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PROGNOSTIC FACTORS IN BREAST CANCER

With Special Reference to Cyclins A, B1, D1 and E, MMP-1 and Decorin

Pia Boström

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To my family

ABSTRACT

Pia Boström
PROGNOSTIC FACTORS IN BREAST CANCER
With Special Reference to Cyclins A, B1, D1 and E, MMP-1 and Decorin

Department of Pathology, University of Turku, Turku, Finland (2014)

Breast cancer is a highly heterogenous malignancy, which despite of the similar histological type shows different clinical behaviour and response to therapy. Prognostic factors are used to estimate the risk for recurrence and the likelihood of treatment effectiveness. Because breast cancer is one of the most common causes of cancer death in women worldwide, identification of new prognostic markers are needed to develop more specific and targeted therapies.

Cancer is caused by uncontrolled cell proliferation. The cell cycle is controlled by specific proteins, which are known as cyclins. They function at important checkpoints by activating cyclin-dependent kinase enzymes. Overexpression of different cyclins has been linked to several cancer types and altered expression of cyclins A, B1, D1 and E has been associated with poor survival. Little is known about the combined expression of cyclins in relation to the tumour grade, breast cancer subtype and other known prognostic factors. In this study cyclins A, B1 and E were shown to correlate with histological grade, Ki-67 and HER2 expression. Overexpression of cyclin D1 correlated with receptor status and non-basal breast cancer suggesting that cyclin D1 might be a marker of good prognosis.

Proteolysis in the surrounding tumour stroma is increased during cancer development. Matrix metalloproteinases (MMPs) are proteolytic enzymes that are capable of degrading extracellular matrix proteins. Increased expression and activation of several MMPs have been found in many cancers and MMPs appear to be important regulators of invasion and metastasis. In this study MMP-1 expression was analysed in breast cancer epithelial cells and in cancer associated stromal cells. MMP-1 expression by breast cancer epithelial cells was found to carry an independent prognostic value as did Ki-67 and bcl-2. The results suggest that in addition to stromal cells MMP-1 expression in tumour cells control breast cancer progression.

Decorin is a small proteoglycan and an important component of the extracellular matrix. Decorin has been shown to inhibit growth of tumour cells and reduced decorin expression is associated with a poor prognosis in several cancer types. There has been some suspicion wheather different cancer cells express decorin. In this study decorin expression was shown to localize only in the cells of the original stroma, while breast cancer epithelial cells were negative for decorin expression. However, transduction of decorin in decorin-negative human breast cancer cells markedly modulated the growth pattern of these cells. This study provides evidence that targeted decorin transduction to breast cancer cells could be used as a novel adjuvant therapy in breast malignancies.

KEY WORDS: breast cancer, extracellular matrix, prognostic factor, MMP-1, decorin, cyclin A, B1, D1 and E

TIIVISTELMÄ

Pia Boström
Rintasyövän ennusteelliset tekijät
– erityisesti sykliinit A, B1, D1 ja E, MMP-1 ja dekoriini

Patologian oppiaine, Kliinis-teoreettinen laitos, Turun yliopisto, Turku (2014)

Rintasyöpä, joka on naisten yleisin syöpä, on äärimmäisen monimuotoinen sairaus. Rintasyövän on ymmärretty olevan joukko erilaisia kasvaimia, jotka poikkeavat toisistaan huomattavasti morfologialtaan, ennusteeltaan ja hoitoon reagoinniltaan. Rintasyövän kliinisen kulun ennustamisessa sekä hoitolinjojen valinnassa käytetään apuna prognostisia eli ennusteellisia tekijöitä. Käyttökelpoisten ennusteellisten tekijöiden löytäminen auttaa kohdentamaan aggressiivisemmat hoidot suuremman uusiutumisriskin omaaville potilaille.

Syöpä johtuu kontrolloimattomasta solujen jakautumisesta. Solunjakautuminen tapahtuu solusyklissä, jota säätelevät erilaistuneet proteiinit. Solusyklissä on useita tarkastuspisteitä, joissa säätelyjärjestelmän pääkomponentteina toimivat sykliinit ja sykliiniriippuvaiset proteiinikinaasit. Sykliinien ilmentyminen on poikkeavaa syövässä. Sykliinien A, B1, D1 ja E:n esiintymistä verrattiin rintasyövän erilaistumisasteeseen, molekulaarisiin rintasyövän alatyyppeihin ja käytössä oleviin ennusteellisiin tekijöihin. Tutkimuksessa todettiin sykliinien A, B1 ja E:n korreloivan kasvainkudoksen erilaistumisasteeseen, jakaantumisnopeuteen Ki-67 ja HER2 positiivisuuteen eli rintasyövän huonompaan ennusteeseen. Sykliini D1 sen sijaan korreloi hormonireseptoristatukseen ja ns. ei- basaalityypin rintasyöpään liittyen parempaan ennusteeseen.

