Research – DPCR
Annales Universitatis Turkuensis, Medica-Odontologica, Turku, 2018

The study focuses on proteins Securin, Pttg1IP and Separase, involved in metaphase-anaphase transition of mitosis, and microRNAs -494, -205, -21 and -126 in association with cell proliferation. The aim is to demonstrate the expression patterns of the studied proteins and levels of microRNAs, and to assess their applicability in predicting the outcome of patients diagnosed with invasive breast cancer. In addition, the influence of fixation delay and time on the staining results of fresh breast cancer tissue is studied.

The study comprises 447 invasive breast carcinomas and a total of 143 triple-negative breast carcinomas diagnosed in Central Hospital of Central Finland during 1987 – 1997 and Turku University Hospital, Turku, Finland, during 2005 – 2015 with complete clinical data and a maximum follow-up period of 22 years. The tissue material was arranged in tissue microarrays. The tissue sections were prepared by immunohistochemical and double and triple immunofluorescence stainings for the investigation of the proteins. An automated *in situ* hybridization method was developed for identifying the studied miRNAs.

Securin, Pttg1IP and Separase showed divergent expression patterns in normal breast epithelium as compared to breast carcinomas, and between specific cancer subgroups. The studied proteins were associated with decreased disease-specific outcome at a statistically significant level. In multivariate analysis involving traditional prognostic features of breast cancer, Securin and Separase were independently associated with higher breast cancer mortality (HR 2.4, p <0.0001 and HR 1.8, p=0.002, respectively). In some breast cancer subgroups abnormal levels of -494 and -205 were detected. In local disease negative for microRNA-494 significantly higher breast cancer mortality was observed (HR 8.5, p=0.04). Also multivariate analysis involving traditional prognostic features of breast cancer suggested independent prognostic value for microRNA-494 (HR 2.1, p=0.04). Variations in tissue fixation and processing were tested in sets of breast specimen revealing the profound impact of pre-analytical incidents on detecting proteins and microRNAs histochemically.

Keywords: breast cancer, prognosis, cell cycle, mitosis, histotechnology

TIIVISTELMÄ

Natalia Gurvits

Metafaasi-anafaasi-siirtymän säätelijöiden kudosopillinen osoittaminen ja ennuste-arvo rintasyövässä

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Patologia, Turun kliininen tohtoriohjelma – TKT
Annales Universitatis Turkuensis, Medica-Odontologica, Turku, 2018

Tutkimuksen kohteena olevat proteiinit, Securin, Pttg1IP ja Separase, osallistuvat solunjakautumisen metafaasi-anafaasi siirtymään ja mikro-RNA:t -494, -205, -21 and -126 liittyvät solujen proliferaatioon. Tavoitteena on osoittaa tutkittavien proteiinien ilmenemistapoja ja mikro-RNA:iden ilmenemistasoja sekä arvioida niiden vaikutusta rintasyöpäpotilaiden ennusteeseen. Lisäksi tutkitaan tuoreen rintarauhaskudoksen fiksaatioviiveen ja fiksaatioajan vaikutusta värjäystolulokseen.

Tutkimus perustuu 447 invasiivisen ja yhteensä 143 kolmois-negatiivisen rintasyövän aineistoon. Tutkimusmateriaali on koottu Keski-Suomen Keskussairaalasta vuosina 1987 – 1997 ja Turun yliopistollisesta keskussairaalasta vuosina 2005 – 2015. Aineistoon liittyvät kliiniset tiedot ja pisimmillään 22 vuoden seurantatiedot, jotka on koottu potilaiden sairauskertomuksista, Auria Biopankista ja Suomen Syöpärekisteristä. Rintasyöpä-näytteistä valmistettiin monikudosblokit. Niistä leikatuillelle kudosleikkeille tehtiin immunohistokemillisia värjäyksiä sekä, kaksois- ja kolmoisimmunofluoresenssivärjäyksiä proteiininen tutkimiseksi. Automatisoitua in situ hybridisaatio–menetelmää käytettiin tutkittavien mikro-RNA:iden tunnistamiseen.

Securinin, Pttg1IP:n ja Separasen ilmenemistavat vaihtelivat normaalin maitorauhasen pintaepiteelin ja rintasyöpäsolujen sekä rintasyövän eri alatyyppien välillä. Tutkittavien proteiinien todettiin liittyvän tilastollisesti merkittävästi rintasyövän huonompaan ennusteeseen. Monimuuttuja-analyysissä yhdessä rintasyövän vakiintuneiden ennuste-tekijöiden kanssa Securin ja Separase liittyivät itsenäisinä ennustetekijöinä korkeampaan rintasyöpäkuolleisuuden riskiin (riskikerroin Securinille 2.4, $p<0.0001$ ja Separaselle 1.8, $p=0.002$). Osassa rintasyövän alaryhmiä todettiin poikkeavia mikro-RNA:iden -494 ja 205 ilmenemistasoja. Paikallisessa rintasyövässä, jossa ei esiintynyt mikro-RNA-494:ää, kuolleisuus oli tilastollisesti merkittävästi korkeampi (HR 8.5, $p=0.04$). Myös monimuuttuja-analyysi yhdessä rintasyövän vakiintuneiden ennustetekijöiden kanssa osoitti mikro-RNA-494:n merkityksen itsenäisenä ennustetekijänä rintasyövässä (HR 2.1, $p=0.04$). Kudosnäytteiden fiksaatioon ja prosessointiin liittyviä muutoksia testattiin rinta-näytesarjoissa, jolloin todettiin pre-analyyttisten tekijöiden keskeinen vaikutus proteiinien ja mikro-RNA:iden histokemiallisiin värjäyksiin.

Avainsanat: rintasyöpä, ennuste, solusykli, solunjakautuminen, kudostekniikat

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ABBREVIATIONS

2-ME	2- mercaptoethanol
APC/C	anaphase-promoting complex
ATM	ataxia-telangiectasia mutated
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BRIP1	BRCA1-interacting protein 1
CDC20	cell-division cycle protein 20
CDH1	cadherin 1
CDK1	cyclin-dependent kinase 1
CEP17	centromeric probe for chromosome 17
CHEK2	checkpoint kinase 2
c-Myc	cancer myelocytomatosis
CT	chemotherapy
DAB	diaminobenzidine
DIG	digoxigenin
DNA	deoxyribonucleic acid
EGFL7	epidermal Growth Factor like domain 7
ER	estrogen receptor
ESPL1	extra spindle poles like 1
ET	endocrine therapy
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
HE	hematoxylin-eosin
HER2	human epidermal growth factor receptor 2

HRPO	horseradish peroxidase
IF	immunofluorescence
Ig	immunoglobulin
IHC	immunohistochemistry
ISH	*in situ* hybridization
LKB1	liver kinase B1
LNA	locked nucleic acid
MCT8	monocarboxylate transporter 8
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
NBF	neutral buffered formalin
ncRNA	non-coding ribonucleic acid
NGS	new generation sequencing
PAK1	p21 activating kinase 1
PALB2	partner and localizer of *BRCA 2*
PBF	Pttg1-binding factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHH3	phosphohistone H3
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
Pttg1	pituitary tumor-transforming gene 1
Pttg1IP	pituitary tumor-transforming gene 1 protein interacting protein
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SAC	spindle assembly checkpoint
SDS	sodium dodecyl sulfate

STK11	serine/threonine kinase 11
TDLU	terminal duct lobular unit
TNBC	triple-negative breast cancer
TP53	tumor protein 53
TRITC	tetramethylrhodamine
TSA	tyramide signal amplification

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals I-IV:

I **Gurvits N**, Repo H, Löyttyniemi E, Nykänen M, Anttinen J, Kuopio T, Talvinen, Kronqvist P. Prognostic implications of Securin expression and sub-cellular localization in human breast cancer.
Cell Oncol (Dordr). 2016 Aug; 39(4):319-331.

II Repo H, **Gurvits N**, Löyttyniemi E, Nykänen M, Lintunen M, Karra H, Kurki S, Kuopio T, Talvinen K, Söderström M, Kronqvist P: PTTG1-interacting protein (PTTG1IP/PBF) predicts breast cancer survival.
BMC Cancer. 2017 Oct; 17(1):705-712.

III **Gurvits N**, Löyttyniemi E, Nykänen M, Kuopio T, Kronqvist P, Talvinen K: Separase is a marker for prognosis and mitotic activity in breast cancer.
Br J Cancer. 2017 Oct; 117(9):1383-1391.

IV **Gurvits N**, Autere TA, Repo H, Nykänen M, Kuopio T, Kronqvist P, Talvinen K. Proliferation-associated miRNAs-494, -205, -21 and -126 detected by *in situ* hybridization: expression and prognostic potential in breast carcinoma patients.
J Cancer Res Clin Oncol. 2018 Apr; 144(4):657-666.

1 INTRODUCTION

Cell division is one of the prime hallmarks of life. Impeccable cell division is the critical requirement for growth, repair, development and reproduction of every organism, both normal and neoplastic (Hannahan and Weinberg, 2011). Missegregation of chromosomes leads to genetic instability and aneuploidy, the characteristics of malignant disease (Wenzel and Singh, 2018, Giam and Rancati, 2015). Abnormal genetic segregation has been associated with several malignancies, including breast cancer, and thus cell cycle control is a potential target of personalized cancer treatments (Cai and Liu, 2017). Metaphase-anaphase transition is one of the phases of the cell cycle where the cell is at its most vulnerable to genetic disorders caused by incomplete sister chromatid segregation (Dominguez-Brauer et al., 2015). Metaphase-anaphase transition is controlled by a complex network of regulating proteins, including Securin (Pituitary tumor-transforming gene 1 protein, Pttg1), Pttg1IP (Pituitary tumor-transforming gene 1 protein interacting protein) and Separase (Extra spindle poles-like 1 protein, ESPL1) (Kumar, 2017). In addition, epigenetic regulation of the cell cycle is further complicated by involvement of inhibitory microRNAs (miRNAs) (O'Bryan et al., 2017).

After becoming duplicated in the S phase of mitosis, the sister chromatids remain intact and together through the metaphase until initiation of the anaphase. The basic mechanisms regulating the timing and order of the events leading to sister chromatid segregation are highly protected and tightly controlled in all normal cells. Premature or untimely loss of cohesion predisposes the cell to uncontrolled centriole duplication, chromosomal missegregation and aneuploidy, the hallmarks of malignant progression (Haaß et al*.,* 2012, Pati, 2008). Uncontrolled chromatid segregation can be triggered by abrupt activation of Separase, an important regulator of the metaphase-anaphase transition of the cell cycle. Separase is tightly and in a mutually exclusive manner controlled by complex inhibitory interactions in pathways involving either Securin or cyclin B1/CDK1 (G2 mitotic-specific cyclin-B1/cyclin-dependent kinase 1) (Zhang and Pati, 2017). Securin belongs to a class of cell cycle regulators preventing metaphase-to-anaphase transition until sister chromatid separation is complete. In the normal cell, Securin acts as an anaphase inhibitor, forming with Separase a complex with the ability to arrest the cell cycle at the metaphase. In an uncontrolled cell cycle, ubiquitin-dependent degradation of Securin triggers untimely activation of Separase and predisposes the cell to premature sister chromatid separation (Waizenegger et al., 2002, Hornig et al., 2002, Holland and Taylor, 2006). Another key actor in the regulation of the metaphase-anaphase transition is Pttg1IP, also responsible for the timed and controlled release of the sister chromatids. Pttg1IP is believed to promote the activation of Securin by facilitating the shift of Securin from

the cell cytoplasm to the nucleus, thus, enabling the activation of Separase by Securin (Salehi et al., 2008). In addition in epigenetic regulation, aberrant miRNA expression is known to function in proliferation and metaphase-anaphase transition (Chivukula and Mendell, 2008, Carleton et al., 2007). MiRNAs -494, -205, -21 and -126 are examples of miRNAs which have previously been suggested with a role in cell cycle regulation and metaphase-anaphase transition (He et al., 2014, Piovan et al., 2012, Zhang et al., 2016, Ebrahimi et al., 2014).

Breast cancer is the most prevalent cancer and the main cause of cancer deaths among women around the world. Presently, some 1.7 million cases and over half a million breast cancer deaths are reported annually, accounting for 25% of all cancer patients and 15% of total cancer deaths among females (Torre et al., 2012). Over the last decades, survival rates have improved for all age groups worldwide and, in Finnish population, the disease-specific 5-year survival is presently 91% (www.cancer.fi). However, despite the average survival rate, breast cancer deaths still occur among patients diagnosed with an advanced and aggressive disease.

The purpose of this study is to examine the protein expressions of a set of regulators of the metaphase-anaphase transition, ie Securin, Pttg1IP and Separase. Moreover, the expression of a set of miRNAs known to be involved in the same event of cell division, i.e. miRNAs -494, -205, 21 and -126, are evaluated. Based on a large amount of material of invasive breast carcinomas diagnosed and treated in two separate Finnish centers for breast cancer diagnosis and treatment, the prognostic impacts of the studied proteins and miRNAs have been evaluated. The study applied some specific histotechniques developed for detecting the proteins and miRNAs under investigation and evaluates chosen quality aspects for potential influence on the prognostic associations. The expression patterns, sub-cellular localizations and prognostic roles of the proteins and miRNAs under investigation have, thus far, not been systematically investigated using extensive amounts of materials of breast cancer patients with long-term follow-up data.

2 REVIEW OF LITERATURE

2.1 Breast cancer

2.1.1 Prevalence and course of disease

Breast cancer is the most common form of malignancy and comprises a serious risk for cancer death. Worldwide, breast cancer counts for approximately 25% of all malignancies and approximately 15% of cancer deaths among women (Torre et al., 2015). Previously, the majority of breast cancer cases were diagnosed in the Western world (GLOBOCAN, 2008). Recently, however, the incidence of breast carcinoma in developing countries of South America, Africa and Asia has exceeded that of the Western world (Green et al., 2008). The explanation for this trend is not completely understood but may reflect the improved diagnostic opportunities in developing countries as well as increased hormonal burden among women, possibly related to e.g. expansion of hormone replacement therapies (Leong et al., 2010). Meanwhile, developments in diagnostic and therapeutic methods have improved the outcome of breast cancer worldwide although some subgroups of breast cancer are still associated with a significant risk of cancer-related death (Gong et al., 2017).

Based on studies with long-term follow-up, the incidence of breast cancer in Finland is among the highest in the world (Forouzanfar et al., 2011). The annual incidence of breast cancer has steadily increased since the 1950's and is presently more than 2.5-fold as compared to the incidence levels 40 years ago (www.cancer.fi). In Finland, every 8th woman develops a breast cancer during her lifetime while the risk is similar in Sweden but somewhat lower in Denmark (www.cancer.fi) (Table 1). Prognosis of breast cancer in Finland is among the best in Europe (Tryggvadóttir et al., 2010). According to the latest statistics from 2005-2017, cancer-specific 5-year survival rate for breast cancer was 90% (www.cancer.fi). At the moment, the annual risk of breast cancer-death among Finnish women is approximately 2% (www.cancer.fi, Forouzanfar et al., 2011). In the Nordic countries, the relative decline in breast cancer mortality between 1985 - 1989 and 2009 - 2013 has been most distinct among premenopausal women, with reductions in mortality rates ranging from 38% in Finland to 55% in Denmark (Kvåle et al., 2017).

Table 1. Summary statistics on breast cancer incidence and mortality in Finnish population (www.cancer.fi).

		Incidence 2015	Incidence 2011 – 2015
New cases	**female**	5161	24679
	male	30	130
Per 100 000*	**female**	185.5	178.6
	male	1.1	1.0

In Russia, breast cancer is also the most common form of malignancy and the leading cause of cancer deaths among women. According to the latest statistics, the overall breast cancer mortality in Russia has reached 50 – 60% (Garin, 2004). Thus far, the high breast cancer mortality rates have been explained by delayed cancer diagnosis since only 60% of breast cancer cases in Russia are diagnosed at a localized stage. However, introduction of mammographic screening programs during the last decades can be expected to increase early detection and improve prognosis of breast cancer in Russia in the near future (www.oncoforum.ru).

2.1.2 Risk factors

Several risk factors have been suggested for breast cancer with somewhat different relevances among the different cancer subtypes (Joy et al., 2005). The overall main risk factors in breast cancer are hormone-related and are believed to depend on the woman's reproductive lifestyle (Cotterchio et al., 2003). Increased risk of breast cancer is usually associated with early menarche, late menopause and few pregnacies considered to reflect physiological endogeneous hormone responses. In addition, obesity, lack of physical activity and dietary factors have been related to increased risk of breast cancer, probably due to cumulative exposure to endogeneous estrogens (Ferrini et al., 2015). Concerning exposure to exogeneous sex hormones, postmenopausal hormone replacement therapy is considered to establish the most serious risk factor for breast cancer. Long-term medication with oestrogens and/or progesterones has especially been evaluated to lead to increased risk for breast cancer, particularly for grade I tumors (Roman et al., 2016). Recently, the use of oral contraceptives has also been proposed to slightly increase the risk for breast cancer (Mørch et al., 2017). Additionally, high alcohol intake and long-term tobacco smoking have been significantly associated with increased incidence of breast cancer (Hamajima et al., 2002, Catsburg et al., 2015).

A genetic background may be identified for a large fraction of breast carcinomas, most commonly for patients under 35 years of age. According to recent evidence, approximately 30 % of breast carcinomas can be detected with a defected high-penetrance gene of which up to 10% are caused by mutations in a single gene (Bevers et al., 2015). Breast cancer genes BRCA1 and BRCA2 are known to be associated with a significantly increased lifetime cancer risk (84% and 39%, respectively) (Antoniou et al., 2003, Chen et al., 2006). The majority of BRCA1-mutated breast carcinomas also exhibit a specific histological basal-like phenotype. Futhermore, rare autosomal dominant mutations in TP53, STK11 and PTEN resulting in Li-Fraumeni, Peutz-Jeghers and Cowden's syndromes have been established with a significant risk of malignant disease, including breast carcinoma (van der Groep et al., 2011). In addition, a large number of low-risk mutations have been identified in genome-wide association studies, eg. in CDH1, LKB1, CHEK2, BRIP1, ATM and PALB2 (Shiovitz and Korde, 2015).

In clinical practice, inherited breast cancer screening is performed based on the patient's family history of breast and/or ovarian carcinomas and on specific biological characteristics of the tumor (Claus et al., 2003). However, probably due to the multi-genetic nature of the disease, the particular risk factors of many individual patients remain undetermined (Boyd, 2014). The clinical applications of many of the detected mutations vary between countries and centers and are often limited to genetic counselling and follow-up procedures. According to present understanding, most known genetic defects, including mutations in BRCA1/2, do not significantly differ from the average prognosis of breast cancer patients (Lakhani et al., 2012).

2.1.3 Histological subtypes

In histological investigation of breast cancer, the international classification criteria by the WHO are applied for invasive as well as *in situ* carcinomas. In the most recent classification (Lakhani et al., 2012), morphological criteria are used to allocate invasive breast carcinomas into fifteen subgroups and six variants. The two major histological subtypes have been nominated invasive breast carcinomas of no special type (N.O.S) and special subtypes. Invasive carcinoma N.O.S, previously known as invasive ductal carcinoma, comprises the largest subgroup (50 – 80 %) of all breast carcinomas. The name reflects the present understanding of the origin of this tumor subtype since the majority of breast carcinomas are believed to derive from the terminal lobular unit (TDLU) (Lakhani et al., 2012). Invasive breast carcinoma N.O.S exhibits a slightly worse prognosis than all breast carcinomas on average (Tot, 2016). The most common breast cancer of the special subtype, invasive lobular carcinoma, comprises approximately 10-15 % of all invasive carcinomas and is characterized, in addition to the typical morphology, by the loss of expression of the

cell-adhesion molecule E-cadherin (Younis et al., 2007). In invasive breast cancer N.O.S and in invasive lobular carcinoma the patient's disease outcome is influenced by the traditional features of breast cancer prognosis, ie. histological grade, tumor diameter, lymph node status and the biological predictors of therapeutic response, estrogen (ER) and progesterone receptor (PR) and *Her2* (human Epidermal Growth Factor Receptor 2) status, and the proliferative activity of the tumor as discussed later in this book (Lakhani et al., 2012). The other special subytypes, eg. tubular, cribriform, medullary, metaplastic, are very rare and generally exhibit a favorable outcome for the disease (Lakhani et al., 2012).

2.1.4 Biological subtypes

In clinical practice, breast carcinomas are divided into four subgroups, i.e. luminal A, luminal B, *Her2*-amplified and triple negative/basal-like (Lakhani et al., 2012). These subtypes represent the molecular and genetic origins of breast carcinomas derived from studies involving gene expression profiling and hierarchical cluster analyses (Perou et al., 2000). In clinical practice, instead of gene expression analyses, surrogate immunohistochemical markers for ER, PR, Her2 and Ki-67 can be used to determine the so-called intrinsic subtype of breast carcinomas (Table 2).

Table 2. Summary of surrogate criteria for intrinsic classification of breast carcinomas.

Subtype	**Criteria**
Luminal A-like	ER and PR positive, HER2 negative, Ki-67 low
Luminal B-like (HER2-negative)	ER positive, HER2 negative, Ki-67 high or PR low
Luminal B-like (HER2-positive)	ER positive, HER2 positive, any Ki-67, any PR
HER2-positive	HER2 positive, ER and PR negative
Triple-negative	ER and PR negative, HER2 negative

ET endocrine therapy, CT chemotherapy.
Modified from ESMO Clinical Practice Guidelines (Senkus et al., 2013).