Soluväliaineen hajotus on lisääntynyt rintasyövässä. Matriksin metalloproteinaaseiksi (MMP) nimitetään entsyymiperhettä, joka osaltaan vastaa tästä solun ulkoisen väliaineen pilkkomisesta. Näiden proteolyyttisten entsyymien ilmentymisellä saattaa olla ennusteellista arvoa eri syövissä. MMP-entsyymeillä on perinteisesti ajateltu olevan osuutta syöpäsolujen leviämisessä. Tässä tutkimuksessa analysoitiin MMP-1:n ilmentymistä sekä rintasyöpäsoluissa että niitä ympäröivien väliaineen soluissa. MMP-1:n ilmentyminen syöpäsoluissa todettiin olevan uusi itsenäinen syövän uusiutumista ennustava tekijä kuten myös Ki-67 ja bcl-2. Tutkimustulokset osoittavat, että MMP-1 osallistuu syövän säätelyyn sekä tumatasolle että solun ulkopuolella.

Dekoriini on sidekudosväliaineessa esiintyvä proteoglykaani, jolla on kykyä säädellä solujen jakautumista. Pienentynyt dekoriini-ekspressio on liitetty huonoennusteisiin syöpiin. Dekoriinin ilmentymisestä erilaisissa syöpäkudoksissa ei kuitenkaan ole täyttä varmuutta ts. ekspressoivatko varsinaiset syöpäsolut sitä vai eivät. Tutkimuksessa rintasyöpäsolujen ei todettu tuottavan dekoriinia. Dekoriinin saattaminen virusvälitteisesti dekoriini-negatiivisiin rintasyöpäsoluihin sai aikaan merkittäviä muutoksia syöpäsolujen morfologiaan ja kasvunopeuteen. Tutkimustulosten perusteella dekoriinia voisi tulevaisuudessa mahdollisesti hyödyntää rintasyövän hoidossa.

Avainsanat: rintasyöpä, solunulkoinen väliaine, ennustetekijä, matriksin metalloproteinaasi - 1, dekoriini, sykliini A, B1, D1 ja E

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I Boström P, Söderström M, Palokangas T, Vahlberg T, Collan Y, Carpen O, Hirsimäki P: Analysis of cyclins A, B1, D1 and E in breast cancer in relation to tumour grade and other prognostic factors. BMC Res Notes 2009; 2:140.

II Boström P, Söderström M, Vahlberg T, Söderström K-O, Roberts PJ, Carpén O, Hirsimäki P: MMP-1 expression has an independent prognostic value in breast cancer. BMC Cancer 2011; 11:348.

III Boström P, Sainio A, Kakko T, Savontaus M, Söderström M, Järveläinen H: Localization of decorin gene expression in normal human breast tissue and in benign and malignant tumors of the human breast. Histochem Cell Biol 2013; 139:161.

In addition, some unpublished data are included in the thesis. The original publications have been reproduced with the permission of the copyright holders.

ABBREVIATIONS

AR	androgen receptor
ALH	atypical lobular hyperplasia
BRCA	breast cancer gene
CAF	cancer associated fibroblast
CAK	cyclin-dependent kinase (CDK) activating kinase
CCND1	cyclin D1 gene
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase (CDK) inhibitor
CISH	chromogenic in situ hybridization
CI	confidence interval
CK5/6	cytokeratin 5/6
CMV	cytomegalovirus
DCIS	ductal carcinoma in situ
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ER	estrogen receptor
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HR	hazard ratio
HE	haematoxylin and eosin
HER2	human epidermal growth factor receptor 2
HPF	high power field
HGF	hepatocyte growth factor
ICC	intraclass correlation coefficent
ILC	invasive lobular carcinoma
IGF-IR	insulin-like growth factor I receptor
ISH	in situ hybridization
LCIS	lobular carcinoma in situ
LIN	lobular intraepithelial neoplasia
LMW-E	low molecular weight cyclin E

LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
Met	hepatocyte growth factor receptor
MMP	matrix metalloproteinase
MMPIs	matrix metalloproteinase inhibitors
NGS	next-generation sequencing
NST	breast carcinoma of no special type
PAI	plasminogen activator inhibitor 1
PAR	protease-activated receptor
PDCD4	programmed cell death 4
PEG3	paternally expressed gene 3
PP	propeptide
PR	progesterone receptor
PG	proteoglycan
pRb	retinoblastoma protein
RT-qPCR	real-time quantitative polymerase chain reaction
SMI	standardized mitotic index (mitosis/mm^2)
SP	signal peptide
TGF	tumour growth factor
TIMP	tissue inhibitor of matrix metalloproteinase
TMA	tissue microarray
TLR	toll-like receptor
VEGFR	vascular endothelial growth factor receptor
WHO	World Health Organization

1. INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women in Europe and it is the first cause of women´s cancer death in Europe. In Finland in 2011 about 4900 new breast cancers were found. The risk of breast cancer in women has increased continuously and the rate of cancer patients is growing further. The incidence of breast cancer is very low among women under 30 years, but the risk of developing cancer increases after 45 years of age. One in nine women will have breast cancer at some point in her life and one in thirty will die from the disease. Even if the number of new breast cancer cases has multiplied during the past decades, breast cancer survival has improved over time possibly due to early diagnosis by effective screening programs and advances in treatment.

The aim of breast cancer treatment is complete removal of tumour without damage to the rest of the body. Surgery is accomplished by radiotherapy and systemic treatment including adjuvant chemotherapy, hormone therapy and targeted therapy (sometimes called biological therapies). Treatment for breast cancer will depend on a number of factors including the size and the grade of the breast cancer, axillary lymph node status, hormone receptor status and HER2 status. Several well-established prognostic and predictive factors are used to guide the clinical management of breast cancer. Difficulties remain in identifying those patients who are likely to benefit the most from treatment.

Breast cancers are highly heterogenous tumours, which only happen to originate in the same anatomical site. Breast tumours of the similar histological type can show remarkably different clinical behaviour, response to the therapy and prognosis. A prognostic factor is a clinically or biologically measurable variable that correlates with the cancer disease in an untreated patient. A prognostic factor can be thought as a measure of the natural history of the disease.

Cyclins are proteins that function at important checkpoints in cell cycle. Several cancer types have abnormal cyclin expression stimulating the cells to divide too fast. The aim of this study was to analyse the expression of cyclins A, B1, D1 and E in breast cancer in relation to the well-known traditional prognostic factors. In addition, immunohistochemically detected expression of cyclins were correlated with the molecular subtypes of breast cancer.

While stromal density of cancer tissue is known to be a breast cancer risk factor, the other aim was to study the stromal microenvironment. Matrix metalloproteinases are endopeptidases with the ability to cleave a wide range of extracellular matrix components. MMP-1 expression in both stromal and tumour epithelial cells was evaluated in breast cancer and the results were correlated with classical prognostic factors and long follow-up time for cancer specific survival in different breast cancer subtypes.

Decorin, a small leucine-rich proteoglycan, is an important multifunctional molecule of the extracellular matrix. Decorin has been shown to be present in various amounts in the stroma of different cancers. However, identification of the origin of decorin expression in cancers has remained open. In this study we studied decorin mRNA expression in benign breast lesions and various histological types of human breast cancers using in situ hybridization. One additional goal of this study was to evaluate the effect of decorin transduction on the behaviour of cultured human breast cancer MCF7 cells.

2. REVIEW OF THE LITERATURE

2.1 Epidemiology and risk factors of breast cancer

Cancer is one of the leading causes of death worldwide. The main types of cancer are lung, prostate, colorectal and breast cancer (WHO, 2011), the last one being the most common cause of cancer mortality among women worldwide (Chodosh, 2011). The annual number of cancer cases in females is predicted to increase in Finland and breast cancer is responsible for one-third of this increase (Figure 1, Finnish Cancer Registry, 2009).

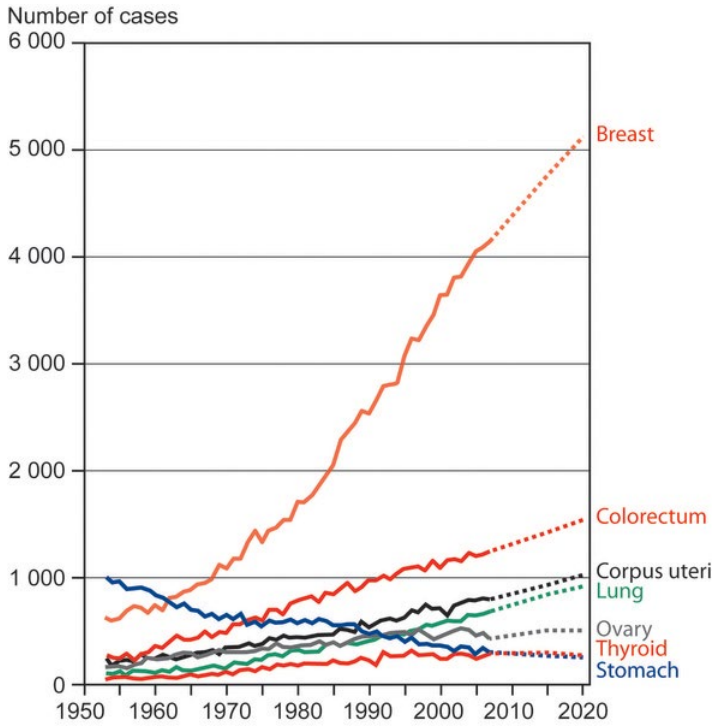

Figure 1. The annual number of cancer cases in females in 2020 is predicted to increase by 3150 cases in Finland. Breast cancer is responsible for one-third of this increase (Finnish Cancer Registry, 2009).

While the amount of new breast cancer cases has increased in many European countries during the past decades, breast cancer mortality has declined or remained stable possibly due to earlier diagnosis and/or improved treatments. Greater public awareness of breast cancer and the promotion of breast self-examination together with effective screening mammography have led the detection of the disease at earlier stages (OECD, 2010).

It has previously been shown that the outcome of breast cancer is significantly better in patients with mammography screen-detected tumours than in patients whose cancer has been found outside of screening (i.e. symptomatic tumours) (Sihto *et al*., 2008; Lehtimäki *et al.,* 2011). In Finland, about nine women out of ten are alive five years after the diagnosis (Figure 2, Finnish Cancer Registry, 2009).