Breast carcinomas of luminal A subtype – and to a lesser extent of luminal B subtype – respond to anti-estrogen treatment and show a favorable course of disease (Bennis el al., 2012). The most aggressive course of disease has been associated with the so-called triple-negative breast carcinomas (TNBC) which lack the expressions of both ER and PR, and amplification of *Her2* (Cheang et al., 2008). TNBC is a biologically aggressive subtype of breast carcinomas frequently diagnosed at a young age and especially common among women of Mediterranean and African origin. TNBC comprises a heterogeneous subgroup of breast carcinomas with different origins of the malignant cells. TNBCs show considerable overlapping with so-called basal-like carcinomas characterized by positivity for basal cytokeratins, such as CK5/6 and/or p63, and p53 (Carvalho et al., 2010). On the other hand, specific histological types, such as medullary or sarcomatoid metaplastic breast carcinomas are also included among TNBCs (Lester et al., 2012).

2.1.5 Prognostic factors

The prognostic factors of breast carcinomas include clinical parameters, i.e. the maximum diameter of the tumor as measured on the microscopic slides, the lymph node status presented as the number of metastatic in relation to all studied nodes (minimum of 12 lymph node studied), the histological type and grade of tumor, possible lympho-vascular invasion and extent of *in situ* tumor component (Lakhani et al., 2012). Biological prognostic features are determined with IHC and ISH and include detection of the expression levels of ER, PR and Ki-67, as well as *Her2* amplification status summarized into intrinsic classification. In determining the prognostic and therapeutic features of the tumor, managing the quality and sources of variation in the preanalytical and analytical procedures are crucial. Commercial gene expression screening technologies are promising, but further research is needed to optimally apply these expensive methods in clinical treatment decisions (Cuzick et al., 2011).

2.2 The cell cycle

The cell cycle is a highly controlled and meticulously coordinated series of events leading to DNA replication, nuclear division and cell fission (Fig. 1). The duration of the cell cycle varies greatly from one cell type to another. However, the M (mitosis) phase always occurs rapidly whereas the interphase lasts a considerably longer period. In the normal cell, the cyclical progression of the cell cycle is dependent on a highly organized feedback signaling system capable of delaying or stopping the next downstream phase of the cell cycle in case the previous phase is not perfectly completed (Alberts et al., 2015).

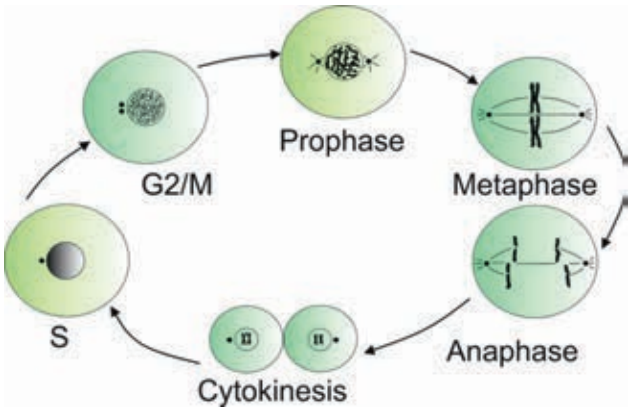

Figure 1. The cell cycle

2.2.1 Metaphase-anaphase transition

The most crucial events of cell division occur in mitosis where the genetic information is passed on to the next generation of cells. During mitosis, one of the most demanding and vulnerable steps is the precise execution of metaphase-anaphase transition (Fig. 1). During this event, the genetic material which during the early phases of mitosis was duplicated with high accuracy, now segregates into two identical daughter cells. The transition is initiated by metaphase where the chromosomes are being aligned at the equatorial plane and attached to the opposite poles of the mitotic spindle. This association is mediated by a highly specialized structure, the kinetochore, which joins each chromosome to the opposite pole by microtubules. At the beginning of anaphase, the cohesion of the sister chromatids is rapidly released and the sister chromatids are pulled apart from each other and travel to the opposite poles of the mitotic spindle. This event is a point-of-no-return as segregation of the chromatids from the bipolar spindle attachments will result in loss of sister chromatid identity. As a consequence of uncontrolled metaphase-anaphase transition, unattached sister chromatid binding may fail and lead to missegregation of genetic material. Thus, uncontrolled or untimely metaphase-anaphase transition may result in dramatic genetic defects in the daughter cells (Alberts et al., 2015).

The metaphase-anaphase transition is controlled by a ubiquitin-dependent degradation process involving a network of driver proteins. Amongst these, the best known are the cyclin-dependent kinases (CDKs), a set of regulators each acting at a specific stage of the cell cycle. Degradation of mitotic cyclin starts at around the time

of metaphase-anaphase transition where the cyclin-specific ubiquitin ligase Anaphase promoting complex (APC/C, cyclosome) forms an intrigue balance between inhibitors and activators of anaphase. The activity of APC/C is regulated by numerous protein kinases and, ultimately, the spindle assembly checkpoint (SAC). SAC prevents premature anaphase until all chromosomes have been securely attached in the mitotic spindle to the poles. The exact functions and interactions occurring at the metaphase-anaphase transition remain to be discovered. However, it is known that defects in regulation at this transition are associated with malignant transformation and gross chromosomal instability, or cell death (Thompson et al., 2010). Several proteins have been shown to be involved in the function of the spindle assembly checkpoint, among them Securin, Pttg1IP and Separase.

2.2.2 Proteins involved in metaphase-anaphase transition

2.2.2.1 Securin

Securin, the protein of the Pituitary tumor transforming gene 1 (*Pttg1*), is a 24kDa protein composed of 202 amino acids. It belongs to a Pttg protein family including also Pttg2 and Pttg3 (Salehi et al., 2008). Securin was first isolated from cells of a growth hormone-secreting pituitary tumor in rats. Initially, its overexpression was shown to induce cellular transformations *in vitro* and tumor formation in nude mice (Pei and Melmed, 1997). In humans, Securin was detected in functioning and non-functioning pituitary tumors where its expression was also correlated with clinical tumor invasiveness (Zhang et al., 1999). Initially, mRNA and protein expressions of Securin have been associated with cell cycle progression, peaking at the S-G2 transition of the cell cycle (Yu et al., 2000, Vlotides et al., 2007). Subsequently, securin has also been shown to critically participate in metaphase-anaphase transition (Fig. 2) (Waizzenegger et al., 2002).

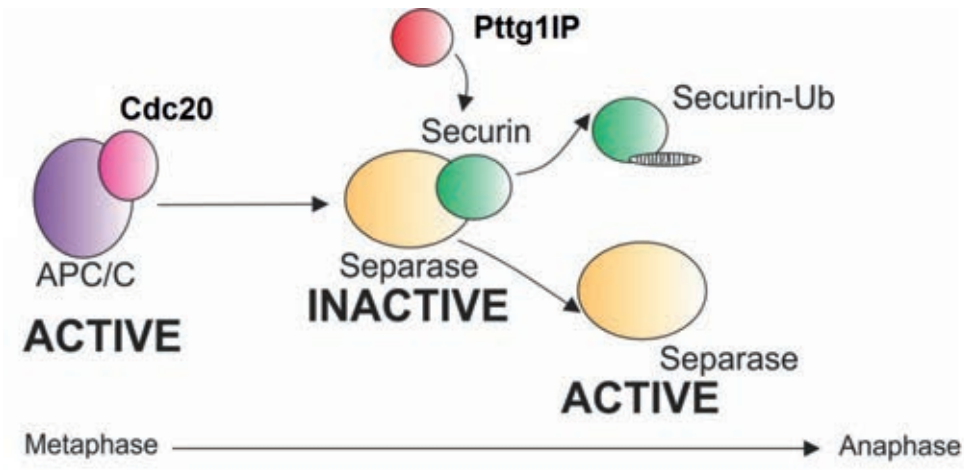

Figure 2. Schematic representation of protein interactions in metaphase-anaphase transition. To begin with, the APC/C^{Cdc20} complex activates Securin which has been nuclearized by Pttg1IP. Degradation of Securin by ubiquination favors the activation of Separase which, eventually, leads to sister chromatid separation.

The exact sequence of all mechanisms involved in Securin-related cell cycle regulation is presently not clarified. However, it is known from literature that in the metaphase-anaphase transition Securin is responsible for monitoring the quality of the genetic material allowing chromosome segregation to happen only after complete and timely duplication of DNA. After a perfect alignment of chromosomes at the equatorial plate ensured by the regulation of SAC, Securin is degraded producing an abrupt stimulus that induces the synchronous separation of chromosomes. Premature chromosome separation is hindered by tight Securin-Separase complexes inhibiting activation of Separase (Jensen et al., 2001, Hornig et al., 2002, Jallepalli and Lengauer, 2001). In this fase, Securin acts under the close regulation of APC/C which, catalyzed by the protein Cdc20, targets the ubiquination and degradation of Securin and releases Separase which, consequently, facilitates the dissociation of sister chromatids by cleaving the Cohesin complex (Fang et al., 1998, Wang et al., 2015a). In abnormal cell cycle, inhibited degradation or/and overexpression of Securin can lead to uncontrolled sister chromatid separation (Peters, 2002), and cancer (Han and Poon, 2013). In the case of an abnormal metaphase, such as an incorrect DNA duplication or dysfunctional mitosis spindle, Securin has the ability to block sister chromatid separation, inhibit anaphase and stop cell cycle progression (Peters et al., 2002). However, transfection experiments have resulted in contradictory conclusions as regard the role of Securin in cell cycle progression; this is due to the fact that, Securin overexpression has been suggested to both promote (Heaney et al., 2002, Hamid et al., 2005) and inhibit proliferation (Mu et al., 2003, Bernal et al., 2002).

In addition to the Securin-dependent mechanism, metaphase-anaphase transition can also be triggered independently from Securin, as apparent from experiments showing that *Pttg1*-knockout mice and human cultured cells survive and proliferate in the absence of Securin (Mei et al., 2001, Wang et al., 2001). On the other hand, Securin has also been suggested to have other roles in mitosis than that of the inhibition of anaphase. In G1/S transition, Securin has been proposed to act along with CyclinD3 as a transcription factor in driving the cell towards the S-phase (Tong et al., 2008). In DNA damage, Securin has been suggested to participate in blocking the cell cycle progression until DNA break repair is completed (Romero et al., 2001, Vlotides et al., 2007). In regulation of apoptosis, and interaction of Securin and p53 has been speculated with both pro- and anti-apoptotic effects in tumorigenesis and malignant progression (Bernal et al., 2002, Cho-Rok et al., 2006).

In neoplasia, abnormal expression of Securin has been associated with the induction of chromosomal instability and aneuploidia (Jallepalli and Lengauer, 2001, Karra et al., 2012). In association with chromosomal instability, both over- (Kim et al., 2007) and underexpression of Securin have been observed (Jallepalli et al., 2001, Bernal et al., 2002, Yu et al., 2003). In addition, a single mutation in the *Pttg1* gene has been reported to predispose cancer cells to chromosomal instability (Mora-Santos et al., 2013). In malignancy, Securin has been reported to be overexpressed both in endocrine and non-endocrine tumors, including neoplasias of the pituitary gland (Vlotides. 2007), colon (Zhou et al., 2014), lung (Zhang et al., 1999), thyroid (Kim et al., 2005, 2007), endometrium (Feng et al., 2012), ovary (Panguluri et al., 2008), prostate (Huang et al., 2014), head and neck (Heikkinen et al., 2016) and the central nervous system (Yan et al., 2015), including the meninges (Iliadis et al., 2018). More recently, Securin has also been discovered as a potential marker of recurrence, invasiveness, metastasis and survival in different types of malignancies (Demeure et al., 2013, Salehi et al., 2013, Genkai et al., 2006, Talvinen et al., 2013, Karra et al., 2012, Karra et al., 2014). Securin overexpression has been shown to predict the outcome of diseases in thyroid (Saez et al., 2006), pulmonary (Rehfeld et al., 2006), colorectal (Talvinen et al., 2006) and esophageal (Shibata et al., 2002) carcinomas, melanomas (Winnepenninckx et al., 2006) and gliomas (Genkai et al., 2006). Futhermore, Securin has been suggested as being potentially useful in predicting chemotherapy response in prostate carcinomas (Castilla et al., 2014). However, others studies have not been able to detect prognostic correlations or have, in contrast, associated Securin overexpression with the improved outcome for malignancy (Ishitsuka et al., 2013, Rehfeld et al., 2006).

In the breast, Securin has been suggested as being essential for morphogenesis of the mammary gland. Loss of Securin has been particularly associated with abnormal proliferation of mammary epithelial cells and spontaneous mammary gland tumors (Hatcher et al., 2014, Obgbagabriel et al., 2005, Solbach et al., 2004). In humans, overexpression of Securin has been associated with aggressive morphology, metastatic spread and relapse of breast carcinomas (Ogbagabriel et al., 2005, Solbach et al., 2004). Securin has also been suggested as a potential therapeutic target for breast cancer (Grizzi et al., 2013). In clinical materials, Securin overexpression has predicted aggressive behavior, nodal involvement and distal metastases, as well as resistance to radiation therapy (Tong et al., 2011, Liao et al., 2014, Grizzi et al., 2013).

Securin is known to be regulated in an estrogen-dependent manner, a finding originating from experiments in the pituitary gland where estrogens were shown to induce increased *Pttg1* mRNA levels in rats (Yin et al., 2001). Moreover, insulin and insulin-like growth factor (IGF-1) have been reported to increase Securin expression

in several cell lines (Thompson and Kakar, 2005). Furthermore, p53, c-Myc and fibroblastic growth factor (FGF) have been suggested as having roles in the regulation and transactivation of Securin (Hamid and Kakar, 2004, Kim et al., 2006, Tfelt-Hansen et al., 2006). According to previous literature, interactions and the activation of Securin are also mediated by heat shock proteins, transcription factor Sp1 and Pttg1-interacting protein (Salehi et al., 2008, Chen et al., 2000).

2.2.2.2 Pttg1-interacting protein

Pttg1-interacting protein (Pituitary tumor-transforming gene 1 interacting protein, Pttg1IP, also Pttg1-binding factor, PBF) is a relatively uncharacterized 22 kDa protein composed of 180 amino acids. It was first isolated and characterized from the pituitary cells of the rats (Yaspo et al., 1998). It is a ubiquitously expressed and highly conserved protein sharing no significant homology with other human proteins suggesting that it has a unique and evolutionarily important physiological role (Smith et al., 2011). Despite this, very little has been reported on its functions. In the normal cell, it has been described to bind to the sodium symporter and monocarboxylate tranporter 8 (MCT8), participating in modulation of cell growth and radioiodine uptake in thyroid cells (Boelaert et al., 2007, Read et al., 2011). Recent evidence from NGS has suggested that *Pttg1IP* has oncogenic characteristics (Melloni et al., 2014). However, as its main role, Pttg1IP functions in the cell cycle through mediating the interaction between Securin and Separase in the metaphase-anaphase transition (Fig. 2).

In vitro experiments demonstrating parallel increases in the expression levels of Pttg1IP and Securin have suggested complex combined regulatory mechanisms for the two proteins (McCabe et al., 2003). It has been demonstrated that Pttg1IP overexpression activates Securin by facilitating the shift of Securin from the cell cytoplasm to the nucleus (Chien et al., 2000). This event is believed to occur in a cell cycle-dependent manner acting as a key regulator in unleashing anaphasis onset (Straford et al., 2005). It appears that also FGF-2 (fibroblast growth factor 2) may be involved in interacting with Pttg1IP to target the subcellular localization of Securin (Ramos-Morales et al., 2000). In malignancy, Pttg1IP has been suggested to promote aberrant nuclearization of Securin leading to premature sister chromatid separation, promoting aneuploidy and subsequent genetic instability (Wang et al., 2001, Kim et al., 2005). However, the range of functions of Pttg1IP are still, for the most part, obscure. Similarly, the mechanisms regulating the expression of Pttg1IP have only partly been determined although the role of inhibitory microRNAs, such as miRNAs -583 and -122, have been suggested to produce a down-regulation of *Pttg1IP* activity and suppression of tumor proliferation (Wang et al., 2014, Li et al., 2013). Recently, mutations in *Pttg1IP* were mapped across 33 types of human

malignancies without identifying clinically relevant associations with outcome (Imruetaicharoenchoke et al., 2017).

Pttg1IP has been shown to be overexpressed in different human tissues including placenta, thyroid gland, lymph node and bone marrow (Chien and Pei, 2000), and testis (Pei and Melmed, 1997). In neoplasia, Pttg1IP has been connected with multiple mechanisms promoting malignancy. In cell cultures, Pttg1IP overexpression has been associated with cell transformation and *in vivo* with promotion of cell invasion in thyroid and colorectal carcinomas, suggesting a role in malignant progression, possibly in association with p53 dysregulation (Read et al., 2016, Hsueh et al., 2013). In papillary and anaplastic thyroid carcinomas, *Pttg1IP* has been described as an oncoprotein which both independently and in collaboration with *Pttg1* elicits cell transformation and tumorigenesis (Stratford et al., 2005). Literature demonstrating the prognostic significance of Pttg1IP in different malignancies is sparse. Overexpression of Pttg1IP has been associated with an unfavorable outcome in colorectal carcinoma (Read et al., 2016, Ren and Jin, 2017), multiple myeloma (Noll et al., 2015), a shorter disease-specific survival time and recurrent disease in thyroid carcinoma (Read et al., 2014, Hsueh et al., 2013).

In the normal breast, Pttg1IP expression is weak and present in only single ductal epithelial cells (Watkins et al., 2010). In breast carcinomas, Pttg1IP overexpression has been observed in association with ER-positivity in both cell lines and in tissue samples - a logical finding considering that the promoter of *Pttg1P* includes an element sensitive for the estrogen receptor alpha (Watkins et al., 2010). This association has also been demonstrated in cell cultures where increased expression of Pttg1IP has been observed in breast cancer cells exposed to oestrogen (Watkins et al., 2010). Pttg1IP has been proposed with a possibly critical role in breast cancer cell invasion (Watkins et al., 2010) but the prognostic role of Pttg1P in breast cancer has not previously been investigated.

2.2.2.3 Separase

Separase (Extra Spindle Poles Like 1, ESPL1) is a highly conserved site-specific multidomain endopeptidase which, in humans, is present as a 190kD protein (Uhlmann et al., 2000). Separase belongs to the family of cysteine proteases responsible for cleaving the keisin subunit of the Cohesin complex during the cell cycle (Uhlman et al., 2000). In humans, Separase is encoded by a single gene but the protein is expressed in several isoforms, the first of which was published in 1988 (Baum et al., 1988). In the cell, expression of Separase has been reported in several subcellular locations, including nuclear, cytoplasmic and mitochondrial, in addition to expression in the mitotic spindle (Jensen et al., 2001, Tkach et al., 2012).

In the normal cell, Separase remains inactive - or possibly minimally active - during the cell cycle only to be irreversibly activated at metaphase-anaphase transition and at mitotic exit of the cell (Fig. 2). The activity of Separase is constantly governed either due to binding to its inhibitory chaperone, Securin, or phosphorylation by CyclinB1-dependent Cdk1 (Cyclin-dependnet kinase 1) (Kumar, 2017, Jallepalli and Lengauer, 2001, Hornig et al., 2002). At the metaphase-anaphase transition, Separase is activated through a rapid ubiquination and destruction of Securin mediated by the active form of the ubiquitin protein ligase APC/C, APC/C^{Cdc20} (Zachariae and Nasmyth, 1999). An alternative route of activation for Separase occurs through phosphorylation when inhibition by CyclinB1-dependent Cdk1 releases Separase activity (Leismann et al., 2000). Both Securin and CyclinB are targets of the active form of the ubiquitin protein ligase APC/C, APC/C^{Cdc20}, ensuring that the peak of Separase activity is carefully controlled at anaphase onset (Hatano et al., 2016). In addition to the mechanism involving Securin and CyclinB1-Cdk1, Separase may also employ self-proteolytic autocleavage activity although the biological implications of this mechanism are not completely understood (Chestukhin et al., 2003, Kumar 2017). In addition to its role in anaphase initiation, regulation by Separase has been implicated in the spindle assembly (Baskerville et al., 2008) and duplication and separation of the centrioles in the mitotic spindle (Tsou and Sterns, 2006). It has also been suggested that Separase may be involved in DNA repair (McAleenan et al., 2013). In conclusion, the preconditions for maintaining the integrity of genetic material during cell division is that Separase remains at optimal level throughout the cell cycle and is rapidly activated only at anaphase onset triggering cleavage of the Cohesin complex and, ultimately, sister chromatid separation (Sun et al., 2009).

Uncontrolled activation of Separase has been shown to cause chromosome missegregation due to premature cleavage of the Cohesin complex (Uhlman, 2001). Thus, Separase has been implicated with oncogenic properties predisposing the cell to a dysfunctional cell cycle and aneuploidy, the major characteristics of malignancy (Shepard et al., 2007). In cancer, both reduced and increased expression of Separase have been reported (Kumar, 2017). Increased expression of Separase in mouse mammary epithelium has been shown to lead to tumorigenesis (Pati et al., 2004, Zhang et al., 2008, Mukherjee et al., 2014). In humans, mutations in Separase gene leading to loss or decrease in Separase protein expression have been reported in lung and kidney carcinomas (Sak et al., 2008, Xu et al., 2011, Mukherjee et al., 2011). Overexpression of Separase has been reported in a number of small patient materials comprising different types of human malignancies, including breast and prostate carcinomas, and osteosarcomas (Zhang et al., 2014a, Meyer et al., 2009). Association between Separase-overexpression and aneuploidy has also been demonstrated in human breast cancer (Zhang et al., 2008, Meyer et al., 2009). Particularly, increased Separase expression has previously been reported in the

luminal B subtype of breast carcinomas (Finetti et al., 2014). In addition, the Oncomine database has revealed strong association between Separase mRNA levels and the histological tumor grade and survival in breast carcinomas (Mukherjee et al., 2014). Thus far, knowledge of the expression patterns, clinical or prognostic associations of Separase in breast cancer are far from complete. However, based on the present understanding Separase may, in the future, prove a potential molecular target for cancer therapy (Kumar, 2017).