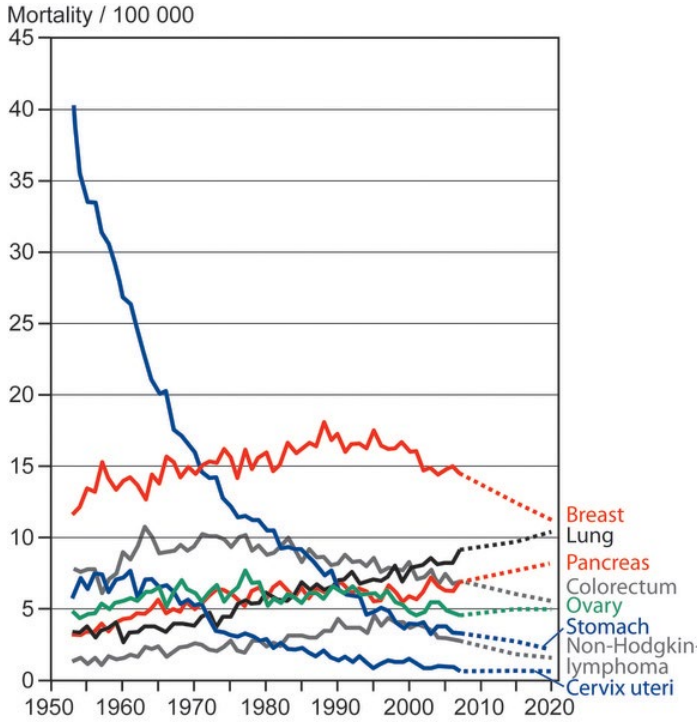

Figure 2. Age-adjusted mortality trends of common sites in Finland, with prediction, females (Finnish Cancer Registry, 2009).

Many risk factors for breast cancer are well known. These include increasing age, family history, young age at menarche, late menopause, overweight after menopause, null parity or late age at first birth, breast density, long-term use of combined estrogen-progestin hormone therapy after menopause, certain types of benign breast diseases and alcohol consumption (Table 1). However, only a small number of women that develop breast cancer carry the above mentioned risk factors (Antonova *et al*., 2011; Endogenous Hormones and Breast Cancer collaborative Group, 2011; Iwasaki and Tsugane, 2011; Stojadinovic *et al.,* 2011). Evidence is growing that physical activity may reduce women´s risk of breast cancer by decreasing body fat and estrogen level (Wu *et al*., 2013), although not all studies show this benefit (Borch *et al*., 2014). High saturated fat intake is reported to increase particularly the risk of hormone receptor-positive breast cancer (Sieri *et al*., 2014). Central body fat distribution has been associated with an increased risk of pre- and postmenopausal breast cancer compared to the risk associated

with a more peripheral distribution of body fat (Amadou *et al*., 2013). High saturated fat is also associated with greater risk of HER2 negative breast cancer (Sieri *et al*., 2014). While most risk factors seem to be common for both estrogen positive and negative tumours (Suba 2014), there are studies that support the hypothesis that breast cancer risk factors vary between the cancer subtypes (Yang *et al*., 2007). Moreover, the stronger the risk factor, the higher the association for poorly differentiated, steroid receptor-negative tumours, such as triple-negative breast cancers is observed (Suba 2014). The most important preventive strategy against breast cancer is suggested to be the strict control of hormonal stability for women during their whole lifetime (Suba 2014).

Table 1. Selected risk factors for invasive breast cancer.

Moderate to high risk factors	**Effect on breast cancer risk**
Ageing	very strong increase in risk
Gender	very strong increase in risk
Lobular carcinoma in situ	7-10 times greater
BRCA1 or *BRCA2* gene mutation	5-14 times greater
Dense breast tissue	3-6 times greater
Family history of breast cancer	
-two immediate family members with breast cancer	3-4 times greater
-mother diagnosed before age 60	2-3 times greater
-mother diagnosed after age 60	1 times greater
Personal history of cancer (including breast cancer, DCIS, other cancers)	2-6 times greater
Benign breast condition (hyperplasia)	
-atypical	2-4 times greater
-usual	1.5-2 times greater

Low risk factors	**Effect on breast cancer risk**
Hormonal therapy use (current or recent use for 5 years)	
-combined estrogen and progestin	1.5-2 times greater
Using oral contraceptives (current or recent use)	1-1.5 times greater
Not having children (compared with a woman who has her first child before 35)	1.5 times greater
Alcohol use (2-4 drinks/day)	1.5 times greater
High socio-economic status	1-2 times greater
Obesity	1-1.5 times greater
Not breastfeeding	1 times greater
Lack of exercise	1 times greater
High bone density	2 times greater
Age 55 or older at menopause	2 times greater
First period before age 12	1-1.5 times greater
Being tall	1 times greater

(Modified from Singletary 2003 and Canadian Breast Cancer Foundation webpages).

About 5% to 10% of all breast cancer cases are estimated to be strongly hereditary (Thull and Vogel, 2004). Breast cancer genes 1 and 2 (*BRCA1* and *BRCA2*) are the most commonly mutated genes, but additional genes associated with the hereditary breast cancer are appearing (Petrucelli *et al.,* 2010) (Table 2). The risk of developing breast cancer of *BRCA1* mutation carriers is around 80% and that of *BRCA2* mutation carriers 26% to 84% (Apostolou and Fostira, 2013). Five percent to 10% of men with *BRCA2* mutations (and a smaller proportion of those with *BRCA1* mutations) develop breast cancer (Ruddy and Winer, 2013). *BRCA1* and *BRCA2* are responsible for approximately 30% of hereditary breast cancer cases worldwide, but only for about 20% in Finland (Pylkäs *et al*, 2008). *BRCA2* mutation has been found to be more common than *BRCA1* mutation in the Finnish population (Syrjäkoski *et al*, 2000). *PALB2* that has an important function in the regulation and localization of *BRCA2* has recently been identified as a breast cancer susceptibility gene (Haanpää *et al*., 2013). *PALB2* c.1592delT founder mutation can be found in about 1% of Finnish breast cancer patients and these patients have been shown to have a 40% increased breast cancer risk by the age of 70 (Haanpää *et al*., 2013). This mutation increases the risk of breast cancer in a way comparable to *BRCA2* mutations (Erkko *et al.,* 2008).

Other inherited cancer genes that predispose to breast cancer include *TP53* mutations in Li-Fraumeni syndrome, *STK11* mutation in Peutz-Jeghers syndrome and *PTEN* mutations in Cowen syndrome (Apostolou and Fostira, 2013*).* Somatic (not-inherited) mutations in the *PIK3CA* oncogene are common in human breast cancer; mutations are observed in 20% to 40% of cases (Cizkova *et al*., 2012). While there is increasing evidence that different mutations play an important role in breast cancer tumourigenesis, most breast carcinomas occur in women with only two non-specific disease-associated risk factors, namely age and female gender (Stojadinovic *et al*., 2011).

Table 2. The most important breast cancer associated genes and risk for breast cancer.

Gene	Name	Risk for mutation carriers for breast cancer %
BRCA1	breast cancer gene 1	80% (Apostolou and Fostira, 2013)
BRCA2	breast cancer gene 2	26-84% (Apostolou and Fostira, 2013)
TP53	tumour protein p53	49% (Masciari *et al*., 2012)
PTEN	phosphatase and tensin gene	25% (Tan *et al*, 2012)
ATM	ataxia-telangiectasia mutated gene	20-40% (Ripperger *et al.,* 2009)
PALB2	partner and localizer of *BRCA2*	35% by 70 years of age (Antoniou *et al*., 2014)
CDH1	E-cadherin	39-52 % risk for lobular breast cancer (Schrader *et al*, 2011)
STK11	serine/threonine kinase 11	32-54% (Apostolou and Fostira, 2013)
CHEK2	checkpoint kinase 2	25% (Cybulski *et al*, 2011)
RAD50	RAD50 homolog	a small risk in Finnish breast cancer families (Tommiska *et al.,* 2006)

2.2 Histopathological classification of breast cancer

Classification of breast tumours is based on histological appearance and growth pattern. According to the WHO classification of invasive breast cancers at least 18 distinct histologic types exist (Ellis *et al*., 2003) (Table 3). The most common type of invasive breast carcinoma (50% to 80%) is called invasive breast carcinoma of no special type (NST), commonly known as ductal carcinoma NST (Ellis *et al*., 2012a). This heterogenous group of adenocarcinoma does not have sufficient characteristics to achieve classification as a specific histological type, such as lobular or tubular carcinoma (Weigelt *et al*., 2010; Ellis *et al.*, 2012b). Morphological features vary considerably from tumour to tumour showing malignant cells invading the stroma diffusely, in trabecular or tubular configurations with extremely variable stromal component in background (Brachtel, 2012).

Table 3. Prevalence of histological types of invasive breast cancer.

Histological type	Prevalence (%) (Rosen, 2001)	Prevalence (%) (Ellis et al., 2003)	Prevalence (%) (Rakha et al., 2006)
Invasive ductal carcinoma (NST)	65-80	50-80	56.4
Carcinoma with osteoclastic giant cells	0.5-1-2		
Invasive lobular carcinoma	5	5-15	8.2
Classical			7.4
Alveolar			0.1
Solid			0.3
Tubulo-lobular			0.4
Pure tubular carcinoma	<2	<2	4.4
Invasive cribriform carcinoma	<4	0.8-3.5	0.6
Medullary carcinoma	<5-7	1-7	2.6
Typical			0.3
Atypical			2.3
Mucinous carcinoma	<2	2	1.4
Neuroendocrine carcinoma		2-5	
Invasive papillary carcinoma	1-2	1-2	0.4
Invas. micropapill. carcinoma	<2.7	<2	
Apocrine carcinoma	<1-4	<4	
Metaplastic carcinoma	<5	<1	
Lipid-rich carcinoma	<1	<1-6	
Secretory carcinoma	few cases	<0.15	
Oncocytic carcinoma		few cases	
Adenoid cystic carcinoma	few cases	0.1	
Acinic-cell carcinoma		few cases	
Glycogen-rich clear cell ca	<1-3	1-3	
Sebaceous carcinoma		few cases	
Mixed cases			25.9
NST and invasive lobular carcinoma			3.3
NST and special type			2.4
Invasive lobular mixed			3.4
Tubular carcinoma mixed			16.8