2.2.3 MiRNAs involved in metaphase-anaphase transition

MicroRNAs (miRNAs) are small, approximately 20 nucleotides long non-coding RNAs (ncRNAs). They act post-transcriptionally regulating gene expression by binding to the target messenger RNAs (mRNAs) and, consequently, inducing translational inhibition or transcript degradation (Filipowicz et al., 2008). After the first miRNA were discovered in nematode in 1993 (Lee et al., 1993, Wightman et al., 1993), new miRNAs have been constantly detected in vertebrate, including humans, in the majority of tissues at variable expression levels (Pasquinelli et al., 2000, Lagos-Quintana et al., 2001). MiRNAs comprise one of the most abundant classes of regulatory genes in humans and, each miRNA is capable of targeting multiple genes within a pathway, they are evaluated to regulate at least 30% of human protein coding genes (Lim et al., 2005). Currently, thousands of miRNAs have been identified in humans and the vast majority of them are deregulated in cancer cells (Di Leva et al., 2013). It has been presented that in carcinomas over half of miRNA genes are located at fragile genomic sites, emphasizing their important and complex roles in malignancy (Calin et al., 2004).

In the literature, miRNAs have been associated with several functions, the most important described as inhibitors of protein-coding of genes. This is achieved either by inhibition of translation, destabilization or activation of mRNA (Guo et al., 2010). Interestingly, in the regulation of miRNAs, a bidirectional connection has been observed so that, on the one hand, epigenetic mechanisms control miRNAs and, on the other hand, miRNAs can target essential epigenetic functions (Chuang and Jones, 2007). Regulation by miRNAs has been associated with numerous biological processes, especially apoptosis, cell differentiation and tumor metastasis (Filipowicz et al., 2008). Particularly, miRNAs have been reported to regulate the cell cycle and control cell proliferation through the mediating activity of genes involved in the cell cycle progression (Croce, 2009).

The role of miRNAs in cancer was first addressed in chronic lymphocytic leukemia where a number of miRNAs were located at fragile regions of the cancer genome, including sites of amplification or loss of heterozygosity (Calin et al., 2004). Since

then, NGS techniques have enabled the screening of the entire known miRNAome in carcinomas and, to date, altered miRNA expressions have been reported for almost all types of malignancies (Li et al., 2009). According to the present knowledge, miRNAs can act as oncogenes (oncomirs) or tumor suppressors and are involved in deregulation of a number of different cellular functions in cancer (Garzon et al., 2009). For example, one of the most investigated processes with miRNA involvement is metastasis where miRNAs are known to have dual roles both as promotors or inhibitors (Nicoloso et al., 2009).

In the clinical context, miRNAs are expected to become extremely valuable in diagnostics, prognostics and treatment of cancer. In addition to detection in tissue specimen, deregulated miRNAs have been detected in the serum of cancer patients diagnosed with several types of malignancies, including leukemia and lymphoma, and carcinomas of the breast, prostate and colon (Cortez and Calin, 2009). Due to their important role in malignanct, miRNAs comprise an attractive target for therapeutic interventions in several types of malignancy.

2.2.3.1 MiRNA-494

Literature on the functional role of miRNA-494 is limited and complex since it has been presented primarily as a tumor suppressive (Shen et al., 2018, Chi et al., 2015, Zhan et al., 2017) but occasionally also as oncogenic miRNA (Nie et al., 2016, Sun et al., 2014). These discrepancies have been explained by variation in cell and tumor types and tumor microenvironments, targeted by miRNA-494 (Yang et al., 2017). However, the role of miRNA-494 in cell cycle regulation is well established, particularly in inducing an arrest at G_2/M and metaphase–anaphase transition triggering sister chromatid separation (Kapanidou et al., 2017). In this, miRNA-494 has been described as modulating the protein levels of CyclinB1, Securin and Cdc20 and downregulate *Pttg1IP* (Yamanaka et al., 2012). Dependent on the type of malignancy, miRNA-494 has been described as both promoting (Liu et al., 2015) and inhibiting proliferation (Zhao et al., 2016, Kim et al., 2011). In cervical carcinoma cell lines, inhibition of miRNA-494 was demonstrated to suppress cell proliferation by inducing cell cycle arrest (Yang et al., 2015a, Yang et al., 2017, Hou et al., 2014). In addition to functions in cell proliferation, miRNA-494 has been described as participating in apoptosis, migration and angiogenesis (Shen et al., 2014, Mao et al., 2015b). Based on data from several malignancies, miRNA-494 has been reported to participate in malignant pregression, invasion and metastasis by directly targeting PTEN, SIRT-1, c-Myc and KIT (Lu et al., 2012, Yang et al., 2017, Yuan et al., 2016, Yun et al., 2018). In breast cancer, ectopic expression of miRNA-494 has been reported to significantly inhibit cellular proliferation, cancer progression and invasion through the Wnt/β-catenin and PAK1 signaling pathways (Zhan et al., 2017).

Aberrant expression of MiRNA-494 has been reported in various types of cancer. In cell lines, specific miRNA expression profiles have been identified in cervical cancer cell lines (Zhu et al., 2012, Dong et al., 2015). In human carcinomas, upregulation of miRNA-494 has been demonstrated in hepatocellular (Liu et al., 2015), colorectal (Sun et al., 2014) and non-small-cell lung carcinomas (Wang et al., 2015b) and downregulation in gastric (He et al., 2014), prostatic (Shen et al., 2014), esophageal (Zhang et al., 2015a), pancreatic (Ma et al., 2015) and breast carcinomas (Song et al., 2015). MiRNA-494 has been demonstrated to promote proliferation in hepatocellular carcinoma (Liu et al., 2015a) and cervical cancer (Yang et al., 2015b).

In addition, reduced levels of miRNA-494 have been demonstrated in association with clinical prognostic factors, such as histological grade, tumor size, lymph node metastasis, clinical stage and recurrence (Yang et al., 2017, Hou et al., 2014). In prognostic evaluations unfavorable outcome has been associated with decreased levels of miRNA-494 in epithelial ovarian, pancreatic and gastric carcinomas (Yang et al., 2017, Ma et al., 2015, He et al., 2014) and with increased levels in colorectal, breast and ovarian cancers (Sun et al., 2014, Marino et al., 2014, Yang et al., 2015). Multivariate survival analyses have demonstrated the independent prognostic value of miRNA-494 for progression-free and overall survival rates in colorectal carcinomas (Sun et al., 2014). Additionally, it has been suggested that miRNA-494 induces drug resistance in malignancy (Tian et al., 2016). It has been suggested that upregulating miRNA-494 or developing analogous pharmaceutical compounds could be utilized in anti-proliferative cancer therapies.

2.2.3.2 MiRNA-205

MiRNA-205, first identified from zebra fish and humans (Lim et al., 2003, Landgraf et al., 2007), is a highly conserved microRNA described as participating both in normal and pathological physiological processes (Vosgha et al., 2014, Adhami et al., 2018). In normal physiology, miRNA-205 has been associated with wound healing (Yu et al., 2010), adipogenesis (Yu et al., 2014) and placental and fetal development (Mouillet et al., 2010). In mouse fetus, reduction of miRNA-205 expression has been shown to lead to neonatal fatality (Wang et al., 2013). In human cancer, the role of miRNA-205 is complex and controversial manifesting as various tissue type-dependent alterations in expression patterns between benign and malignant epithelial cells (Zhang et al., 2015b). The majority of studies suggest that miRNA-205 is a tumor suppressor (Hulf et al., 2013, Hou et al., 2013, Hagman 2013, Hanna et al., 2012, Childs et al., 2009) while some studies present miRNA-205 as an oncogene (Kalogirou et al., 2013, Karaayvaz et al., 2013). In both roles, miRNA-205 has been reported to effectively modulate cancer progression through a variety of cellular processes, particularly cell proliferation, (Wu et al., 2009) apoptosis (Wang et al., 2013) and epithelial-mesenchymal transition (Xu et al., 2016).

MiRNA-205 has been found upregulated in several malignancies, including carcinomas of the ovary (Su et al., 2013), endometrium (Ma et al., 2016) and bladder (Kriebel et al., 2015). Instead, down-regulation has been detected in breast (Elgamal et al., 2013), prostate (Srivastava et al., 2013), thyroid (Salajegheh et al., 2015), bladder (Ganji et al., 2017) and esophageal carcinomas (Gu et al., 2013). In prognostic analyses, miRNA-205 has been discovered to be a promising biomarker for patients with lung (Li et al., 2017), gastric (Tao et al., 2017) and endometrial carcinomas (Ma et al., 2016). As a regulator of several crucial cellular pathways, miRNA-205 comprises a promising candidate for therapeutic applications in tumor suppression (Adhami et al., 2018).

In breast cancer, down-regulation of miRNA-205 expression has been shown in cell lines (Wu et al., 2009, Elgamal et al., 2013) as well as in human tumor tissues (Piovan et al., 2012, Elgamal et al., 2013, Wang et al., 2013, Sempere et al., 2007), and in the serum of breast cancer patients (Shaker et al., 2015). In prognostic analyses, miRNA-205 has been associated with disease progression (Markou et al., 2014) and metastasis (Berber et al., 2014) as well as with disease-free interval and overall survival (Quesne et al., 2012, Huo et al., 2016). The observed prognostic implications of miRNA-205 in breast cancer have been explained by inhibited cell proliferation and cell invasion, as well as by induced cellular senescence (Wu et al., 2009, Elgamal et al., 2013, Piovan et al., 2012). Aberrant miRNA-205 expression has been witnessed in all subtypes of the intrinsic classification of breast carcinomas (Teoh and Das, 2017). However, the most significant downregulation has been detected in TNBC (Savad et al., 2012) and in inflammatory breast carcinoma (Huo et al., 2016). Based on observations from cell lines, miRNA-205 has been suggested with a central role in inhibiting proliferation in TNBC (Piovan et al., 2012, Liu et al., 2013).

2.2.3.3 MiRNA-21

MiRNA-21 was among the first to be identified and presently one of the most extensively studied miRNAs with crucial roles in several biological processes. Despite the accumulating data on the role of miRNA-21 in diseases, the precise mechanisms driving transcriptional regulation of miRNA-21 are not completely understood. However, the most common understanding is that miRNA-21-expression is maintained by both transcriptional and post-transcriptional regulation (Davis et al., 2008). In addition, a non-transcriptional mechanism implicating gene amplification of miRNA-21 has been proposed (Haverty et al., 2008).

In carcinoma cell lines, miRNA-21 has been shown to vitally participate in oncogenesis (Li et al., 2016). The so called oncogenic miRNA addiction has been demonstrated as miRNA-21-dependent tumor development in pre-B-cell lymphoma in mice (Medina et al., 2010). In humans, miRNA-21 is one of the most commonly observed oncomirs and associated with almost all hallmarks of carcinogenesis, including promoting the cell cycle, increasing cell proliferation and avoiding apoptosis (Feng and Tsao, 2016, Folini et al., 2010). Aberrant miRNA-21 expression has been associated with invasive and metastatic potential in human malignancies (Zhang et al., 2016) and linked with the up-regulation or inhibition of specific cancer-related target genes, such as PTEN and TP53 (Fang et al., 2017, Papagiannakopoulos et al., 2008). In addition to malignancy, miRNA-21 upregulation has been reported in other pathological conditions, particularly in cardiovascular diseases (Huang et al., 2013).

MiRNA-21 is one of the most abundant miRNAs in solid tumors and has been reported upregulated in many different malignancies, including carcinomas of the cervix (Deftereos et al., 2011), lung (Zhang et al., 2015c), kidney (Lu et al., 2013), colon (Tao et al., 2015), liver (Mao et al., 2015a), esophagus (Hiyoshi et al., 2009) and pancreas (Dillhoff et al., 2008). Particularly in esophageal squamous cell carcinoma, miRNA-21 has been described to act as promotor of proliferation and migration (Wu et al., 2016) and in glioblastoma it has been reported to have an antiapoptotic function (Chan et al., 2005).

The oncogenic role of miRNA-21 has also been demonstrated in breast carcinomas. Several authors have reported on a higher prevalence of miRNA-21 in cancerous versus normal breast tissue (Qi et al., 2009, Ozgun et al., 2013, Lee et al., 2011, Si et al., 2013). MiRNA-21 up-regulation has been reported both in breast tumor epithelial cells (Hug et al., 2015, MacKenzie et al., 2014, Sempere et al., 2007, Mar-Aguilar et al., 2013) and in tumor-associated fibroblasts (Sempere et al., 2007). Over-expression of miRNA-21 has been demonstrated in all intrinsic subgroups of breast cancer (Savad et al., 2012). In prognostic studies of breast carcinomas, expression of miRNA-21 has been associated with poor clinical outcome (MacKenzie et al., 2014). In addition, miRNA-21 overexpression has been associated with decreased response to oncologic therapies, particularly resistance to chemotoxic agents (Chen et al., 2014), trastuzumab (Gong et al., 2011) and neoadjuvant therapy (Yadav et al., 2016). According to recent literature, circulating miRNA-21 could be applied as a potential new non-invasive biomarker for diagnosis and prognosis of breast cancer as well as an indicator of invasiveness of the tumor (Han et al., 2016, Heneghan et al., 2010). High concentrations of miRNA-21 in serum of breast cancer patients have been correlated with the stage of the disease, also with occurrence of visceral metastasis (Asaga et al., 2011). Unfortunately, the reported sensitivity and specificity levels vary considerably between reports (Swellam et al., 2018, Gao et al., 2013),

hampering the clinical applications of measuring circulating miRNA-21 in cancer patients. Nevertheless, miRNA-21 is considered a promising novel biomarker for diagnostic and prognostic use and a potential future target for cancer treatments (Fang et al., 2017).

2.2.3.4 MiRNA-126

MiRNA-126 has been detected with important cellular roles enhancing angiogenesis, proliferation and migration (Meister and Schmidt, 2009). The primary role of miRNA-126 is in angiogenesis where it has diverse properties in fetal and adult organisms. While supporting proangiogenic signaling in embryonic vessels (Fish et al., 2008), in adult tissues it participates in maintaining homeostasis of mature endothelial cells and inhibiting angiogenesis. However, in hypoxia or endothelial damage, the expression of miRNA-126 is recovered in specific well-vascularized tissues, such as heart, lung and liver, inducing activation of endothelial progenitor cells and angiogenesis (van Solingen et al., 2015). In several malignancies, miRNA-126 has been demonstrated to promote proliferation, invasion and cell survival, partly through interaction with *EGFL7* (Chistiakov et al., 201, Zhou et al., 2013, Sun et al., 2010, Salajegheh et al., 2013). In addition, in pancreatic cancer cells miRNA-126 has been shown to be involved in epithelial-mesenchymal transition inducing malignant progression and cancer cell proliferation (Hamada et al., 2012).

MiRNA-126 is one of the most commonly observed cancer-related microRNAs and dysregulated in most malignancies. As one of its the major functions, miRNA-126 has been shown to inhibit proliferation in several carcinomas, including breast (Ebrahimi et al., 2014, Tavazoie et al., 2008), colon (Yuan et al., 2016b), gastric (Wang et al., 2015c) and lung carcinomas (Yang et al., 2015a). However, several reports using a variety of carcinoma types have described divergent roles for miRNA-126 suggesting both oncogenic and tumor suppressor effects (Otsubo et al., 2011, Barshack et al., 2010). MiRNA-126 has been described as a tumor suppressor in breast (Tavazoie et al., 2008), endometrial (Zhao et al., 2016b), colon (Li et al., 2013), pancreatic (Jiao et al., 2012) and gastric carcinomas (Feng et al., 2010). In clinical materials, miRNA-126 has been associated with tumor size and metastatic rate (Tavazoie et al., 2008, Huang et al., 2016). In prognostic analyses, patients with reduced levels of miRNA-126 have been identified with a worse prognostic outcome in colon (Yuan et al., 2016, Hansen et al., 2014, Liu et al., 2014, Li et al., 2015), cervical (Yang et al., 2014), prostate (Watahiki et al., 2011), gastric (Li et al., 2010) and esophageal carcinomas (Hu et al., 2011). It has also been speculated that miRNA-126 expression could influence sensitivity to chemotherapy in colon cancer (Huang et al., 2016).

In breast carcinoma cell lines, miR-126 over-expression has been demonstrated to inhibit cell proliferation by repressing cell cycle progression from G0/G1 to S phase (Zhang et al., 2008). In breast carcinoma patients, miRNA-126 expression was associated with increased metastasis, recurrent disease and survival (Tavazoie et al., 2008, Hafez et al., 2012). Analyzing breast cancer subtypes, miRNA-126 expression was suggested to have prognostic associations in luminal subtypes but not in TNBC (Tavazoie et al., 2008). Altered expression of miRNA-126 has also been detected from blood samples of breast carcinoma patients to be associated with histological grade and hormone receptor status (Wang et al., 2010). Presently, miRNA-126 comprises a promising therapeutic target in cardiovascular diseases, e.g. in treatment of post-infarction heart failure (Chistiakov et al., 2016). It could be foreseen, that cancer cell growth could, in the future, be inhibited by restoring the expression of the epigenetically silenced tumor suppressor gene miRNA-126 (Ebrahimi et al., 2014).

2.3 Histological techniques

The aim of histological techniques is to preserve the structural integrity of the specimen and maintain their cellular and sub-cellular structures so that they can be evaluated microscopically. Apart from frozen sections applied for rapid microscopic analysis particularly in oncological surgery, fixation is used to preserve biological specimen from decay caused by autolysis. The purposes of fixation are to disable the intrinsic biomolecules and proteolytic enzymes in the tissue from digesting the specimen, to protect the sample from extrinsic damage due to microorganisms and to provide mechanical stability to help preserve the morphology of the tissue during processing. After fixation and various other treatments including dehydration in alcohols and infiltration with paraffin, sectioning of the tissue block allows microscopic analysis of the specimen based on transmission of light through the tissue section. In recognizing various cell types and cellular components of the specimen, a variety of stainings reacting either chemically or physically are available to accentuate identification of tissues, cells or organelles. In immunohistochemistry (IHC), specific antibodies are used to visualize selected proteins, carbohydrates or lipids. For a similar purpose, fluorescent stains are applied in immunofluorescence (IF) stainings. Other advanced techniques, such as *in situ* hybridization (ISH) are used to identify specific DNA or RNA molecules with variously labelled probes. In case of fluorescently labelled probes, signals can be detected by fluorescent or confocal microscopes whereas chromogenic signals or silver deposits can be detected using a common light microscope. Scanning of stained sections is

increasingly used to capture, analyze and store the histological image. (Gartner, 2017).

In research, the technical details in the abovementioned processes are known to cause variations in tissue morphology, in the interpretations of IHC, IF and ISH, as well as in the genomic studies (Hewitt et al., 2012, Chung et al., 2008, Sapino et al., 2014). Recently, the active developments in research biobanks have emphasized the need for standardizing the conditions of tissue processing within and between institutions (Olson et al., 2014, Zhou et al., 2015). In the following, selected steps in processing tissue for histological evaluation are addressed and their potential influences on prognostic evaluations of breast carcinomas are evaluated.

2.3.1 Aspects of tissue processing and preservation methods

Preanalytical variations in tissue handling have been shown to essentially influence the detection of biomarkers and their cellular and proteomic profiles (Shabinkhani et al., 2014). By definition, preanalytical factors include the type and size of the biospecimen, the duration of cold ischemia, the choice of preservative and the type and duration of fixation, the details of tissue processing concerning dehydration, clearing and paraffin embedding, and the treatment of tissue blocks during sectioning and storage. According to the literature, the effects of these sources of variation may also vary considerably within and across different medical institutions and individual laboratories and as regard the different types of specimen and histological techniques applied (Engel and Moore, 2011).

The variety of preanalytical factors known to affect the accurate assessment of biomarkers within a specimen illustrates the complexity of challenges faced by basic and translational research (Agrawal et al., 2017). Moreover, in clinical pathology practice, the effect of preanalytical variation has been acknowledged worldwide e.g. in breast carcinoma specimen it has been estimated that 20% of immunohistochemical ER and PR determinations may be inaccurate (Hammond et al., 2010). Delayed fixation particularly presents a challenge in the determination of diagnostic biomarkers. For instance, in breast carcinoma specimen it has been shown that a 24-hour cold ischemia time already causes a considerable increase the risk of false-negative interpretation of ER-IHC (Nkoy et al., 2010). Conversely, increased levels of IHC-expression have been reported after a delayed fixation at least for the apoptosis-related protein Caspase-3 (lznerowicz et al., 2011) and transcription factor STAT- 1 (Gündisch et al., 2012). Particularly in detection of phosphorylation sites and phosphoproteins, significantly altered interpretations have already been observed after very short cold ischemia times of 5 and 10-20 minutes (Mertins et al., 2014, Ugner et al., 2016).

2.3.1.1 Fixation delay

Fixation delay refers to the time from removal of the tissue specimen from the patient to the initiation of fixation which will cause the tissue to suffer from interruption of oxygen supply. Fixation delay can be divided into warm and cold ischemia (Hammond et al., 2010). Warm ischemia refers to the time when the blood supply to the tumor is interrupted by the surgeon during the the excision of the tissue specimen. Cold ischemia is the time from excision to the initiation to tissue fixation. During these periods the cells progressively loose blood flow and encounter ischemia, acidosis, loss of molecular activities, eventually, ending in enzymatic degradation of cellular structures (Hammond et al., 2010).

In the literature, controversial interpretations have been presented concerning the influence of fixation delay on tissues. According to some researchers, a delay of less than 12 hours does not significantly alter the morphology or the extent or intensity of various different immunostainings (Khoury et al., 2012, Portier et al., 2013, Arima et al., 2016, Pinhel et al., 2010, Apple et al., 2011). In contrast, Lee and co-workers (2014) observed significant reduction of Her2-immunoexpression already after a 1-hour fixation delay. It has been suggested that the effects of delayed fixation may vary between antibodies, eg. Arima and co-workers reported (2016) that delayed fixation caused reduction in Ki-67 but not ER immunopositivity in breast carcinomas. Moreover, it has been speculated that fixation delay may affect in different ways the immunohistochemical antigeneity in different subcellular localizations. It has been particularly suggested that membranous markers, such as Her2 and E-cadherin might be more vulnerable to alterations in IHC-expression after fixation delay (Khoury et al., 2012). According to the literature, detection DNA by ISH may be more susceptible to cold ischemia than IHC. Khoury and co-workers (2012) reported significant artifacts in samples after a period longer than 1-hour cold ischemia - nevertheless, they did not report a statistically significant effect on the registered *Her2*/CEP17 ratio. According to others, up to 3-hour cold ischemia did not cause adverse effects on *Her2*-ISH in different human carcinomas (Portier et al., 2013, Zeng et al., 2014). For genomic studies, delayed fixation has been suggested to be the major quality problem causing increased proteolysis and nucleic acid degeneration (Hewitt et al., 2008).

2.3.1.2 Fixatives

While preserving the structural integrity of tissues, fixatives modify the biological functionality of the proteins, and modify and degrade cellular DNA and RNA (Kanai et al., 2018). Optimal fixation conditions also depend on the type of tissue, method of detection and antigen of interest or type of genomic analysis (von Wasielewski et al.,

2002, Atkins et al., 2004). Although established in use, the detailed effects of the fixation process and paraffin impregnation are still quite poorly understood.

In histology practice, the two main types of fixatives commonly used are formalin-based cross-linking fixatives and alcohol-based coagulating fixatives (Bussolati and Leonardo, 2008). Formalin (neutral-buffered 10% formaldehyde, NBF) is presently, the most common fixative for light microscopy for research purposes as well as in the practice of clinical pathology. Formalin-fixation is a multistep chemical reaction which is dependent on the time and temperature used during the process; as the result, proteins become cross-linked and create chemical inter- and intramolecular bridges (Helander et al., 1994). NBF has the advantages of rapid penetration into the tissue causing little distortion or shrinkage of tissue and hardly any destruction of the cellular constituents (Titford and Horenstein, 2005). Moreover, formalin is well-known, easy to use and low-cost (Nykänen and Kuopio, 2010). Conversely, the methylene bridges formed by formalin fixation mask epitopes necessary for antigen detection. As compared to other formaldehyde solutions, NBF phosphate salts ensure better tissue preservation and the neutral pH inhibits accumulation of formalin pigment in the tissue (Drury and Wallington, 1980, Kiernan, 1999).

Historically, a variety of different fixatives have been tested and used, with particularly solutions being named after eg. Zenker, Helly, Bouin, Hollande, Gendre, Clarke and Carnoy. According to the literature, some of these solutions have been suggested to have better applications to specific research settings, eg. Bouin's solution in the detection of breast biomarkers or Carnoy's solution in examining lymph node status. (Gagnon et al., 2011, Dias et al., 2016).

Recently, numerous alternative fixatives have been introduced as substitutes to the use of formalin (Perry et al., 2016, Bussolati et al., 2017). These have been developed with the purpose of avoiding the known toxicity and carcinogenecity of formalin, as well as to tackle technical problems related to fragmentation and cross-linking of proteins and chemical modification of nucleic acids (Dotti et al., 2010, Masuda et al., 1999). Various commercial alternative fixatives are increasingly available and recommended particularly for automated tissue processors (Titford and Horenstein, 2005, Yaziji and Barry 2006, Nykänen and Kuopio 2010), for biobanking approaches (Braun et al., 2011) and for parallel molecular and morphological analysis (Kap et al., 2011).

2.3.1.3 Duration of fixation

In the literature, there are controversial reports on how the duration of fixation, both under- and overfixation, influence detection of biomolecules (Goldstein et al., 2007,

Yaziji et al., 2008, Kao et al., 2018). According to most papers, inconsistencies in cell morphology and expressions of biomarkers can be demonstrated after overfixation while underfixation is more poorly understood (Chung et al., 2018, Dapson et al., 2007, Kalkman et al., 2015, Kalkman et al., 2014a and 2014b). In contrast, others reports state that overfixation is likely to be less problematic than insufficient fixation (Arima et al., 2016). Concerning IHC, it is believed that overfixation may potentially lead to excessive protein co-linkages and, consequently, false-negative IHC (Hammond et al., 2010). Prolonged formalin fixation is known to proportionally add masking of epitopes (Kao et al., 2018, Hewitt et al., 2008). It has been reported that particularly the quality of RNA is susceptible to increased duration of fixation leading to loss of detection of nucleic acids (Chung et al., 2008).

In the literature, the recommended duration of fixation varies significantly, from 6 hours (Yamashita-Kashima et al., 2014) to up to one week (Sato et al., 2014). Recommendations for duration of formalin fixation have been published for several different types of tissues and malignancies (Kanai et al., 2018). The duration of fixation has been seen as particularly critical for large heterogeneous tumors where penetration of formalin may be uncertain (Moatamed et al., 2011). Additionally, variations in tolerance of duration of fixation have been reported between antibodies so that e.g. in breast carcinomas prolonged fixation has been shown to lead to gradual reduction in Ki-67 but not in ER or HER2 immunopositivities (Arima et al., 2016).

2.3.1.4 Preservation and storage of tissue specimen

In order to achieve adequate quality and reproducibility of IHC, it is imperative that the preservation of archival tissue material is carefully considered. It has been demonstrated that aging of FFPE material has variable and time-dependent effects on the immunoreactivity of the specimen (Economou et al., 2014). According to some recommendations, in order to achieve high and reproducible quality of IHC, FFPE blocks should be used within one year from processing (Ramos-Vara et al., 2013). However, others have shown that stable antigenicity is retained in tissue blocks considerably longer, even for up to 68 years of storage (Dowsett et al., 2014, Vis et al., 2000, Camp et al., 2000). Nevertheless, the quality of IHC and IF may be dependent on the biomarker applied as some antibodies may lose their antigeniety considerably earlier (Combs et al., 2016, Ramos-Vara et al.. 2013, Malmstrom et al., 1992). Some researchers have also reported loss of integrity of mRNA in archival material (Beaulieu et al., 2010, Ribeiro-Silva et al., 2014, Cronin et al., 2004) while others have observed no adverse effect on the quality of mRNA in FFPE blocks during 21 years of storage (Chu et al., 2006, Godfrey et al., 2000).

It is also well known that slide-mounting FFPE sections decreases the quality of ICH in an antigen-dependent manner (Kanai et al., 2018, van den Broek and van de Vijver, 2000, DeVito et al., 2004, Jacobs et al., 1996, Wester et al., 2000). Therefore, it has been recommended that slide-mounted FFPE sections should be stored in the dark at 4C° and optimally stained within a week from sectioning (Bass et al., 2014, Ramos-Vara et al., 2013).

2.3.2 Aspects of histological detection methods

2.3.2.1 Immunohistochemical and immunofluorescent stainings

Immunohistochemistry (IHC) and immunofluorescence (IF) of FFPE are ancillary histological techniques used to complete histochemical stains in identifying specific cellular biomarker molecules. Both techniques can be used to demonstrate and semi-quantitatively assess the presence and location of antigens in tissue sections. IHC is most widely used in research, and also in clinical practice to provide evidence in support of the morphological interpretation of the origin and nature of the cells and tissues, and to evaluate the progression and the predicted course of disease, prognosis and treatment responses of neoplastic disease. Immunofluorescence (IF) technique is more commonly applied for research purposes but also has applications in clinical pathology providing additional evidence for the classification and evaluation of the course of disease e.g. in autoimmune, renal glomerular and bullous skin diseases. (Makki et al., 2016).

Technically, both IHC and IF are based on specific binding reactions of tissue antigens with the antibody of interest and visualization of this binding on the tissue section (Fig. 3). In more detail, the basic procedure of both methods usually includes a combination of the following steps. After preparing the tissue sections additional antigen retrieval by heat or protease treatment may be required in order to make the epitopes available for antibody binding. Often blocking of endogeneous biotin or enzymes is also necessary to reduce unspecific staining. Thereafter, sample labeling occurs by the incubation of the antibodies as primary or secondary reagents in specific concentrations. This can be performed in direct binding where the primary antibody specifically recognizes the target protein. More commonly, however, an indirect method is applied where the primary antibody is recognized with a secondary antibody raised against the immunoglobulins of the primary antibody (Taylor et al., 2013, Makki et al., 2016).

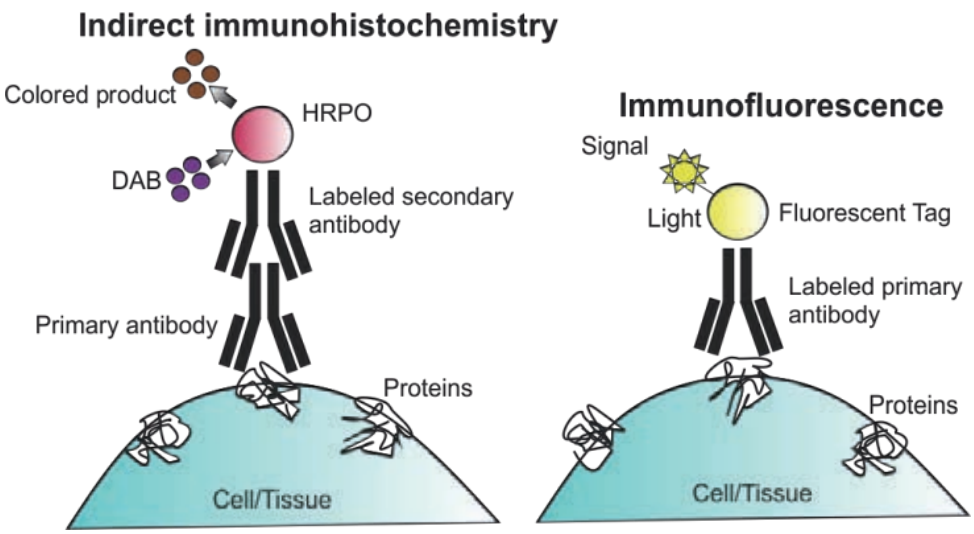

Figure 3. Examples of indirect and direct antibody binding in immunohistochemistry and immunofluorescence methods
DAB 3'-Diaminobenzidine, HRPO horseradish peroxidase

The principal difference between IHC and IF is the selection of a reporter molecule for detection. In the case of chromogenic detection in IHC, an enzyme conjugated to the secondary antibody cleaves a substrate containing chromogen and produces at the location of the antigen a colored precipitate which can be visualized under light microscopy. IHC is completed by counterstaining in order to contrast the antibody reaction. In IF, detection is performed applying fluorescence, i.e. fluorochrome dyes that absorb ultraviolet rays and emit light visible in fluorescence microscopes. (Odell and Cook, 2013).

Multi-staining techniques are used for simultaneous visualization of two or more antigens in the same section. The advantage of this method is the ability to observe the co-expressions and localizations of specific proteins within the same cell or tissue section under visual control of the morphology of the specimen. In clinical practice, multi-stainings are routinely utilized e.g. in diagnosis of prostatic and pulmonary lesions. Furthermore, multistaining techniques have potential in combining the biological and pathological understanding of breast lesions (Robertson et al., 208).

Both IHC and IF share similar technical challenges which most commonly involve unspecific background staining and weak target signal; both of which hamper the microscopic interpretations of the specimen. In IHC, staining methods based on biotin, or peroxidase and phosphatase reporters are typically prone to result in high background staining unless endogenous forms of these molecules have been quenched (Odell and Cook, 2013). Unspecific binding of primary antibody to non-

target epitopes is often enhanced by a high concentration of primary antibody (www.thermofisher.com). Paradoxially, extremely high concentrations of secondary antibody have also been shown to decrease the intensity of the achieved signal (www.thermofisher.com). In IF, autofluorescence, the natural emission of light from biological structures, constitutes a specific problem in FFPE material. Autofluorescence may be caused by an inherent signal from the tissue itself, e.g. from the blood vessel walls, or be enhanced by paraffin or fixatives, such as aldehyde (www.thermofisher.com, Davis et al., 2014). In addition, one significant problem of IF is photobleaching, i.e. loss of intensity during exposure of the staining to light (Makki et al., 2016). To a certain extent, photobleaching can be avoided by the choice of robust fluorochromes less prone to bleaching. Another common problem of chromogenic multi-stainings is that, antigens localizing in the same subcellular structure cannot easily be distinguished from each other. To prevent this, IF labeling may provide a solution because, due to their smaller size, fluorescent labels seldom prevent each other's binding in cell organelles (Mason et al., 2000, Brouns et al., 2002). Moreover, the merging of different fluorescent labels in the same localization allows interpretation of co-expressions as regards both visual assessment and spectral imaging (van der Loos, 2010).

2.3.2.3 MiRNA in situ hybridization

The *In situ* hybridization (ISH) method applies labeled probes composed of complementary DNA, RNA or modified nucleic acids for detecting a specific DNA or RNA sequence in tissues or single cells. In research, ISH is used to reveal the location of specific nucleic acid sequences in order to provide insights into on the organization and physiological processes of genes. In clinical practice, ISH has established applications in tumor pathology, e.g. in lymphoma, sarcoma and breast cancer diagnostics and prognostication. For miRNAs, ISH technology is a means to detect individual miRNAs in specific tissue compartments and cell populations under visual morphological control. (Nielsen, 2012).

Technically, after pretreatments of FFPE material similar to those described for IHC and IF, the hybridization process applies modified RNA-nucleotides called LNA (locked nucleic acid) probes. LNAs are the gold standard of miRNA-ISH methodology because of their special hybridization specificity based on elevated melting temperatures. The use of LNAs enables distinction between the minimal differences in nucleotide sequences of miRNAs (Nuovo, 2010). Finally, detection in miRNA-ISH is performed using a selected reporter molecule, as previously explained for IHC and IF.

MiRNA-ISH has special advantages as compared to the more commonly used methods, Northern blot analysis, qPCR and microarray requiring extraction of RNA

(Zhang et al., 2015c). In ISH, miRNAs can be detected under visual spatial control and this provides a unique opportunity to locate and quantify the expressions within tissue compartments, individual cells and subcellular locations of neoplasms (Warford, 2012).

2.3.3 Aspects of microscopic and digital imaging

Since its invention in the 18th century, the microscope has remained the most commonly used optical device used to observe a magnified image of a histolocial specimen. However, recently digital pathology, an image-based information environment where computer technology is applied to collect and manage data generated from a digital slide, is being increasingly applied for research and clinical use for archiving and sharing, consultation, quality assurance and telepathology (Pantanowitz, 2010). Digital pathology enables “virtual” microscopy i.e. converting glass slides into digitalized slides which can be viewed, managed, shared and analyzed on a computer monitor (Robertson et al., 2018, Weinstein et al., 2009), including comprehensive commercial systems such as the Pannoramic system (www.3dhistech.com). In the future, high-resolution digital images registered at multiple magnifications and focal planes in virtual microscopy can be expected to provide a more intensive analysis of the data by using computer assisted image analysis systems (Feldman, 2009).

3. AIMS OF THE STUDY

This study aims at developing selected histopathological methods to detect specific proteins and miRNAs functioning during cell division, and assess their potential in predicting the prognosis of breast carcinoma patients.

The specific aims of this thesis are:

1. To evaluate selected histotechnical aspects and develop methods for IHC, double and triple IF, and ISH for detecting proteins and miRNAs in breast carcinoma specimen (I-IV)
2. To demonstrate the expression patterns of Securin, Pttg1IP and Separase and discuss their possible biological roles in invasive breast cancer (I-III)
3. To demonstrate altered levels of miRNAs -494, -205, -21 and -126 and discuss their possible biological roles in invasive breast cancer (IV)
4. To evaluate the prognostic potential of Securin, Pttg1IP and Separase in invasive breast carcinomas (I-III)
5. To evaluate the prognostic potential of miRNAs -494,- 205, -21 and -126 in invasive breast carcinomas (IV)

4. MATERIALS AND METHODS

4.1. Patient materials (I-IV)

Table 3 summarizes the description of the patient materials applied in publications I-IV. The patients comprise women diagnosed and treated with unilateral invasive breast carcinoma in two Finnish specialized centers. The first cohort (Cohort 1) comprises patients diagnosed in the Department of Pathology, Central Hospital of Central Finland, Jyväskylä, Finland, during 1987 – 1997 (I-IV). The second cohort (Cohort 2) was obtained from the Auria biobank and represents patients diagnosed with TNBCs in the Department of Pathology, Turku University Hospital, Turku, Finland, during 2005 – 2015 (III-IV).

All patients were surgically treated with resection or mastectomy with axillary evacuation. In addition, radiation and/or adjuvant treatments with anti-estrogen and/or cytostatic drugs were administered depending on the patient's age, hormone receptor and lymph node status following the international guidelines for treatment of invasive breast cancer at the time of diagnosis (Goldhirsch et al., 2009). None of the patients received neoadjuvant treatments. The clinico-pathological and intrinsic classifications of the patients were determined according to the criteria of the WHO classification (Lakhani et al., 2012) and St. Gallen International Expert Consensus (Coates et al., 2015). Complete clinical and follow-up data was deduced from patient files. Causes of death were obtained from autopsy reports, death certificates and from the Finnish Cancer Registry, Cancer Society of Finland, Helsinki, Finland. Prognostic associations were tested for patients with complete clinico-pathological and follow-up data. The maximum follow-up period was 22 years and 9 months (mean 12.4 years) (III) for Cohort 1 and 11 years and 9 months (mean 5.1 years) for Cohort 2 (II).

The research was performed in accordance with the ethical standards of institutional and national research committees approved by the Regional Ethical Review Boards of Turku University Hospital and Auria Biobank, Turku, Finland, Central Hospital of Central Finland, Jyväskylä, Finland, and Finnish Cancer Registry, Cancer Society of Finland, Helsinki, Finland (permit numbers 6/2002, AB15-9859 and TK-53-716-16). All research procedures involving human participants were performed in accordance with the ethical standards of the World Medical Association code of ethics (www.wma.net/policies-post/wma-international-code-of-medical-ethics), the 1964 Helsinki declaration and its later amendments (www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-

subjects), and the Biobank Act (688/2012, Ministry of Social Affairs and Health, Helsinki, Finland).

Table 3. Summary of patient materials comprising all breast cancer subtypes (Cohort 1) and TNBCs (Cohort 2) with clinico-pathological data included in prognostic analyses. Follow-up period 22 years for Cohort 1 and 5 years for Cohort 2.

	I	II	III	IV
	Cohort 1	**Cohort 1 Cohort 2**	**Cohort 1**	**Cohort 1 Cohort 2**
Mean age at dg (range) (years)	58 (39-78)	56 (39-78)	56 (39-78)	60 (34-87)
		62 (32-93)*		60 (32-90)*
Node positive (%)	51	49	46	35
		35*		38*
Mean tumor size (range) (cm)	2.4 (0.2-10.0)	2.4 (0.2-10.0)	2.4 (0.2-10.0)	2.2 (0.2-10.0)
		2.7 (0.8-18.0)*		2.9 (0.9-8.5)*
Histological type (%)				
Infiltrating ductal	80	82	82	71
		100*		100*
Special type	20	18	18	22
Intrinsic subtype (%)				
Luminal	67	70	69	53
Her2-amplified	16	15	15	13
Triple-negative	17	15	17	11
				100*
Dead of breast cancer (%)	34	34	35	27
		22*		33*

*TNBCs

4.2 Tissue materials (I-IV)

Table 4 summarizes the specimen of breast carcinomas and benign breast tissues used in papers I-IV. All breast carcinoma specimen used in prognostic analyses were prepared according to the standard practice of a clinical histopathology laboratory including fixation in buffered formalin (pH 7.0) and embedding in paraffin (formalin-fixed paraffin embedded, FFPE). Tissue microarrays (TMAs) were constructed by identifying in HE stainings two representative cancer cell areas from the center and periphery of the tumor and, thereafter, punching the paraffin block in these areas to

obtain two tissue cores of each tumour (diameter of tissue cores 0.6 mm in Cohort 1 and 1.5mm in Cohort 2).