Invasive lobular carcinoma (ILC) is the most common special type of breast cancer and the second most common type of all breast cancers representing 8% to 15% of all invasive cancers (Rakha *et al*., 2008b; Vandorpe *et al*., 2011). Infiltrating lobular carcinoma has a different pattern of metastatic spread with a tendency for unusual sites such as the retroperitoneum and serosal surfaces (Rakha *et al*., 2011). In its classical form, small non-cohesive tumour cells disperse through a fibrous stroma or infiltrate as individual rows of cells (Indian file pattern) (Brachtel, 2012; Lakhani *et al*., 2012a). The lack of cell cohesion or dissociated cell pattern occurs as a result of alterations in E-cadherin function (Faleiro-Rodrigues *et al*., 2004). In addition to the loss of membranous E-cadherin expression in ILC, simultaneous loss of α, β and γ catenin expression has been observed and it has been shown to be associated with cytoplasmic localization of p120 catenin (Yu *et al*., 2010; Rakha *et al*., 2010a; Brachtel, 2012; Lakhani *et al*., 2012a).

Other, relatively uncommon types of breast cancer, e.g., tubular carcinoma, cribriform carcinoma, carcinoma with medullary features, invasive micropapillary carcinoma, mucinous carcinoma, carcinoma with apocrine differentiation, carcinoma with signet-ring-cell differentiation and metaplastic carcinoma of no special type have particular microscopic features of the tumour cells, growth pattern or extracellular material. Various subtypes of breast carcinoma may indicate the distinct behaviour of breast cancer and, in addition, some subtypes have a lower recurrence risk than would be suggested by their histological grade (Li, 2010a; Rakha and Ellis, 2011; Ellis *et al*., 2012b).

Non-invasive cancer is called carcinoma in situ. It is a neoplastic proliferation characterized by the replacement of the native epithelial cells by atypical cells within the ducts (ductal carcinoma in situ) or lobules (lobular carcinoma in situ) without invasion through the basement membrane into the surrounding stroma. The cancer cells in situ carcinoma can have cytological atypia, different architectural features and a tendency for progression to invasive breast cancer (Schnitt *et al*., 2012). Ductal carcinoma in situ (DCIS) is more common than lobular carcinoma in situ (LCIS) and is divided into three grades on the basis of nuclear features: low, intermediate and high (Schnitt *et al*., 2012). According to architectural pattern DCIS is sub-classified into solid, cribriform, papillary and micropapillary subtypes (Virnig *et al.,* 2010). In addition, the presence or absence of necrosis and the type of necrosis (punctuate, comedo) is often included in pathologists report (Schnitt *et al*., 2012).

Lobular intraepithelial neoplasia (LIN) includes the entire spectrum of so-called atypical lobular hyperplasia (ALH) and LCIS (Lakhani *et al*., 2012b). ALH is diagnosed when fewer than 50% of the acini are involved by the luminal proliferation and no significant distension of the involved acini is noticed. LCIS is diagnosed when more than 50% of the acini of the affected lobular unit are distended and distorted by cellular proliferation (Malley 2010; Lakhani *et al.*, 2012b). Several variants of LCIS have recently been recognized. These include pleomorphic LCIS, pleomorphic apocrine LCIS, LCIS with areas of comedo necrosis and carcinoma in situ with mixed ductal and lobular features.

In addition, clear cell and signet ring cell variants of LCIS have been described (Malley, 2010). The clinical significance of these LCIS variants is still uncertain (Lakhani *et al.*, 2012b).

2.3 Prognostic and predictive factors in breast cancer

A prognostic factor provides information on clinical outcome at the time of surgery, independently of systemic adjuvant therapy. Such factors show the intrinsic biologic characteristics of tumours which are usually indicators for growth, invasion and metastatic potential (Subramaniam and Isaacs, 2005). The classical clinical prognostic factors that are considered to be independent variables in breast cancer include age, axillary lymph node status, tumour size, histopathologic features including tumour type and grade, lymphovascular invasion, expression of proliferation marker Ki-67, estrogen receptor (ER) and progesterone receptor (PR) status (Cianfrocca and Goldstein, 2004; Subramaniam and Isaacs, 2005; Weigel and Dowsett, 2010; Ly *et al*., 2012).