For developing histological detection methods, FFPE specimens of malignant and benign breast tissue were used (I-IV). In addition, fresh tissue was applied in fixation experiments in order to evaluate the influence of delay and duration of formalin fixation on the histological methods used (IV). Fresh tissue material was collected from the operation theater of Turku University Hospital, Turku, Finland. Malignant tissue specimens were obtained from patients submitted to oncoplastic surgery due to invasive breast carcinoma and benign tissue specimen from breast reductions of healthy young individuals (Table 4). For fixation experiments, fresh specimen were dissected into pieces of similar size (app. 5mm in diameter) and each tissue piece was submitted to fixation in buffered formalin (pH 7.0) according to a systematic protocol exposing each tissue piece for a different pre-fixation delay and a different duration of exposure to formalin. After completing the fixation, each tissue piece was processed according to standard procedure, embedded in paraffin and cut at 3µm. The specimens were evaluated as whole sections and as TMAs (diameter of tissue cores 4mm).

Table 4. Summary of specimen of malignant and benign breast tissue applied for IHC, IF and ISH (I-IV).

Type of tissue	IHC (I-IV)	IF* (I-III)	ISH (IV)
Benign	62 - 68	28	22
Cancer**	349 - 497	23	285
Intrinsic subgroup			
Luminal	154 - 281	11	129
***Her2*-amplified**	37 - 51	4	31
TNBC	39 - 143	8	68

*including double and triple IFs, **including Cohorts 1 and 2

4.3 Histological detection methods

4.3.1 Immunohistochemical stainings (I-III)

Table 5 summarizes the antibodies and immunohistochemical (IHC) procedures applied for detection of Securin, Pttg1IP and Separase, including details of primary antibodies, antigen retrieval and detection. Briefly, Securin-IHC and Separase-IHC were performed using a Lab Vision Autostainer 480 (Thermo-Fisher Scientific, Fremont, CA, USA) and the signals were detected using a PowerVision + polymer kit, according to the manufacturer's protocol (DPVB + 110HRP Immunovision Technologies, Vision Biosystems, Norwell, MA, USA), with diaminobenzidine as chromogen. For IHC of Pttg1IP an automated immunostaining Discovery XT machine

(Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) was used in conjunction with an OmniMap HRP and ChromoMap DAB Kit (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) for detection.

Table 5. Details of IHC for Securin, Pttg1IP and Separase immunohistochemistry.

	origin	clone	source	dilution	antigen retrieval	incubation
Securin	mouse	DCS-280	Abcam ab3305	1:100	MW*	1h RT***
Pttg1IP	rabbit	polyclonal	Abcam ab128040	1:500	on platform**	20min 37°C
Separase	mouse	6H6	Novus Biologicals H00009700-M01	1:300	MW*	1h RT***

*microwave oven, citrate buffer pH6, **mild treatment with Cell Conditioning 2 solution (Roche Diagnostics/Ventana Medical Systems), ***room temperature

4.3.2 Immunofluorescence stainings (I-IV)

Multi immunofluorescence (IF) stainings were initiated with Ki-67 and cleaved Caspase-3 because of their different cellular expressions. Antibodies raised in the same host species were tested for selected physical and chemical pretreatments. These included heat (microwave treatment and boiling), pH (pH6 and pH 9) and chemical compounds Riboclear (Riboclear®, Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) and 2-ME/SDS (2-mercaptoethanol/sodium dodecyl sulphate) with PBS (Phosphate-buffered saline) washing and Triton X-100 washing. Detections of Securin, Pttg1IP and Separase were performed using antibodies raised in different species or representing different immunoglobulin subtypes. Control stainings were performed to demonstrate that no cross reactions were observed between the used primary and secondary antibodies.

The details of multi IF are summarized in Table 6. For double IF, sections were stained manually using a tyramide signal amplification system for the sequential detection of rabbit and mouse primary antibodies (TSA™ Kits #41 and #2 with Alexa Fluor® 555 and 488 tyramides, Molecular Probes, LifeTechnologies, Eugene, OR, USA). Detection of triple IF was performed using TSA™ Kit #41 (LifeTechnologies), 1070-05 and 1080-05 (SouthernBiotech, Birmingham, AL, USA) with Alexa Fluor® 555, 488 and 647 tyramides (LifeTechnologies) were used. Enzymatic activity used for the detection of the first primary antibody was destroyed after the first tyramide labelling by boiling the slides in water for 1 min. Finally, the slides were mounted using ProLong® Gold antifade reagent with DAPI (LifeTechnologies/Molecular Probes, Thermo Fisher Scientific, Eugene, OR, USA).

Table 6. Details of the applied double and triple IF methods.

	origin	clone	source	dilution
Double IF				
Securin	mouse	DCS-280	Abcam ab3305	1:100
	rabbit	EPR3240	Abcam ab79546	1:500
Pttg1IP	rabbit	polyclonal	Abcam ab128040	1:500
Separase	mouse	6H6	Novus Biologicals H00009700-M01	1:100
Ki-67	rabbit	polyclonal	Chemicon International AB9260	1:1000
PHH3	rabbit	polyclonal	Cell Marque 369A-14	1:100
Caspase-3	rabbit	D3E9	Cell Signalling Technology #9579	1:100
Triple IF				
Securin	rabbit	EPR3240	Abcam ab79546	1:500
Separase	mouse	6H6	Novus Biologicals H00009700-M01	1:1600
CyclinB1	mouse	V152	MA5-13128	1:600

4.3.3 *In situ* hybridization (IV)

Development of miRNA-ISH method was initiated by manual detection of U6 with chromogenic staining (Fig. 4). The manual choromogenic ISH method applied previously published protocols with slight modifications (Jørgensen et al., 2010). The modifications concerned performing overnight hybridization and using anti-DIG (double digoxigenin) Fab-fragments in the detection of the probes. Also, the optimization of the protocol was started applying single-labelled probes. However, signal intensity obtained using these probes was not satisfactory and double-labelled probes were applied in further experiments. Next, for detecting U6 signal, an automated chromogenic ISH was tested (https://www.biocompare.com/Application-Notes/43455-Automated-in-Situ-Hybridization-Of-MicroRNA-On-The-DISCOVERY-ULTRA/).

When tested with protease pretreatment, the automated chromogenic method produced uneven signal over the specimen leaving many nuclei unstained. Therefore, the more sensitive immunofluorescent method modified with manual deparaffinization was applied. The slides were loaded wet into Ventana Discovery XT automated slide staining instrument (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA). DIG-labeled LNA-modified probes were designed by Exiqon (Exiqon A/S, Vedbaek, Denmark) and treated according to the recommendations by the manufacturer. All steps of hybridization and fluorescent detection were optimized for each miRNA separately to achieve best possible signal to background ratio (Table 7). Tissue permeabilization was optimized for breast tissue under study and finally performed treating the samples for 20min at 37 C applying ISH Protease 3 (Roche Diagnostics/Ventana). For the fluorescent detection of the probes, anti-DIG antibody (Roche Diagnostics/Ventana, clone 1.71.256) and OmniMap anti-ms HRP (Roche Diagnostics/Ventana) were used. Amp HQ Kit and Amplification anti-HQ HRP Multimer (Roche Diagnostics/Ventana) were used to amplify the signal before applying rhodamine substrate (Roche Diagnostics/Ventana). Amplification was not used for U6. After immunofluorescent detection slides were collected from the instrument and mounted with ProLong Gold antifade reagent with DAPI (LifeTechnologies/Molecular Probes, Thermo Fisher Scientific, Eugene, OR, USA). Finally, this method was further optimized for detection of miRNAs -21 and -126 by combining it with signal amplification. The possibility of false positive signals was excluded by using control stainings applying either no probe or a scramble probe without homology to any of the miRNA sequences available in the miRBase database.

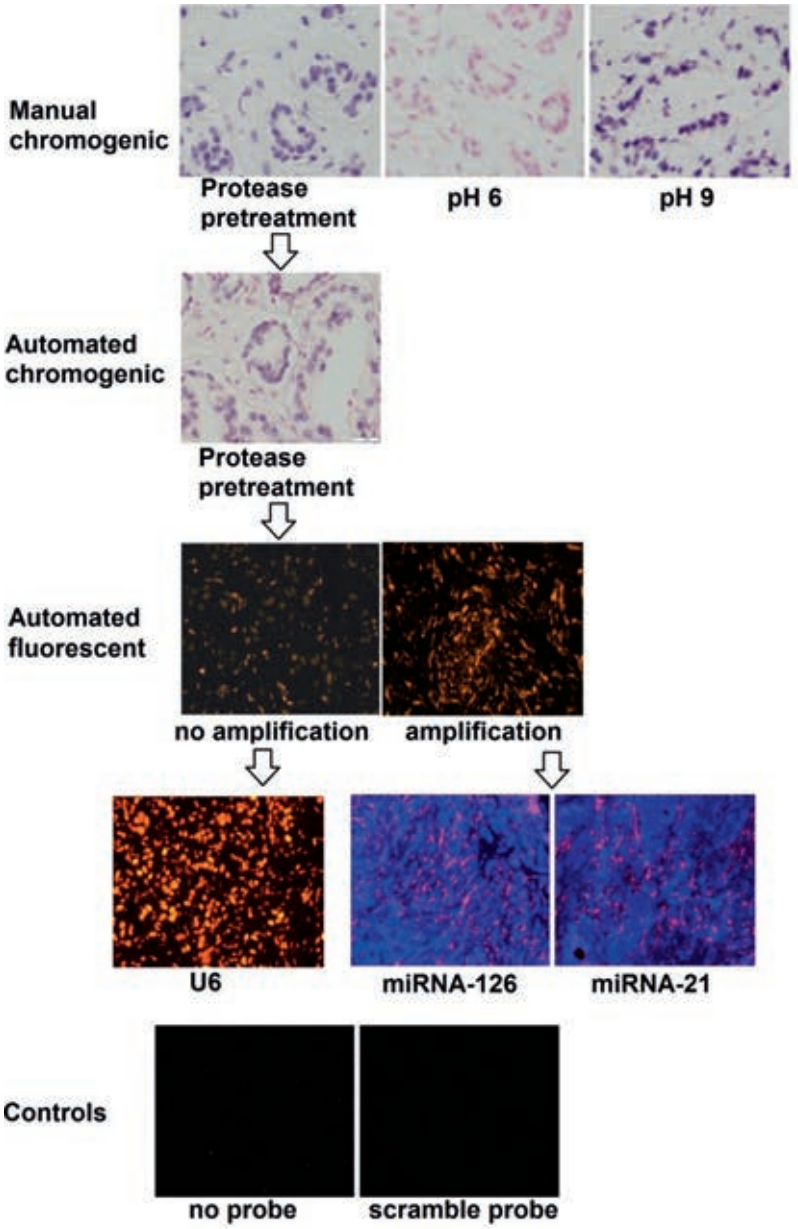

Figure 4. Demonstrative microphotographs resulting from development of ISH method for detecting U6 and miRNAs -126 and -21. In manual chromogenic detection protease pretreatment resulted in acceptable results for U6 and was further applied in an automated chromogenic method. For detection of miRNAs -126 and -21, optimal detection result was obtained using automated fluorescent method with or without amplification of signal. No signal was detected in control stainings without probe or with scrambled probe. (Magnification x40).

Table 7. Details of ISH for U6 and miRNAs -494, -205, 21 and -126.

	source	hybridization and stringent washing temperature	detection, min	2-step amplification	fluorescent substrate, min
U6	99002-15	53C	24	not used	32
miRNA-494	hsa-miR-494-3p 616987-360	54C	16	8+16 min	12
miRNA-205	hsa-miR-205-5p 18099-15	55C	16	12+16 min	20
miRNA-21	hsa-miR-21-5p 38102-15	53C	16	8+16 min	12
miRNA-126	hsa-miR-126-3p 90005	56C	16	12+16 min	20
Scramble probe	99004-15	53C	16	12+16 min	20

Table 8. Summary of the immunohistochemical interpretations of Securin, Pttg1IP and Separase. The table shows for each staining the expression patterns observed, the principles of evaluation and the classification criteria applied for determining the prognostic impacts.

	expression pattern	evaluation principle	prognostic classification
Securin	immunopositive cancer cells	fraction (%)	<10% vs ≥10%
	intensity of immunopositivity	0 - 3+	negative vs positive
	subcellular localization of immunopositivity	nuclear, cytoplasmic, both	cytoplasmic <50% vs ≥50%
			cytoplasmic <10% vs ≥90%
Pttg1IP	immunopositive cancer cells	0%, <10%, 10-50% or ≥50%	<50% vs ≥50%
Separase	nuclear immunopositivity in cancer cells	fraction (%)	<1% vs ≥1%
	diffuse immunopositivity in cancer cells	0 - 2+	negative vs positive

4.3.4 Detection of routine clinical markers of breast cancer (I-IV)

IHC for detecting estrogen (ER, clone SP1, Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) and progesterone (PR, clone 1E2, Roche Diagnostics/Ventana) receptors, Her2 (clone 4B5, Roche Diagnostics/Ventana) and Ki-67 (polyclonal, Chemicon International, Temecula, CA, USA) was performed using BenchMark XT automated staining platform applying an ultraView Universal DAB Detection Kit (Roche Diagnostics/Ventana). On the basis of Her2-IHC (intensity of IHC 2+ and 3+), cases were chosen for gene amplification detection by ISH (Wolff et al., 2014, Goldhirsch et al., 2013). *Her2*/Chr17 double ISH were performed using a BenchMark XT machine (Roche Diagnostics/Ventana), the *Her2* DNA and the Inform Chromosome 17 probe set, and the ultraView SISH detection kit to detect *Her2* (Roche/Ventana) and the ultraView Alkaline Prosphatase Red ISH Detection Kit to detect Chr17 (all from Roche Diagnostics/Ventana) (Roche/Ventana).

4.3.5 Interpretations of IHC, IF and ISH (I-IV)

4.3.5.1 Interpretations of IHC (I-III)

The procedure for interpretation of IHC of Securin, Pttg1IP and Separase are summarized in Table 8. All interpretations were performed in sets of 100 invasive breast cancer cells (in minimum 100 and in maximum three times 100 cells evaluated). TMA cores including less than 100 invasive cancer cells were excluded from the study.

Securin was registered for the fraction, intensity, and subcellular localization of immunopositive cancer cells (I-III). The fraction of Securin-positive cancer cells was registered as a continuous variable (%). The intensity of Securin expression classified as negative, faint, moderate or strong (intensity scores 0, 1+, 2+ and 3+, respectively) as it was the most practical classification method and produced high reproducibility of evaluations (Karra et al., 2012). The sub-cellular localization of Securin was classified as nuclear, cytoplasmic or a combination of both (I). Pttg1IP-IHC was registered as negative, faint, moderate or strong (0%, <10%, 10-50% or ≥50%) (II). Separase-IHC was registered based on the fraction (%) of cancer cells exhibiting mitotic expression and on the most practival scoring (0, 1+, 2+) for the staining intensity of diffuse expression (III). For prognostic evaluations, the patients were allocated into subgroups with low versus high expression of the studied proteins based on statistical analyses.

4.3.5.2 Imaging and interpretation of IF and ISH (I-IV)

Imaging IF and ISH was initiated by observing each section under fluorescence microscope (Axio Scope A1, Carl Zeiss Microscopy GmbH, Jena, Germany) and comparing the expression patterns and intensities with those of the scanned images (Pannoramic Midi FL slide scanner, 3DHISTECH Ltd., Budapest, Hungary) in order to ensure comparability between the microscopic and digitalized images. When scanning double IFs, a range of exposure times was tested for each channel using the Pannoramic Scanner software (www.3dhistech.com/pannoramic_scan). Finally, the optimal exposure time was set manually so that it optimally demonstrated the features of interest. After this adjustment, all specimen were treated with the same exposure time settings in order to ensure comparable images and reliable interpretations throughout the whole series of specimen. In double IF, the exposure time for DAPI_Q channel was determined at 1500msec, and for TRITC_43 and FITC_38 at 2 msec. For triple IFs and microRNA-ISHs the optimal quality of images was achieved using automatically set exposure times for DAPI_Q, TRITC_43, FITC_38 and Cy5_Q channels.

Double IFs combining Separase with Ki-67, PHH3 and cleaved Caspase-3, and triple IFs stainings combining Separase, Securin and CyclinB1 were qualitatively evaluated in virtually merged microphotographs of the digitalized images to determine co-expression and subcellular co-location of the studied proteins in invasive cancer cells as compared to benign breast tissues.

In ISH of U6 (non-coding small nuclear RNA) and miRNAs -494, -205, -21 and -126, the pattern of expression was observed and registered separately for invasive cancer cells and stromal cells. MiRNA-126 detected endothelial – not epithelial – cells both in benign and malignant tissue. The intensity level of U6 was classified into negative, low, moderate and high (intensity scores 0 – 3+) under fluorescent microscope (Fig. 5). Only specimen with intensity scores 2 – 3+ were considered to show sufficient quality of RNA acceptable for miRNA detection. The intensity of miRNA-ISHs were classified semi-quantitatively (0 – 3+) and, for prognostic evaluations, patients were allocated into miRNA negative vs positive subgroups (0 vs 1+ - 3+, respectively).

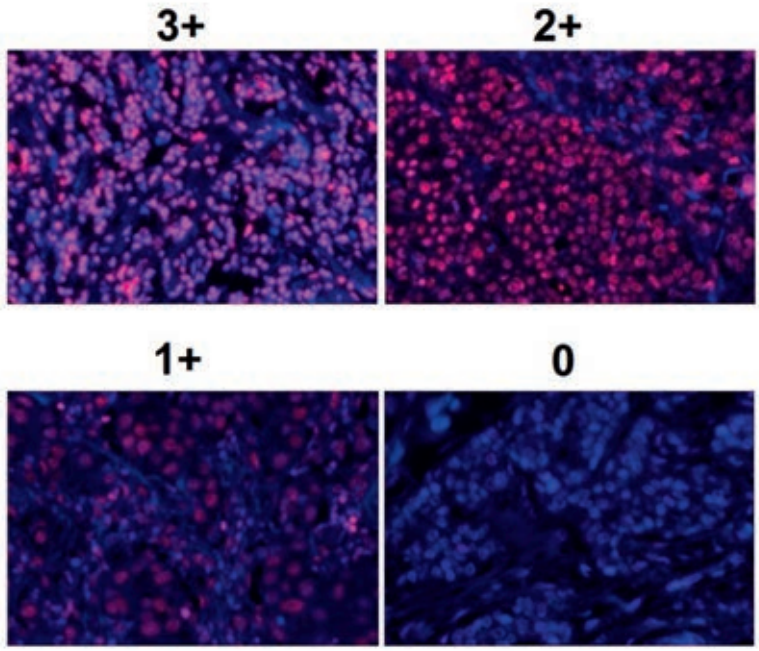

Figure 5. Intensity levels of U6 were graded (0 – 3+) under light microscope and intensities 2 – 3+ were considered acceptable. (Magnification x40).

4.3.5.3 Interpretations of routine markers of clinical pathology (I-IV)

Interpretations for routine IHC markers of clinical pathology, ER, PR, and IHC and ISH for *Her2* and Ki-67 followed previous literature and generally accepted international guidelines (Wollf et al., 2014, Goldhirsch et al., 2013).

4.4 Statistical analyses

For statistical analysis, patients were allocated into different expression groups based on the observed levels of Securin, Pttg1IP and Separase immunopositivities. All cutpoints were set based on mean and median values of continuous variables and prognostic associations involving univariate analyses where the chosen cutpoints produced the strongest predictive value of disease survival in our material. The cut-point for Securin at 10% immunopositive cancer cells (<10% versus ≥10%) was also based on previous literature (Talvinen et al., 2008). In evaluating the subcellular localization of Securin, the cutpoints at 90% versus 10% of cancer cells with cytoplasmic Securin expression were used as criteria for categorizing the specimen. The different expression levels of Pttg1IP were categorized into negative versus

positive. Similarly, mitotic Separase was optimally stratified in univariate prognostic analyses as absence or presence (<1 % versus ≥ 1 %) of Separase-positive cancer cell mitoses. For diffuse Separase expression, the optimal cut point was identified between negative vs. positive expression. The categorizations were supported by morphological observations and associated with high consistencies of interpretations as calculated inter- and intraclass reproducibility.

In this research, survival analyses were performed to identify explanatory variables associated with breast cancer-specific mortality. The analyses were started with one explanatory variable at the time using Kaplan-Meier analysis together with log rank or Wilcoxon tests. This analysis was continued with Cox's proportional hazard model where multiple prognostic factors were included simultaneously. Relations and influence on survival between the studied markers and the established clinical prognostic factors of invasive breast carcinoma (i.e. tumor size, axillary lymph node status, histological and intrinsic subgroups and histological grade) were quantified as hazard ratios (HRs) with 95 % confidence intervals (CIs). The validity of proportional hazards assumptions was assessed both visually and numerically, and no marked deviations for assumptions were observed. Associations between categorical variables were tested with Fisher's exact test and for continuous variables with Pearson, Spearman and Kendall's correlation coefficients. In addition, inter- and intra-observer reproducibilities between different expression levels were assessed using intra-class correlation coefficients. P-values less than 0.05 (two-tailed) were considered statistically significant. All computations were performed with SAS for Windows, Version 9.3 or later or SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC, USA). In addition, R Statistical Software was used (R Development Core Team 2017, www. R-project.org).

5. RESULTS

5.1 Development of histological techniques for detecting Securin, Pttg1IP and Separase, and miRNAs -494, -205, - 21 and -126

5.1.1 Development of triple IF method for detecting Securin, Pttg1IP and Separase (III)

When testing triple IFs with pairs of primary antibodies raised in the same host species, promising immunoreactivity results were obtained using the combination of pretreatments Riboclear® (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) and heating in 98°C, as well as 2-mercaptoethanol/SDS and heating in 56°C with PBS washing (Fig. 6, Table 9). However, the tested pretreatments were not sufficient to elute or denaturate other pairs of primary antibodies but showed cross-reactions between antibodies raised in the same species. When testing different immunoglobulin subtypes of primary antibodies, successful detections resulted using Securin antibody raised in rabbit, and Separase and CyclinB1 antibodies raised in mice (Ig subtypes G2 and G1, respectively).

Table 9. Summary of immunoreactivity results from testing different physical and chemical pretreatments for multi IFs using antibodies produced in the same species. For each pretreatment, the efficiency to avoid cross-reactions was evaluated and the pretreatments with moderate efficiency were considered acceptable for further development.

	Pretreatment	**Result**
Heat	Microwave treatment	low efficiency
	1 min boiling	low efficiency
	3 min boiling	low efficiency
pH	Riboclear	low efficiency
	Riboclear and short boiling	low efficiency
	Riboclear and H2O2	low efficiency
	Riboclear and heating 98°C	moderate efficiency
Chemical compound	2-ME/SDS	background fluorescence
	2-ME/SDS 56°C and PBS washing	background fluorescence
	2-ME/SDS 56°C and Triton X-100 washing	moderate efficiency

*Riboclear® (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA)
**2-mercaptoethanol

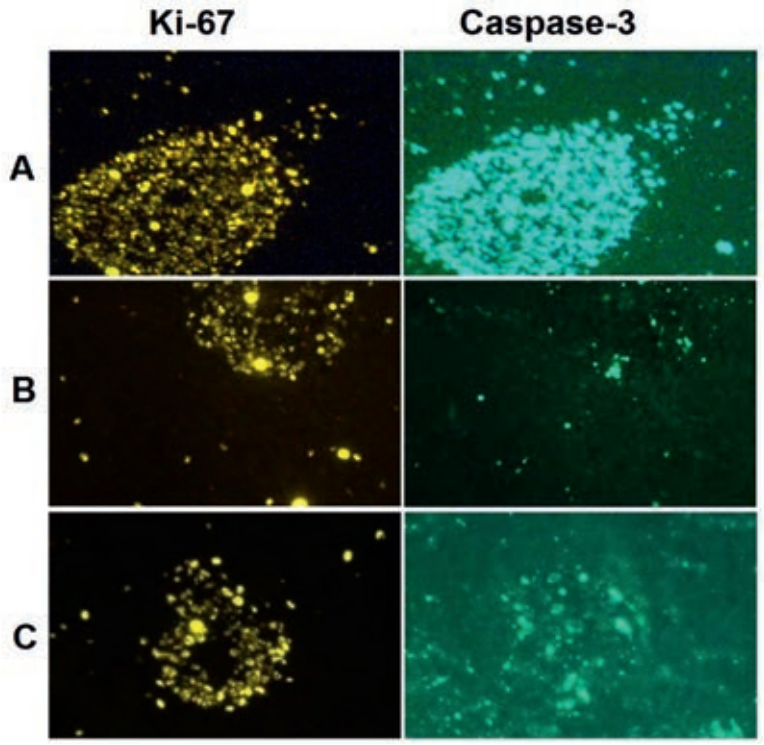

Figure 6. Multi IF using Ki-67 and Caspase-3 antibodies. The pictures show examples of different physical (A. temperature, 1 min boiling) and chemical conditions (B. denaturation with Riboclear® and heating 98°, C. elusion with 2-ME/SDS 56°C and PBS washing). In these treatments combining Riboclear® with heating to 98°C and 2-ME/SDS heated to 56°C with PBS washing showed the most promising immunoreactivity result. (Magnification x40).

5.1.2 Development of semi-automated ISH method for detecting U6 and miRNAs -494, -205-, -21 and -126

When testing several conditions for manual detection of U6, protease pretreatment produced the most promising result (Fig. 4). In an automated chromogenic method, however, protease pretreatment produced an uneven signal over the specimen leaving many nuclei unstained. Instead, the more sensitive immunofluorescent method modified with manual deparaffinization produced acceptable detection of U6. This detection method, further optimized combining it with signal amplification, produced acceptable result for detection of miRNAs -126 and -21 (Fig. 4).

5.1.3 Influence of fixation conditions on IHC and ISH

In fixation experiments, high quality of IHC combined with optimal cellular morphology was achieved in specimen treated with immediate formalin-fixation for 24 hours (Table 10, Fig. 7). Interpretation of IHC, particularly evaluating the subcellular localization of immunoexpression, was considerably hampered when the initiation of fixation was delayed for 24 hours. After a 3-day delay in initiation of fixation, the cellular morphology of both benign and malignant specimen was completely destroyed with total loss of Securin immunoexpression. The influence of extended fixation from 24 hours to 7 days did not markedly influence the morphology or immunoexpression of the specimen. However, artificial Securin-staining with unspecific granular precipitates was observed in association with 7-day exposure to formalin.

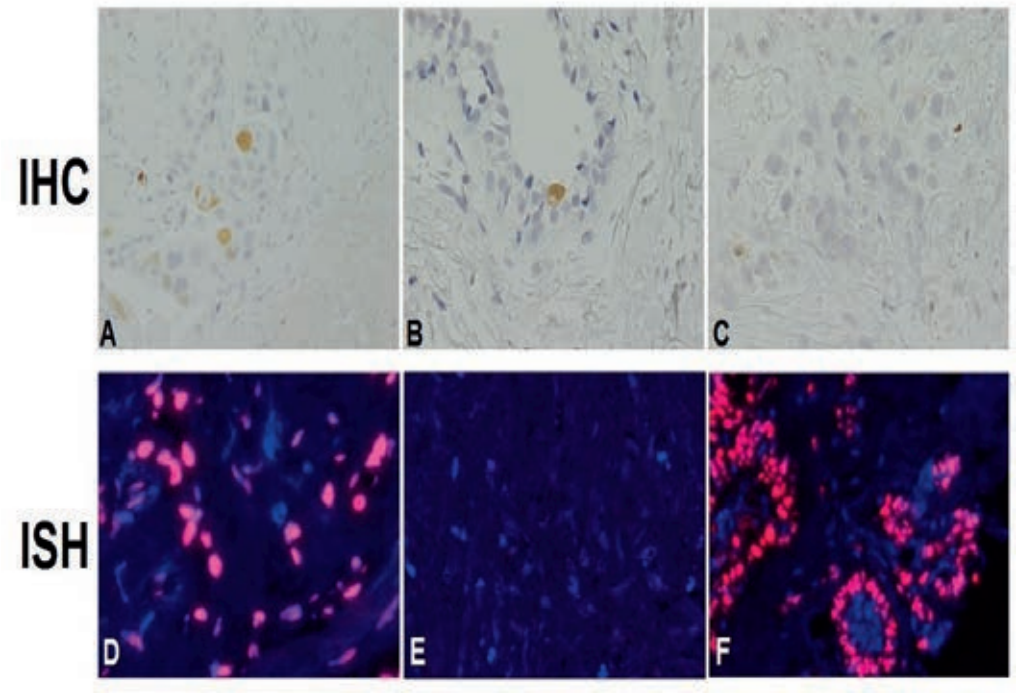

Figure 7. Influence of fixation conditions on IHC and ISH. Immediate fixation for 24 hours produced optimal quality of Securin-IHC in breast carcinomas (A). Securin-IHC could be reliably evaluated in malignant and benign specimen even after a 24-hour (B) but not after a 72-hour delay prior to fixation (C). In ISH, acceptable intensity of U6 in cancer specimen was observed after up to 24-hour fixation delay (D) but not longer (E). However, in benign breast tissue U6 was acceptable even after 6-day fixation delay (F).

In ISH, optimal quality of RNA with intact cellular morphology was established in breast carcinoma cells and adjacent stroma when the initiation of fixation was delayed for no more than 48 hours (Table 10, Fig. 7). Instead, in benign breast epithelial and stromal cell, the quality of RNA was retained even after 6-day delay of formalin fixation.

According to the present experiment, the duration of formalin fixation from 24 to >48 hours did not influence the quality of RNA in benign or malignant breast epithelial cells, or in the adjacent connective tissue stroma.

Table 10. Summary of results from fixation experiments for IHC and ISH. Fresh tissue specimen of benign breast tissue (n=4) and breast carcinomas (n=5) were systematically submitted to a series of fixation delays and durations. For IHC, the quality of staining was determined observing the appropriate immunoexpression for Securin (+ versus -). For ISH, the integrity of RNA was verified based on detection of U6 (+ versus -).

Delay / duration of fixation (d)	Benign	Malignant
IHC		
0/1	+	+
0/7	+	+
1/1	+	+
1/7	+	+
3/1	-	-
3/7	-	-
ISH		
0 / 1	+	+
0 / >2	+	+
…*		
2/1	+	+
2/>2	+	+
…*		
6/>2	+	-
7/>2	-	-

*Only results from the most informative observations points and the respective fixation conditions are shown.

5.2 Securin, Pttg1IP and Separase in invasive breast carcinoma

5.2.1 Expression patterns of Securin, Pttg1IP and Separase

5.2.1.1 Securin (I, II)

In benign breast tissue (n=68), securin expression was observed in less than 1% of luminal epithelial cells, invariably located in the cell nucleus. Occasionally, single myoepitelial and stromal fibroblasts showed faint securin-positivity. However, pure cytoplasmic securin expression was not observed in benign breast (Fig. 8).

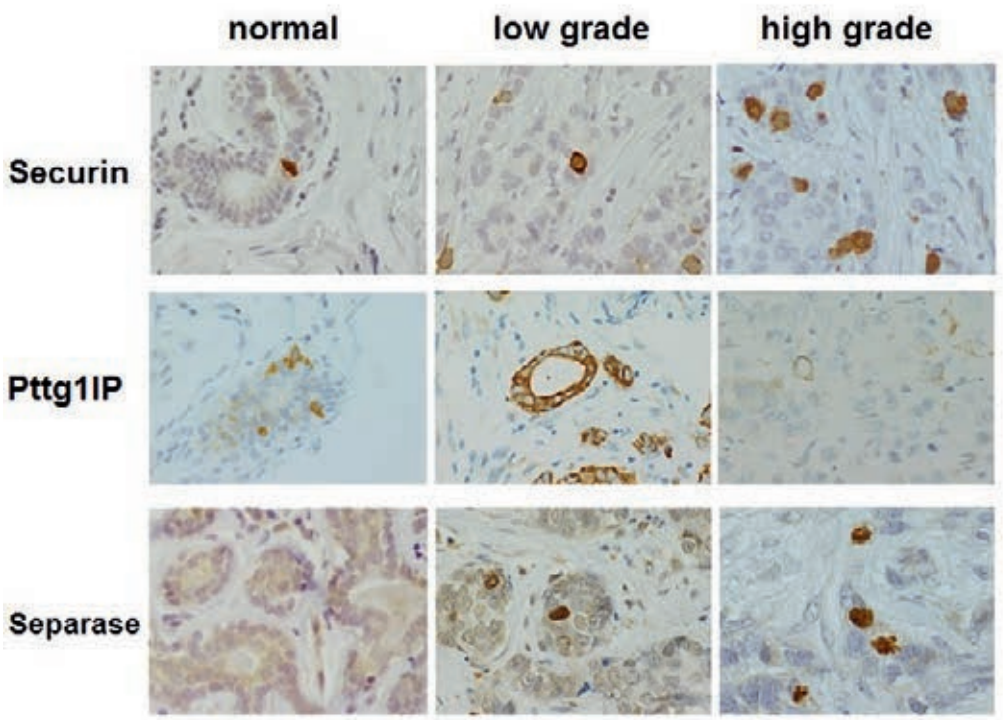

Figure 8. Summary of IHC expression patterns of Securin, Pttg1IP and Separase in normal breast tissue, and low-grade and high-grade carcinomas. (Magnification x40).

In breast carcinomas ($n = 447$) (Fig. 8), Securin expression was observed in an average of 8.3% of malignant cells (median 6%, range 0–57.7%) with varying intensity. In addition, the subcellular localization of Securin expression varied between the carcinoma cases from nuclear (57%) to cytoplasmic (38%), with the majority of cases (64%) showing cytoplasmic staining. Securin-positivity representing high-grade carcinomas. In breast carcinomas divided according to the intrinsic classification, the average fraction of Securin-positive carcinoma cells was 7.2% in luminal carcinomas (median 5%, range 0–51.0%), 10.1% in *Her2*-amplified carcinomas (median 8.6%, range 0.3–40.3%) and 17.2% in TNBC (median 15.9%,

range 0.6–77.5%). The intrinsic subtypes were also strongly associated with the fraction of Securin-positive cancer cells (<10% vs ≥10%) and the subcellular location (nuclear vs cytoplasmic) of Securin-positivity ($p < 0.0001$ and $p < 0.001$, respectively). Nuclear Securin expression was strongly associated with luminal subtype ($p = 0.002$) whereas cytoplasmic localization of Securin expression was typical in TNBC and *Her2*-amplified carcinomas. The intensity of Securin staining did not show a statistical association with any of the breast carcinoma subtypes.

5.2.1.2 Pttg1IP (I, II)

In benign breast tissue (n=62), expression of Pttg1IP was observed only in occasional cells of the luminal epithelium (Fig. 9). No expression was found in myoepitelial or stromal cells.

In breast carcinomas (n=401) (Fig. 9), Pttg1IP-positivity was observed in two thirds (74.3%) of tumors, mostly with moderate or strong intensity (41% and 15%, respectively). Total Pttg1IP-negativity (score 0) was observed in 26% of carcinomas and the majority of these (57%) represented TNBC. The expression of Pttg1IP was significantly associated with the subcellular localization of Securin ($p<0.001$) so that nuclear Securin expression was observed in Pttg1IP-positive tumors while cytoplasmic Securin was observed in absence of Pttg1IP expression. This statistical association could also be verified morphologically in double IF stainings of Securin and Pttg1IP (Fig. 9). Considering the established morphological and clinical features of breast carcinoma diagnostics i.e. tumor size, axillary lymph node status, histological grade and intrinsic classification, the combination of Pttg1IP-negativity and cytoplasmic Securin was significantly associated with aggressive clinical features ($p<0.001$).

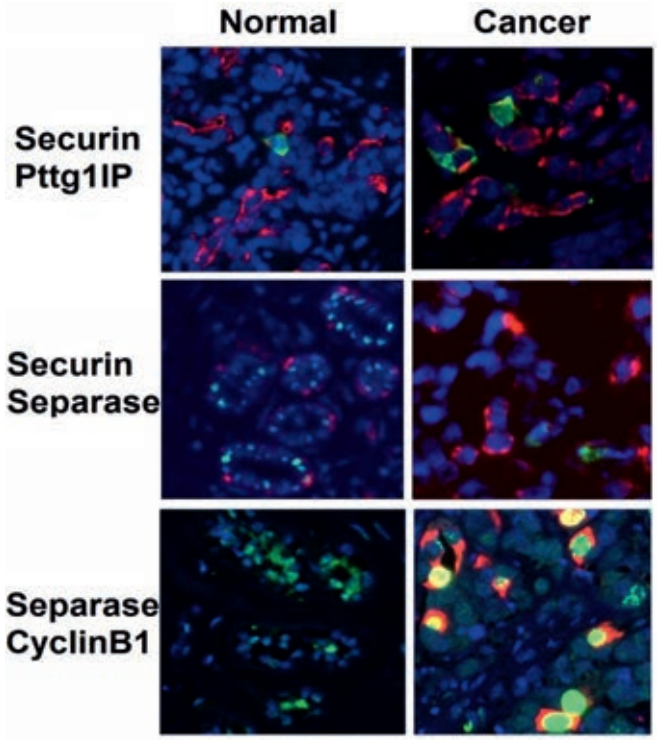

Figure 9. Summary of double IFs showing co-expression of Securin (green) and Pttg1IP (red), Securin (red) and Separase (green), Separase (green) and CyclinB1 (red) in breast carcinoma. (Magnification x40).

5.2.1.3 Separase (III)

In benign breast tissue (n=66), expression of Separase was observed in single luminal epithelial cells (Fig. 10). Only occasional faint Separase-positivity was detected in stromal cells.

In breast carcinoma cells (Fig. 10), Separase was observed as two distinct morphological expression patterns, as strong precipitates in mitotic cells and as diffuse positivity in the cytoplasm and nucleus. Between these expression patterns, an inversed relation was observed so that high mitotic Separase expression was accompanied by lack of diffuse Separase expression and vice versa. Typically, the first expression pattern was observed in carcinomas exhibiting aggressive morphological and clinical features, whereas the latter expression pattern occurred along with favorable histological and clinical characteristics.

The expression of mitotic Separase was statistically strongly associated with expressions of both Securin ($p<0.0001$) and CyclinB1 ($p<0.0001$). The mutual expressions of Separase with Securin and CyclinB1 were, further, evaluated with the

help of double IFs and triple IFs to demonstrate co-expressions of the proteins in breast carcinomas (n=23) and in benign breast specimen (n=28). In most breast carcinoma cases, triple IFs of the breast carcinoma specimen demonstrated co-expression for Separase and cyclinB1, but not for Separase and Securin, corresponding to the expression patterns observed in benign breast epithelium. However, in a minority of cases an aberrant expression pattern was revealed with cancer cells showing either Separase-positivity alone or co-expressed with Securin (Fig. 10).

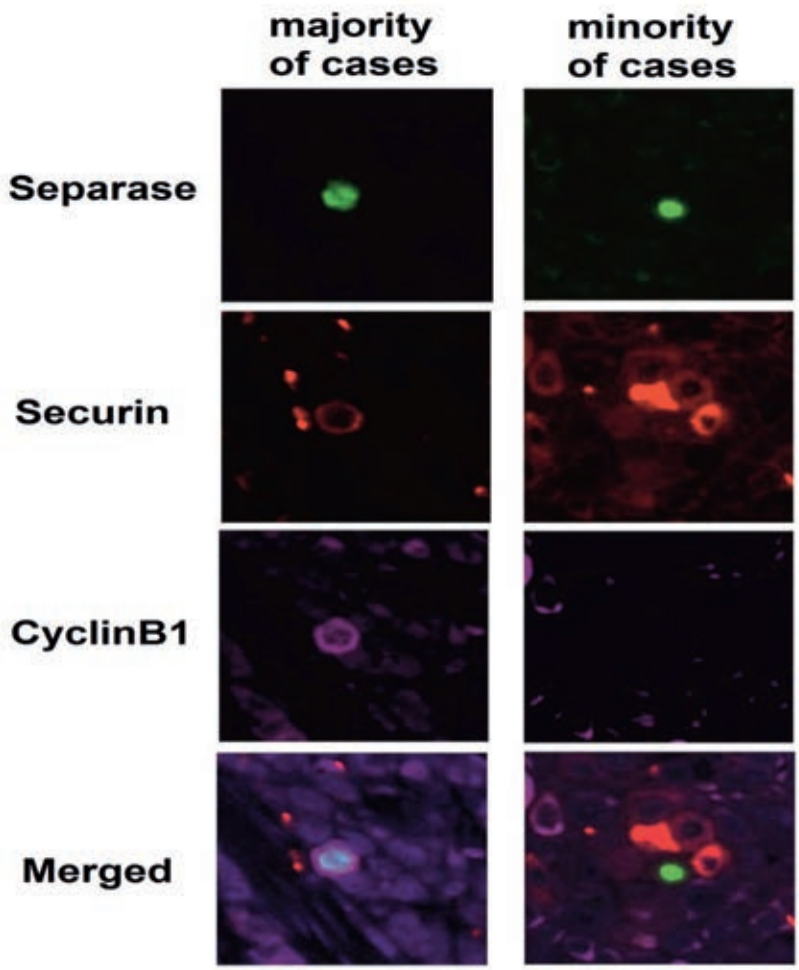

Figure 10. Summary of triple IFs showing co-expression of Separase, Securin and CyclinB1 in the majority of the cases as opposed to aberrant expression in the minority of cases. (Magnification x40).

5.2.2 Prognostic associations of Securin, Pttg1IP and Separase

5.2.2.1 Securin (I, II)

In the present material, both the extent and the sub-cellular localization of Securin were associated with the outcome of breast cancer patients ($n = 447$ (**I**) and n=497 (**II**)). A high fraction (≥10%) of Securin expressing cancer cells predicted up to a 2.7-fold increased risk of breast cancer-specific death ($p<0.0001$, 95%CI 1.6-2.7) (Fig. 11A). Moreover, breast carcinomas showing cytoplasmic Securin were associated with a 5.7-fold increased risk of breast cancer mortality as compared to carcinomas showing nuclear Securin ($p=0.052$, 95%CI 0.8-42). The prognostic value of cytoplasmic Securin expression was further emphasized in statistical quartile analyses where the majority (75%) of patients with sparse (<10% of cancer cells) cytoplasmic securin expression were alive in average 11.2 years after diagnosis, whereas for the majority of patients showing extensive (≥90% of cancer cells) cytoplasmic Securin expression survived only on average 4.2 years after diagnosis (Fig. 11B). The intensity of Securin expression did not show statistical significance in any of the statisical approaches.

Also in multivariate analyses, both extent of Securin expression and extensive (≥90% of cancer cells) cytoplasmic Securin expression were found to be independent prognosticators of breast cancer survival ($p < 0.0001$ and $p < 0.001$, respectively), along with axillary lymph node status and tumor size (Table 11).