A purely predictive factor is any measurement associated with a positive response to a given therapy, but it does not predict the outcome in untreated patients (Cianfrocca and Goldstein, 2004; Subramaniam and Isaacs, 2005). Certain biologic factors, including ER/PR expression status, expression of Ki-67 and HER2 are both prognostic and predictive factors (Cianfrocca and Goldstein, 2004; Fasching *et al.*, 2011). With the exception of ER or PR expression and HER2 gene amplification, there are no clinically useful predictive factors to identify breast cancer patients that will benefit from hormonal or targeted therapy (Pusztai *et al*., 2003; Oldenhuis *et al*., 2008, Hefti *et al*., 2013). Prognostic factors are traditionally used to identify patients, in whom unnecessary adjuvant therapy could be avoided on the risk of relapse. Predictive factors determine which treatment is best for the patient (Lønning, 2007). Prognostic and predictive factors are commonly discussed together and they both are used in deciding on cancer treatment and prognosis. As a positive prognostic factor may have a negative predictive value and in reverse, further studies are needed for the choice of treatment strategies individualized for each patient (Tonini *et al*., 2008). Although more than 100 individual prognostic factors have been reported in the literature, only a few of them such as plasminogen activator inhibitor 1 (PAI) expression can be used in the clinic (Lønning, 2007; Senkus *et al*., 2013). Because of this gene expression profiling may become an important tool to predict the response to a specific treatment. However, large well-controlled studies are required to achieve this goal (Lønning, 2007).

2.3.1 Grading of invasive breast cancer

Breast cancer includes a heterogeneous group of tumours with distinct clinical behaviour, histopathological features, as well as response to therapy and outcomes (Weigelt *et al*., 2010). Despite the advances in molecular pathology, histologic grade and type of breast cancer remains the cornerstone of cancer management (Rosai, 2007). Many studies have

shown that histologic grade provides a strong predictor of outcome in patients with invasive breast cancer (Rakha *et al*., 2008a). The microscopic grading system of breast carcinoma has been developed more than 50 years ago and today the most widely used system is the so called Nottingham combined histologic grade (Elston and Ellis, 1991; Rakha *et al*., 2008b). The degree of differentiation is representative to the aggressive potential of the tumour and ranges from well to poorly differentiated. Well differentiated (grade I) cancer is the least aggressive, while poorly differentiated (grade III) cancer is the most aggressive type and needs a more effective treatment (Rakha *et al*., 2010c).

Method of grading

A reliable grading of breast cancer should be done of the entire tumour tissue. The amount of clear acinar, glandular formation and defined tubular structures with a central luminal space is counted. One point is given, if more than 75% of the tumour cells are forming tubules/glands and three points if tubular formation is less than 10% of the tumour. Two points are given, if acinar formation ranges from 10% to 75%. Nuclear pleomorphism refers to the size and shape of the nucleus and nuclear appearances are evaluated from the area showing the worst degree of pleomorphism. Score 1 nuclei are very similar in size to nuclei in ductal epithelial cells with minimal pleomorfism. Score 2 cells have larger nuclei than normal cells with mild to moderate pleomorphism and with a small, but visible nucleoli. Score 3 has a prominent nuclei exhibiting marked variation in size and shape. Mitotic figures are evaluated from the hot spot area, and special attention should be given to high quality fixation, optimally stained haematoxylin and eosin (HE) sections and size of the high power field (HPF) of the microscope. Total number of mitoses per 10 HPF is calculated and only clear mitotic figures are counted as an indicator of proliferative activity. Scoring categories for the mitotic counts are read from "field diameter in mm" corresponding line of the calibration table (e.g. table in WHO Classification of Tumours of the Breast 2012, p.19). Scores from the acinus formation, nuclear atypia and mitotic count are added together, giving a possible total score of 3 to 9. The histological grade of the breast cancer is assigned on the following basis: scores 3-5 represent grade I, well-differentiated cancer, scores 6-7 represent grade II, moderately differentiated cancer, and scores 8-9 represent grade III, poorly differentiated cancer (Elston and Ellis, 1991).

2.3.2 Histopathological and biological prognostic factors

Estrogen/progesterone receptor status

Estrogen and progesterone hormones play a significant role both in normal glandular development and in breast cancer progression (Cui *et al*., 2005). Estrogen mediates its biological effects on breast tissue by binding to specific intracellular receptors, estrogen receptor α (ERα) and β (ERβ) (Liang and Shang, 2013). The oncogenic effect of estrogen is mainly due to ERα-mediated transcriptional activation of genes that advance cell proliferation or reduce apoptosis (Liang and Shang, 2013). Although the presence of

ER and PR in an invasive breast carcinoma is considered both as a prognostic and a predictive factor (Rakha *et al.*, 2007; Badve and Nakshatri, 2009; Liu *et al*., 2010), both lose their prognostic value after long-term follow-up (Cui *et al*., 2005; Taneja *et al*., 2010; Lindström *et al*., 2012). Changes in the hormone receptor status during tumour progression or after treatment are estimated to happen in 10% to 40% of patients (Cui *et al*., 2005; Lindström *et al*., 2012). Approximately two-thirds of breast cancer cases express ER (Liu *et al*., 2010; Sandhu *et al*., 2010) and the majority of reports shows that ER is the most important single predictive factor identified in breast cancer (Oh *et al*., 2006; Badve and Nakshatri, 2009; Weigel and Dowsett, 2010). More than half of these ER+ tumours also express PR, the expression of which has been considered as a clinical indicator of ER function (Cui *et al.,* 2005; Lanari *et al*., 2009; Rakha *et al*., 2010c, Lanari *et al*., 2012). PR expression has been shown to be an independent factor associated with improved survival of breast cancer patients (Liu *et al*., 2010). ER+/PR-, ER-/PR+ or ER-/PR- tumours are biologically and clinically distinct exhibiting higher risk of mortality compared to the risk of women with ER+/PR+ tumours (Rakha *et al*., 2007; Dunnwald *et al*., 2007). This is confirmed by gene expression arrays showing that ER+ and ER– breast cancers are fundamentally different diseases at the molecular level (Perou *et al*., 2000; Sorlie *et al*., 2001; Geyer *et al*., 2012). Current endocrine therapy for treating ER+ breast cancer involves the use of anti-estrogens that block ER and aromatase inhibitors that decrease local and systemic estrogen production (Nair *et al*., 2011).