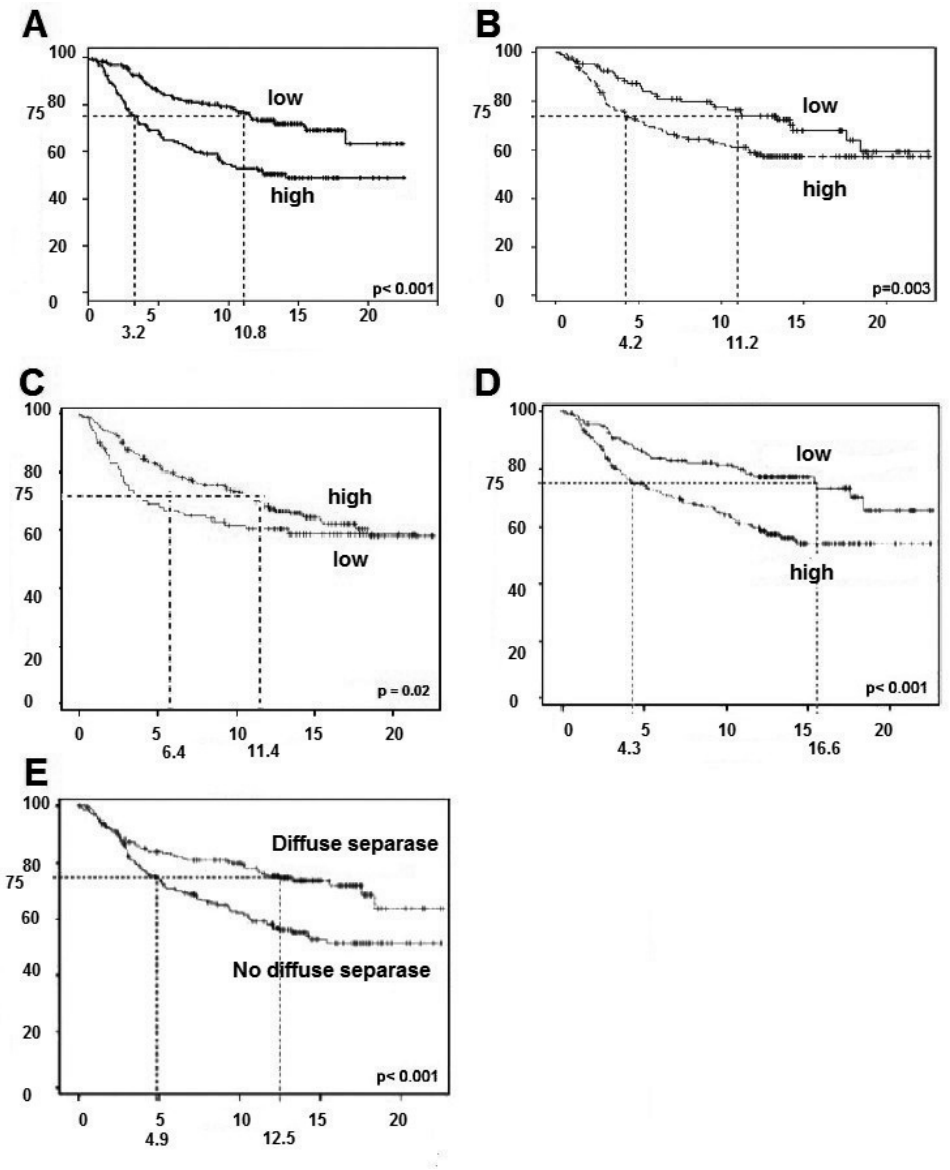

Figure 11. Summary of Kaplan-Meier analyses of breast cancer survival based on immunoexpressions of Securin, Pttg1IP and Separase. **A.** Securin expression in < 10% versus ≥ 10% of cancer cells, **B.** cytoplasmic Securin in < 10% versus ≥ 90% of the cancer cells, **C**. Pttg1IP expression low versus high, **D.** mitotic Separase expression in <1% versus ≥1% of cancer cells, and **E.** diffuse Separase-expression negative vs positive. All comparisons revealed statistically significant prognostic values between the evaluated patient groups. The 75% quartiles of each curve show the survival difference between low (*a*) and high (*b*) expression. X-axis: Disease-specific survival rate (%), Y-axis: years after diagnosis.

Table 11. Summary of multivariate survival analyses involving immunoexpressions of Securin and Separase, as compared with the established prognosticators of invasive breast carcinoma. The prognostic associations for Securin expression are shown separately for high (≥10%) immunopositivity and for cytoplasmic Securin (≥90% of cancer cells). The prognostic associations for Separase expression are shown for high mitotic (≥1%) immunopositivity and lack of diffuse immunoexpression.

		HR	p	95%CI
I, II:	**High fraction (%) of securin**	2,4	<0,0001	1,5–3,8
	Nodal status	2,8	<0,0001	1,8–4,3
	Tumor size	1,2	0,003	1,1–1,4
	Histological grade		ns.	
	Intrinsic classification		ns.	
I:	**Cytoplasmic securin**		ns. (0,6)	
	Nodal status	1,7	0,04	1,1–3,0
	Tumor size	1,3	0,0002	1,1–1,6
	Histological grade		ns.	
	Intrinsic classification		ns.	
III:	**Mitotic separase**	1.6	0,03	1.0-2.5
	Nodal status	3.2	<0,0001	2.1-4.8
	Tumor size	2.1	0,001	1.3-3.3
	Histological grade		NS.	
III:	**Diffuse separase**	1.8	0,002	1.2-3.8
	Nodal status	3.2	<0,0001	2.1-4.9
	Tumor size	2.2	0,0006	1.4-3.5
	Histological grade		NS.	

5.2.2.2 Pttg1IP (II)

Among the 497 breast carcinoma patients, loss of Pttg1IP expression predicted a 1.5-fold risk for disease-specific mortality (p=0.002, 95%CI 0.9-2.7) (Fig. 10C). Further quartile estimations of the Kaplan-Meier survival curves demonstrated in average a 5-year survival difference between the majority (75%) of patients with Pttg1IP-positive versus Pttg1IP-negative carcinomas (11.4 years versus 6.4 years, respectively). The prognostic associations were still intensified when analyzed in combination with Securin comparing patient subgroups with the most favorable versus unfavorable outcomes (high Pttg1IP and low Securin versus low Pttg1IP and high Securin) (p<0.001). As demonstrated in the quartile estimations, the majority (75%) of patients showing the most favorable combination of Pttg1IP and Securin

survived on average 17.6 years after diagnosis as opposed to the majority of patients exhibiting the most unfavorable combination of the protein expressions who survived on average only 3.5 years.

In multivariate analyses Pttg1IP did not show an independent prognostic value over Securin immunoexpression, or over the established prognostic features, nodal status, tumor size, histological grade and intrinsic classification.

5.2.2.3 Separase (III)

Both mitotic Separase and lack of diffuse Separase comprised significant prognostic associations among breast cancer patients (n=349). Simply the occurence of Separase-positive mitotic figures among breast cancer cells (≥1%) indicated a 2.0-fold risk of breast cancer-specific mortality (p=0.0004, 95%CI=1.4-3.0) (Fig. 10D). Applying this prognostic impact into statistical quartile analyses revealed, for the majority (>75%) of breast carcinoma patients, an average of 11.3-year survival difference between patient subgroups detected with and without Separase-positive mitotic figures (4.3-year and 15.6-year survival, respectively).

Correspondingly, significant prognostic associations were observed for loss of diffuse Separase expression indicating a 1.8-fold risk of breast cancer-specific mortality (p=0.0006, 95%CI 1.3-2.6) (Fig. 10E). In statistical quartile analyses, this translated into a 7.5-year average survival difference for the majority (>75 %) of patients with carcinomas with versus without diffuse Separase expression (12.5 versus 4.9 year breast cancer-specific survival, respectively) (p=0.001).

When the patient material was divided into subgroups for intrinsic classification, both mitotic and diffuse Separase showed statistically significant prognostic impact only among luminal breast carcinomas (HR 2.2, p=0.001, 95%CI 1.4-3.4 for mitotic Separase and HR 2.9, p<0.0001, 95%CI 1.7-4.9 for diffuse Separase) whereas no prognostic associations were detected among HER2-amplified carcinomas or TNBC.

In multivariate analyses involving Securin and Ki-67, and the established prognosticators of breast carcinoma, only the loss of diffuse Separase – and not mitotic Separase – remained an independent prognosticator for breast cancer-specific survival (HR 1.6, p=0.02, 95%CI 1.1-2.4) (Table 11).

5.3 MiRNAs -494, -205, -21 and -126 in invasive breast carcinoma (IV)

5.3.1 Expression levels of miRNAs -494, -205, -21 and -126

Acceptable intensity of U6 was observed where intensities of 2+ (67% of all carcinoma specimens) and 3+ (33% of all carcinoma specimen) were considered to ensure sufficient quality of RNA. For both fresh and archival patient material, only specimens with an acceptable U6 level were included in the study. This meant that 42% of the tissue specimens were excluded from the clinical and prognostic analyses based on unacceptable U6. Among *Her2*-amplified U6 showed intensities of 2+ (66%) and 3+ (34%), among luminal - 2+ (63%) and 3+ (37%) and among TNBC - intensities 2+ (79%) and 3+ (21%).

5.3.1.1 MiRNA-494

In benign breast tissue (n=14), miRNA-494 was expressed with varying intensities in all normal cell types, including epithelial, mesenchymal and inflammatory cells (Fig 12).

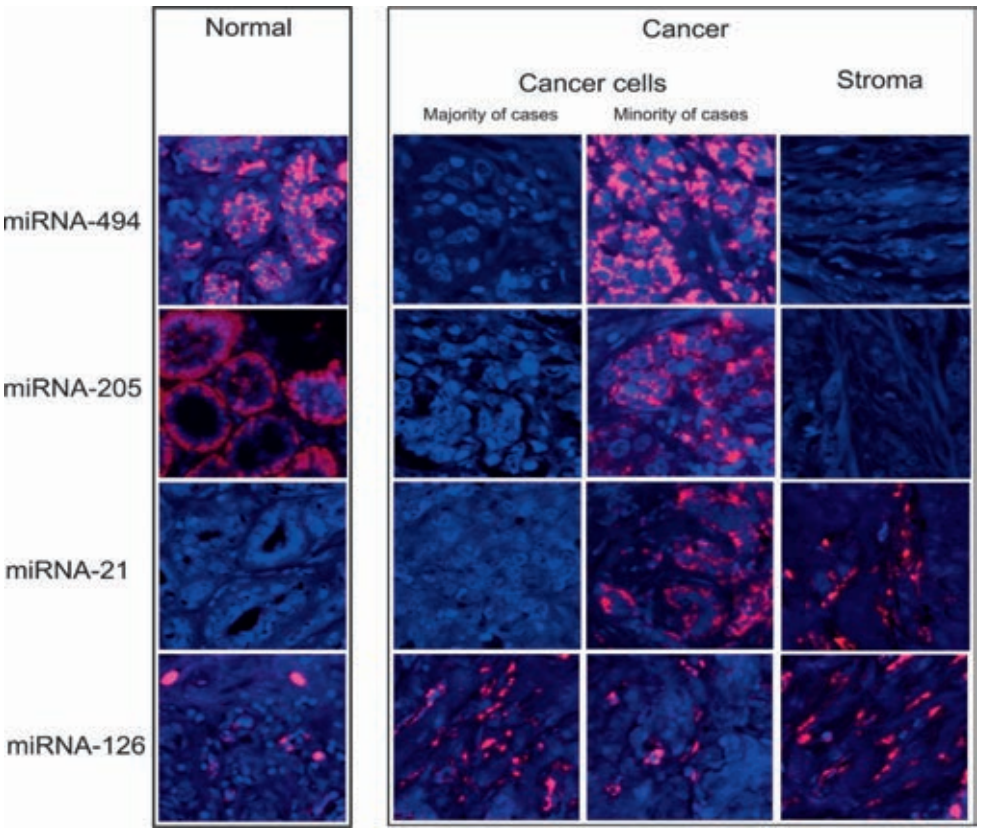

Figure 12. Expression patterns of miRNAs in breast cancer.

In breast carcinomas (n=245), the majority of cases were miRNA-494-negative (69.8%). No associations were detected between miRNA-494-expression and the morphological and clinical features of breast cancer diagnostics, including axillary lymph node status, tumor size, grade or proliferative activity of the tumors. Due to the heterogeneous expression of miRNA-494 in TNBC, a more detailed evaluation of cancer cells areas/populations was performed allowing identification of tumor foci with sub-total or total loss of expression of miRNA-494. This approach revealed that approximately two thirds (67.5%) of TNBC patients showed at least partial loss of miRNA-494. Among the clinico-pathological features including axillary lymph node status, tumor size, grade or proliferative activity of the tumors miRNA-494-negativity in TNBC showed association with tumor size (Fisher's exact test 0.03) but not with nodal status.

5.3.1.2 MiRNAs -205, -21 and -126

In benign breast tissue (n=15), miRNA-205 was positive in basal epithelial/myoepithelial cells of normal mammary ducts, but negative in luminal epithelial cells of normal breast tissue (Fig 12), conversely in breast carcinomas, miRNA-205 was negative in the great majority of cases. The few cases showing miRNA-205-positivity comprised 9 of the studied 203 carcinomas and represented luminal and *Her2*-amplified subtypes of the intrinsic classification (6% and 3% of carcinomas, respectively). No miRNA-205 expression was observed in TNBC.

In benign breast tissue (n=15), miRNA-21 was negative in epithelial and occasionally positive in single stromal cells (Fig. 12), while in breast carcinomas miRNA-21 was variably expressed in malignant epithelial cells and cancer-associated fibroblast-like cells of breast carcinoma. No difference in expression of miRNA-21 was detected between different intrinsic subtypes of breast carcinomas.

In benign (n=14) and malignant breast tissue, miRNA-126 was observed in endothelial cells of both benign and malignant specimen.

5.3.2 Prognostic associations of miRNA-494

Among node-negative patients, MINRA-494-negativity predicted an 8.5-fold increased risk of breast cancer-specific mortality ($p = 0.04$, CI 1.1–62.5) (n=126) (Fig. 13). Similar trends for prognostic value was observed among low grade (I – II) carcinomas (n= 172) although the associations sparsely failed to show statistical significance (p=0.007). Among breast carcinomas subdivided according to the intrinsic classification, miRNA-494 did not show statistically significant prognostic associations.

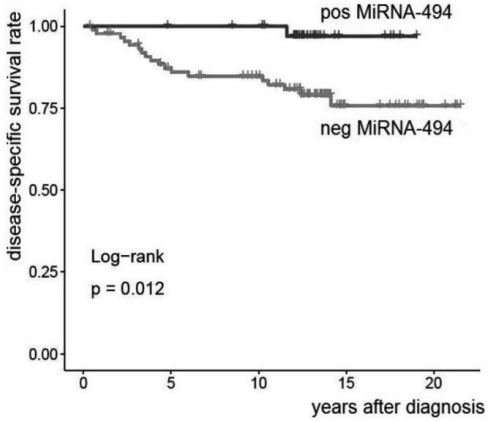

Figure 13. Summary of Kaplan-Meier analysis showing survival of patients with positive vs negative miRNA-494.

In multivariate analysis involving nodal status, intrinsic classification and Ki-67 among all breast carcinomas ($n = 166$), the miRNA-494-negativity showed an independent prognostic value ($p = 0.04$, HR 2.1, CI 1.0–4.1). However, when tumor size (≤ 20 vs > 20 mm) and/or histological grade (grades I–II vs III) were included in the analysis, miRNA-494 scarcely lost its independent prognostic power. In multivariate analysis among node-negative patients, miRNA-494-negativity ($n = 97$) was an independent prognosticator for breast cancer death even when the analysis was repeated for patient subgroups of the intrinsic classification ($p = 0.03$, HR 10.1 CI 1.3–76.9) and small (<2cm in diameter) vs large (≥2cm in diameter) tumor size ($p = 0.03$, HR 9.7, CI 1.2–76.9).

6. DISCUSSION

6.1. Regulation of metaphase-anaphase transition in breast cancer

In malignancy, some of the most fundamental dysfunctional traits involve deregulated cell growth caused by sustained and uncontrolled cell proliferation. During mitosis, the metaphase-anaphase transition is among the most vulnerable steps where failure of alignment of even a single chromosome can prevent the cell from initiating anaphase. As a result, the cell may either degenerate without completing the division or, if the checkpoint mechanism is overridden, cell division may occur regardless of the chromosome error (Malumbres and Barbacid, 2007). Over the last decades, cell cycle regulation has been extensively investigated in several types of malignancies but the mechanisms governing metaphase-anaphase transition in cancer cells are still not completely understood (Maiato et al., 2017).

In this study, Securin, Pttg1IP and Separase, and miRNAs -494 and -205 were examined in human breast cancer. The expression patterns of the studied proteins have not previously been systematically described in breast carcinomas and the existing reports include inconsistent and often controversial descriptions of their expression patterns and, consequently, on their biological roles in malignancy. Similarly, the studied miRNAs -have not either been previously thoroughly investigated in human breast cancer. This study also reports on the prognostic associations of the proteins and miRNAs in breast cancer, for the first time using comprehensive and consistent patient material (n=349 - 497 in papers I-IV) with an up to 22-year follow-up. The value of a long–term follow-up in the prognostic evaluations is emphasized as breast cancer is known to be a disease with unexpected late recurrences (Sestak and Cuzick, 2015). In evaluating the expression patterns and prognostic associations of the studied proteins and miRNAs, special emphasis is placed on adequate histopathological detection methods. The presented results are partly obtained from new methods developed for triple IF and automated ISH and, throughout the investigations, the focus is on the quality of the applied histological techniques.

6.2 Securin, Pttg1IP and Separase in diagnosis and prognosis of breast carcinoma (I, II, III)

6.2.1. Expressions of Securin and Pttg1IP in breast cancer (I, II)

Previously, the localizations of Securin immunoexpression in different cellular compartments have been inconsistently described resulting in contradictory interpretations on the biological roles of subcellular Securin expression. Based on observations from cell cultures as well as from tissue specimen of human neoplasms,

Securin has been described as nuclear (Vlotides et al., 2007), cytoplasmic (Xu et al., 2016b, Zhang et al., 2014b, Ghayad et al., 2009, Lin et al., 2013, Wen et al., 2004) or, occasionally, even membranous (Bacac et al., 2011). Commonly, a combined expression in both nuclear and cytoplasmic locations has been described in different types of carcinomas (Li et al., 2015, Salehi et al., 2013, Piercontiet al., 2015, Wei et al., 2015, Ito et al., 2008, Ramirez et al., 2012). In the present research, breast carcinoma cells showed a variable combination of nuclear and cytoplasmic Securin expression which associated with the subtypes and different outcomes of the disease. In our material, among the low grade carcinomas of the luminal subtype a combination of nuclear and cytoplasmic Securin expression was observed whereas in high grade carcinomas of *Her2*-amplified and TNBC subtypes cytoplasmic expression dominated. Benign breast epithelium showed only nuclear Securin.

According to present knowledge, the main role of Pttg1IP in metaphase-anaphase transition is to facilitate the shift of Securin from the cell cytoplasm to the nucleus, consequently, promoting the activation of Securin in inducing anaphase initiation (Chien et al., 2000). This interpretation originates from observations in a small cohort of colon carcinomas where Pttg1IP was reported to promote nuclearization of Securin (Read et al., 2016). Similar conclusions can be drawn from the present results although the findings are not supported by functional evidence from *in vitro* experiments. In this research, both morphological and statistical approaches show an association between the lack of Pttg1IP expression and cytoplasmic localization of Securin, favoring the interpretation that Pttg1 promotes nuclearization of Securin in breast cancer cells.

According to the literature, the subcellular localization may be decisive for the activity and biological functions of several proteins (Liang et al., 2015). Concerning Securin, it has been speculated that cytoplasmic expression participates in regulation of translation (Pei 2000), whereas nuclear Securin has been suggested with roles in sister chromatid separation and, as a transcription factor, more generally in cell cycle regulation and cell metabolism (Yu et al., 2000, Tong et al., 2009). However, it is known that the subcellular localization of proteins is regulated by complex networks of interactions and post-translational modifications (Larance et al., 2015). Many of these mechanisms, such as deregulated phosphorylation, have also been suggested to manipulate the subcellular localization of Securin (Pei, 2000, Gil-Bernabé et al., 2006).

6.2.2 Prognostic applicability of Securin and Pttg1IP in breast cancer (I, II)

Previously, the prognostic value of Securin has been well documented in several malignancies, e.g. in colorectal (Ren and Jin, 2017), non-small cell lung (Wang et al., 2016b), gastric (Xu et al., 2016b) and clear cell renal carcinomas (Wondergem et al., 2012). In breast carcinomas, Securin has been identified as an independent prognosticator of unfavorable outcome through its role as a promoter of cancer cell

proliferation (Hatcher et al., 2014). In the present investigations (I, II, III) and in previous studies from the same patient material (Karra et al., 2012 and 2014), high expression of Securin in cancer cells serves as an independent predictor of breast cancer-specific mortality ($p < 0.001$).

The role of Pttg1IP in progression and prognosis of human malignancies has been introduced relatively recently and, thus far, there are no previous reports on its prognostic impact in breast carcinomas (Watkins et al., 2010). The prognostic value of Pttg1IP was first identified in thyroid carcinomas (Hsueh et al., 2013, Read et al., 2017) and, more recently, in colorectal carcinomas (Read et al., 2016). In our material of breast carcinomas, Pttg1IP -negativity was associated with a slightly increased risk of disease-specific mortality (HR 1.5, p = 0.002, 95%CI 0.9-2.7) although the prognostic impact of Securin still exceeded that of Pttg1IP.

Previously, the combined prognostic impact of Pttg1IP associated with the subcellular localization of Securin has not been thoroughly investigated. Results from gastric adenocarcinomas (Xu et al., 2016b) and esophageal squamous cell carcinomas (Ito et al., 2008) suggest that cytoplasmic – rather than nuclear - Securin predicts aggressive course of disease and poor outcome. Correspondingly, the present results indicate that loss of Pttg1IP and the associated cytoplasmic localization of Securin intensify the prognostic impact of Securin alone and predict increased mortality among all breast cancer patients and, particularly, among patients with TNBC ($p = 0.003$). Based on these findings, we suggest that, in addition to high expression, cytoplasmic localization of Securin may aid in identifying breast carcinoma patients with an aggressive disease and increased risk of cancer mortality. In particular, the extent and localization of Securin expression may intensify prognostic evaluations for TNBC, a specific treatment challenge due to the limited therapeutic options available. Indeed, Securin has recently been implicated as having therapeutic potential in specific types of malignancy (Chen et al., 2018).

6.2.3 Expression of Separase in breast cancer (III)

In normal cell division, Separase functions in cleaving Cohesin and, consequently, triggering anaphase under the regulation of APC/C (Primorac and Musacchio, 2013). Previously, the normal cellular functions of Separase in mitotic and meiotic divisions as well as its dysfunctional roles in malignant transformation and in inducing aneuploidy and CIN have been thoroughly described (Uhlmann et al., 2000, Zhang and Pati 2017). Similarly, the various expression patterns of Separase have been addressed and reported to reflect a variety of biological roles in oncogenesis (Zhang and Pati, 2017). In previous literature, only nuclear expression of Separase has been described in human carcinomas, including breast cancer (Meyer et al., 2009). In TNBC, it has been speculated that misregulation of sister chromatid segregation by hyperactive Separase may induce CIN and aneuploidy (Yadav et al., 2015, Mukherjee et al., 2014b, Al Ejeh et al., 2014).

According to previous literature, Separase is critically regulated by CyclinB1 and Securin in metaphase-anaphase transition (Chang and Barford, 2014). Moreover, the present findings from double and triple IF stainings suggest specific interactive regulation between Separase, Securin and CyclinB1. Consequently, in the majority of breast carcinomas, Separase-positivity co-expressed with CyclinB1 but not Securin, a finding consistent with the present general understanding of normal Separase-dependent cell cycle control (Chang and Barford, 2014). However, in some breast cancer cases, an aberrant expression pattern with either simultaneous expression of all three markers or Separase-expression alone was observed. These cases with aberrant expression represented high grade and TNBC carcinomas. It remains for further investigations to confirm whether, among aggressive breast carcinomas, Separase may exhibit an independent role in driving proliferation, thus predisposing the cell to aneuploidy and CIN.

6.2.4 Prognostic applicability of Separase in breast cancer (III)

In our patient material, the observation of Separase-positive mitotic cells was associated with a doubled risk of breast cancer mortality (p=0.0004). Furthermore, a lack of diffuse Separase-expression predicted an almost doubled (1.8-fold) risk of breast cancer death (p=0.0006). In addition to all breast cancer patients, prognostic impact was also demonstrated among luminal breast carcinomas for both mitotic (HR 1.3, p=0.02) and diffuse Separase expression (HR 2.9, p<0.0001). Additionally, in multivariate analyses, both mitotic Separase and loss of diffuse Separase showed independent prognostic value over the traditional clinical prognosticators, nodal status and tumor size.

In previous literature, there are no reports on the prognostic value of Separase in human breast cancer. Nevertheless, RNA levels of ESPL1 have been associated with metastatic spread in breast cancer (Meyer et al., 2009, Finetti et al., 2014). However, overexpression of Separase has been demonstrated in breast carcinomas as well as in several other types of human malignancies, such as colorectal carcinomas, osteosarcomas, glioblastomas and chronic myeloid leukemias (Zhang and Pati, 2017) and, in some of these malignancies, Separase has also been identified as having prognostic value (Meyer et al., 2009, Mukherjee et al., 2014a). Moreover, ESPL1 gene expression as evaluated in a meta-selection, has been associated with carcinogenesis and prognosis in several solid tumors (Rouam et al., 2010).

In the present investigations, Separase-IHC appeared to be a promising marker of cancer cell proliferation with potential for clinical applications detected both manually and automatically (Ståhlhammar et al. 2018, Robertson et al. 2018b). Due to the oncogenic functions of Separase in human cancer cells, attempts are being made to modulate its activity for use as a therapeutic strategy (Do et al., 2016). According to resent research, therapeutic options utilizing Separase inhibitors, as well as Securin as an immunotherapeutic target (Mirandola et al., 2015, Demeure et al., 2013), could constitute new strategies for Separase and Securin –overexpressing aneuploid

tumors with resistance to traditional oncological treatments. However, anti-mitotic drugs exploiting microtubule dynamics and interfering with the mitotic spindle have been described as a double-edged sword (Haschka et al., 2018). On the one hand, they could impair the vitality of cancer cells by inducing aneuploidy; while on the other hand, they might promote selection of the most aggressive cancer cell populations which could lead to tolerance of aneuploidy and increased cell survival – both features posing major challenges for safe clinical applications.

6.3. MiRNAs -494 and -205 in diagnosis and prognosis of breast cancer (IV)

6.3.1 Expression of miRNA-494 in breast cancer

In the present results, miRNA-494 was expressed with varying intensity in all normal cell types of the human breast. In breast carcinomas, miRNA-494 was detected in 30.2% of specimen, the negative cases representing the subtypes of luminal and *Her2*-amplified carcinomas. Previously, miRNA-494 has been reported as being significantly downregulated in cell lines and tissues of epithelial ovarian (Yang et al., 2017) and cervical carcinomas (Chen et al., 2015) but overexpressed in colorectal carcinomas (Sun et al., 2014). The few reports on the association of miRNA-494 and proliferation are equally controversial since miRNA-494 has been reported to promote cancer cell proliferation in the cell lines of cervical carcinoma (Yang et al., 2015) as well as suppress proliferation and inducing cancer cell senescence in lung carcinoma cells (Ohdaira et al., 2012).

6.3.2 Prognostic applicability of miRNA-494 in breast cancer

Thus far, the present study is the first to report on the prognostic impact of miRNA-494 in comprehensive well-characterized materials of breast carcinoma patients with a long-term follow-up. The existing evidence on the prognostic value of miRNA-494 in malignancy is incoherent and, in part, controversial. In previous literature, an unfavorable outcome for the disease has been associated with reduced levels of miRNA-494 in epithelial ovarian, pancreatic and gastric carcinomas (Yang et al., 2017, Ma et al., 2015, He et al., 2014) and increased levels of miRNA-494 in sets of colorectal, breast and ovarian carcinomas (Sun et al., 2014, Marino et al., 2014, Yang et al., 2015). The observed discrepant prognostic associations may partly be explained by the small and heterogeneous patient cohorts in the previous papers (n-values ranging from 64 for breast carcinomas to 247 for colorectal carcinomas). However, the manifold and partly contradictory biological roles of miRNAs may also be reflected in the incoherence of the results.

In the present study, decreased levels of miRNA-494 predicted an aggressive course of disease and reduced survival time, so that even 20 years after from diagnosis a clear survival difference could be observed between subgroups of patients with

negative versus positive miRNA-494. In addition, miRNA-494-positivity could provide a means to further intensify the prognostic evaluations among node-negative breast cancer, predicting an 8.5-fold increased risk of disease-specific mortality ($p = 0.04$, 95%CI 1.1–62.5) among this patient subgroup traditionally associated with favorable course for the disease.

Recent research has revealed miRNAs with potential roles in individualized treatment decisions in breast cancer (Dong et al., 2013). An overall decrease in miRNA expression has been demonstrated in advanced as opposed to local breast carcinomas, and benign breast tissue (Dvinge et al., 2013). Based on their roles in the regulation of cell differentiation and division, down-regulation of certain miRNAs may be crucially involved in tumor formation and progression (Lu et al., 2005). Despite the valuable information which miRNA expression profiling has provided on the regulation of gene expression in several malignancies, a common limitation of this line of research is the inconsistency of results within and between cancer types. Recently, there have been attempts to tackle this challenge with the help of microarray platforms simultaneously profiling a large set of miRNAs (Adhami et al., 2018). An optimal miRNA signature could provide a valuable means to detect prognostic and predictive markers in both tissue specimen and serum of breast cancer patients (Thakur et al., 2016, Kodahl et al., 2014, Pourteimoor et al., 2018). In addition, circulating tumor miRNAs could, in the future, be developed into diagnostic support and screening methods for detection of breast cancer at an early stage (Shimomura et al., 2016).

6.3.3. Expression of miRNA-205 in breast cancer

Previous literature provides inconsistent and partly controversial data on the complex biological roles of miRNA-205 in human tumors (Zhang et al., 2015b). According to the present general understanding, miRNA-205 exhibits the characteristics of a tumor suppressor (Hulf et al., 2013, Hou et al., 2013, Hagman 2013, Hanna et al., 2012, Childs et al., 2009) although others have also identified it as an oncogene (Kalogirou et al., 2013, Karaayvaz et al., 2013).

Based on PCR, reduced levels of miRNA-205 have been observed in breast carcinomas as compared to benign breast tissue (Song et al., 2015). However, controversial observations prevail on the expression miRNA-205 in breast carcinomas. Both up- and down-regulation of miRNA-205 has been suggested for the different breast cancer subtypes of the intrinsic classification (Greene et al., 2010). In our material, miRNA-205 was not expressed at all in the great majority of breast cancer cases. However, single cases of luminal and *Her2*-amplified carcinomas showed observable levels of miRNA-205. Similar to our results, previous literature has emphasized the heterogeneity of miRNA-205 in breast carcinoma, reporting increased levels in all intrinsic subtypes (Greene et al., 2010).

6.3.4 Prognostic applicability of miRNA-205 in breast cancer

A meta-analysis of 4827 cancer patients, suggested that miRNA-205 is a promising biomarker for predicting the recurrence and progression of patients with adenocarcinomas, including breast carcinomas (Zhang et al., 2015b). Moreover, loss of miRNA-205 has been associated with progression of breast cancer (Markou et al., 2014), including a reduced overall and metastasis-free survival (Quesne et al., 2012, Huo et al., 2016). In previous research (Quesne et al., 2012, Sempere et al., 2007) as well as in the present results, however, the small numbers of cases with aberrant expression levels do not allow speculation on the biological roles or possible prognostic associations of miRNA-205. Despite this, in some other types of malignancies, such as in carcinomas of the prostate (Hulf et al., 2013, Osipov et al., 2016) and endometrium (Wilczyński et al., 2016), as well as in non-small cell lung carcinomas (Duan et al., 2017) and gliomas (Yue et al., 2016) miRNA-205 has been suggested as a prognostic biomarker, also with potential in cancer management (Hulf et al., 2013).

6.4 Developing histological methods for detecting proteins and miRNAs in breast cancer specimen

6.4.1 IHC and IF method

One of the main challenges in the IF method is managing cross-reactions when detecting antibodies produced in the same species of animals. In this study, one aim was to develop and test protocols to manage cross-reactions in double and triple IF stainings. At the moment, there is no universally applicable method available for elution/denaturation of antibodies of the same species although previous literature introduces several approaches and their limitations to solve this problem (Osman et al., 2013, Nakata and Suzuki, 2012, Chan et al., 2011, Krenacs et al., 2010, Pirici et al., 2009, Tornehave et al., 2000).

In the present study, we tested some physical pretreatments, including temperature and pH, as well as chemical agents, such as detergents, to overcome the problem of cross-reactions. These experiments were performed using the antibodies Ki-67 to demonstrate proliferation and Caspase-3 to stain apoptosis because these markers should not show co-expression in the same cells. Obviously, the use of only two antibodies in the experiments calls for caution when making conclusions on this result. However, some tested methodological variations showed promising results e.g. the commercial acidic Riboclear-solution (www.ventana.com, 2014) combined with heat treatment for detection of Ki-67. In previous literature, the efficiency of microwave heating for elimination of antibodies has been considered controversial (Bauer et al., 2001, Ikeda et al., 2011, Lan et al., 1995). Elution using 2-mercaptoethanol/SDS also appeared a fairly efficient method and has been recommended for many antibodies although some reports suggest that the

conditions required for successful elution may depend on the affinity of the antibody (Gendusa et al., 2014). As expected, the evaluation of the results was often hampered by background fluorescence caused by SDS, although Triton-X 100 was found to partly decrease the problem (Lee et al., 1978).

In conclusion, immediate fixation for 24 hours produced the optimal quality of Securin-IHC in breast carcinomas. In our observations, Securin-IHC could be reliably evaluated in malignant and benign specimen even after a 24-hour delay but not after a 72-hour delay prior to fixation. In multiple IF stainings, no efficient pretreatment could be identified for avoiding cross-reactions between antibodies produced in the same species. To summarize from the experiences in the present study, the only successful solution to manage this problem was using antibodies from the same species but representing different immunoglobulin subtypes and, thus, allowing application of two different secondary antibodies.

6.4.2 ISH method

Among the numerous ISH methods applied in different approaches of medical research, this study presents the development of an ISH method applicable for detecting miRNAs. Previous literature describes numerous methodological variations for miRNA detection but the present study is among the few reporting on results derived from fully automatized ISH (MacKenzie et al., 2014, Sempere, 2014, Singh et al., 2014). Based on literature, in other methods than *in situ* hybridization the detected miRNA expressions vary greatly depending on the applied detection method, emphasizing the impact of methodological aspects on the interpretations of the expression patterns and conclusions on the biological functions of miRNAs. The advantage of the applied automatized ISH method applied in this study is the possibility to observe the expressions of miRNAs under visual control in different locations, cell types and subcellular structures on the tissue sections (Chugh and Dittmer 2012, Pritchard et al., 2012, Urbanek et al., 2015).

To a great degree, the ISH procedure is highly standardized, including the gold standard of using protease pretreatment as opposed to heat treatments in pH buffers applied as a standard procedure in IHC. In the present study, however, we elaborated on the methods of detection and observed that only the most sensitive detection method available for ISH could provide an acceptable miRNA signal. For U6, no amplification of the signal was applied since the evaluation involved semi-quantitative registration of U6 expression levels consistently for the whole material of breast cancer specimens. Instead, when evaluating the studied miRNAs, amplification of the signal was performed and, thereafter, evaluations were performed consistently for each miRNA producing congruent and comparable registration for each specimen in the whole tissue material.

In the present study, only specimens with acceptable U6 signal qualified for analysis, resulting in the exclusion of 42% of the tissue cores of the studied patient material.

This finding emphasizes the importance of the quality of the tissue specimen in detecting miRNAs and also calls for critical evaluation of conclusions drawn from previous literature. It is well known that features of tissue processing may cause considerable variations in the results, also demonstrated in the present research by testing different conditions of tissue processing. According to our findings, particularly a cold ischemia time considerably influences conservation of miRNA. Concluding from U6 detections, up to a 2-day delay from removal of the surgical specimen to the initiation of formalin fixation will result in an acceptable quality of miRNA in the breast cancer tissue. However, in large surgical resections, the penetration of fixative to the tumor may be unpredictable and compromise the integrity of the miRNA. Contrary to previous evidence (Singh et al., 2014), in our observations the duration of fixation did not hamper conservation of miRNA. As a final conclusion, the developed automatized ISH method for miRNA detection is well applicable to research purposes providing that the fixation procedure is optimized for different types of tissues and specimens.

6.4.3 Tissue processing

Despite its universal acceptance both in clinical and research approaches, the usability of FFPE material varies greatly depending on the detailed practices of fixation and tissue processing (Engel and Moore, 2011). There is ample evidence that numerous sources of variation in the preanalytical methods within and between individual laboratories – including the type of the specimen, fixation, tissue processing and, finally, archival storing of specimen range - result in variable and often unexpected impact on the detection of different biomarkers (Table 12) (Vaught and Lockhart, 2012, Shabinkhani et al., 2014).

The complexity of the challenges faced by translational research is illustrated in the variety of preanalytical sources of variation complicating assessment of biomarkers within a tissue specimen (Agrawal et al., 2017). Although tissue fixation is known to be decisive for the application of histological techniques, it is commonly not possible to control the treatment of the specimen prior to fixation. Even in the optimal situation where the exposure to fixative is standardized, numerous variations in fixatives and their application methods vary between laboratories. Many of these variations may, to a certain extent, be compensated by the application of antigen retrieval procedures which methods; however, these methods are not standardized between laboratories either. These uncontrollable preanalytical sources of variation in histological techniques make it difficult to produce reproducible results and perform meaningful comparisons between different biomarkers detected in different research laboratories. (O'Leary, 2001, Leong and Leong, 2011). Even experience from clinical pathology indicates, variation in detection of routine biomarkers due to preanalytical variations. For example, IHC for ER and PR may be inaccurate in up to 20% cancer specimen worldwide (Wolff et al., 2007, Hammond et al., 2010). It has been reported that false negative rates of ER-IHC increase already starting from 24 hours after collection (Nkoy et al., 2010) whereas other biomarkers may be affected even after a

considerably shorter, cold ischemia time 5 to 20 minutes (Mertins et al., 2014, Ugner et al., 2016). The pre-analytical and analytical sources of variation of histological methods in translational research need to be considered in order to develop new methods applicable for validation in clinical settings (Nykänen and Kuopio, 2010).

Table 12. Examples of preanalytical sources of variation and suggested possible solutions to be considered when applying histopathological methods in translational research.

Phase	Source of variation
Prefixation	Duration of cold ischemia
Fixative	Formula
	Concentration
	pH
Fixation	Specimen size
	Volume ratio
	Duration
Processing	Dehydration and clearing
	Paraffin impregnation
	Frequency of cutting
Archival storage	Duration

In treatment of breast cancer patients, most international and national guidelines present recommendations for treatment of tissue materials prepared for morphology, IHC and ISH (Hammond et al., 2010, Fitzgibbons et al., 2017, Leyland-Jones et al., 2008, Cheung et al., 2014). In addition, molecular testing and multigene profiling have become increasingly valuable sources of predictive and prognostic information in breast cancer with separate technical challenges in retrieving high-quality RNA (Koz and Dabbs 2016, Desmedt et al., 2008). Individual treatment of breast cancer patients today, in addition to the clinical characteristics of the patient and tumor, is based on detection of biomarkers of the intrinsic classification has provided guidelines for more accurate prediction of treatment responces, thus, considerably improving patient outcome (Petit et al., 2004). The current treatment recommendations have been obtained from research on biomarkers and patient outcomes from meta-analyses of large patient cohorts a long-term follow-up. The FFPE samples are currently the most widely available source of tissue material for this purpose. The above described methodological challenges of breast cancer prognostication emphasize the importance mastering the histological techniques and combining the obtained results with expertise in biological, morphological and clinical understanding of breast cancer. Standardization of tissue handling and processing are critical prerequisites for development and clinical applications of new prognostic and therapeutic biomarkers (Chung et al., 2018).

7. CONCLUSIONS

The present study reports on the expression patterns and prognostic impacts of a set of proteins involved in the metaphase-anaphase transition and a set of proliferation-associated miRNAs. Securin, Pttg1IP and Separase were detected with the help of IHC and double and triple IFs, and miRNAs -494, -205, -126 and -21 using an automatized ISH procedure. The results were based on patient materials comprising a maximum of 447 invasive breast carcinomas and up to 143 TNBCs with complete clinical data and a maximum follow-up period of 22 years. The specific conclusions of this thesis are:

1. The expression patterns and subcellular localizations of Securin, Pttg1IP and Separase in breast carcinoma cells differ from benign breast epithelium. In addition, the extent and the subcellular localization of the proteins differ between cases of breast carcinomas dependent on the aggressivity of the tumor. Particularly, the observed association between loss of Pttg1IP and sytoplasmic localization of Securin supports previous research on the role of Pttg1IP in regulating nuclearization and activity of Securin. Over all, the findings support the understanding that Securin, Pttg1IP and Separase play a role in in breast cancer progression.
2. Securin, Pttg1IP and Separase are strong prognosticators of breast cancer survival. Securin and Separase also show independent prognostic value along with nodal status and tumor size. The results suggest that Securin and Separase might be developed into clinically applicable prognostic features for breast cancer. In addition, Separase is a promising proliferation marker with practical and prognostic value over PHH3 and Ki-67.
3. The levels of miRNAs -494 and -205 in breast carcinoma cells differ from benign breast epithelium. In addition, specific breast cancer subgroups can be identified based on divergent levels of miRNAs -494 and -205. In breast cancer, sustained level of miRNA-494 is observed in subgroups of aggressive clinical features, supporting the part of previous research presenting miRNA-494 with a role in promoting cancer cell proliferation.
4. Decreased levels of miRNA-494 predict an unfavorable outcome of breast cancer, particularly among patients with a node-negative disease.
5. Several preanalytical sources of variation in the histotechnical process influence the reliability of detecting proteins and miRNAs. Particularly, cold-ischemia time comprises a challenge for both IHC and ISH, suggesting that special caution should be taken when conducting research and when making conclusions from previous literature utilizing FFPE materials.

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