HER2

The human epidermal growth factor receptor 2, HER2, is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases and has been shown to provide both prognostic and therapeutic predictive value (Rakha and Ellis, 2009; Fiszman and Jasnis, 2011). HER2 is overexpressed in approximately 20% to 25% of invasive breast cancers (Fiszman and Jasnis, 2011) and is detected either by gene amplification or overexpression of the HER2 protein product (Madrid and Lo, 2004). HER2 positive breast cancer is associated with tumour aggressiveness, relapse and high rate of mortality (Murphy and Modi, 2009; Weigel and Dowsett, 2010). Recent studies suggest that the crosstalk between the ER and HER2 pathway plays a role in resistance to endocrine therapy and to HER2 directed agents (Gluck *et al*., 2011; Nahta and O´Regan, 2012). Patients with HER2 overexpressing tumour are more likely to benefit from adjuvant anthracycline-based therapy (Cianfrocca and Goldstein 2004; Leo *et al*., 2011) and trastuzumab therapy (Herceptin™) when compared to patients with HER2 negative tumour (Goldhirsch *et al.*, 2011). Trastuzumab is a humanized monoclonal antibody that binds selectively to the HER2 protein. For women with advanced HER2 positive breast cancer that has disease progression during treatment with Herceptin, Lapatinib (Tykerb™) is approved as a next line HER2 therapy (Murphy and Modi, 2009; Abramson and Arteaga, 2011). Labatinib is a small-molecule drug directed at the internal tyrosine kinase portion of the HER2 oncoprotein and can potentially resensitize HER2 positive tumour cells to the action of trastuzumab (Scaltriti *et al*., 2009; Rakha *et al*., 2010b).

Pertuzumab (Perjeta®) is an anti-HER2 humanized monoclonal antibody and works on a different part of the HER2 protein as Herceptin. Pertuzumab prevents HER2 from coupling with other HER receptors and is able to overcome at least some pathways of resistance to standard trastuzumab-based therapy (Baselga *et al*., 2012). Trastuzumab emtansine (Kadcyla®) is an antibody-drug conjugate that connects the inhibition of trastuzumab and the cytotoxic chemotherapy. This targeted medicine is indicated for the treatment of adults with HER2 positive breast cancer who have previously received trastuzumab, taxane-based chemotherapy or lapatinib (FDA, 2013).

Ki-67

Ki-67 is a proliferation marker, whose expression varies throughout the different cell-cycle phases with a peak during mitosis and absence in the resting phase G0 (Yerushalmi *et al*., 2010; Weigel and Dowsett, 2010; Dowsett *et al*., 2011). Near the end of the mitotic phase, a sharp decrease in Ki-67 levels occurs (Yerushalmi *et al*., 2010). The use of Ki-67 as a prognostic and a predictive marker in breast cancer and in other malignancies has been widely studied and it seems that immunohistochemical expression of Ki-67 has an increasing value as a prognostic marker in early breast cancer (Yerushalmi *et al*., 2010). Recent studies further indicate that Ki-67 might have a valuable role in the distinction between luminal A and luminal B subtypes and in predicting the benefit of adjuvant treatment in breast cancer subtypes of poor prognosis (Cheang *et al*., 2009; Yerushalmi *et al.*, 2010; Goldhirsch *et al*., 2011). Since the St. Gallen consensus in 2011, the value of 14% has been regarded as the Ki-67 cut-off value which distinguishes luminal A and luminal B subtypes (Goldhirsch *et al*., 2011). However, these breast cancer subtypes lack the accepted Ki-67 expression cut-off points for prognostic purposes (Dowsett *et al*., 2011), and there are not enough data to find a relationship between the Ki-67 expression and therapy-specific benefit (Perez-Garcia and Cortes, 2012). Many cut-off values for high and low Ki-67 staining in breast cancer have been used, although immunohistochemical staining levels of 10%-20% have been the most common (Dowsett *et al*., 2011; Brown *et al*., 2014). A new study has raised the possibility that luminal HER2-negative breast cancer patients with Ki-67 higher than 35% might benefit from chemotherapy achieving improved survival (Horimoto *et al.*, 2014).

p53

The *TP53* gene codes for a tumour suppressor protein p53 that regulates the growth arrest and apoptosis, when cell damage is irreparable (Lara *et al*., 2011; Suzuki and Matsubara, 2011). Acquired mutations in the *TP53* gene are observed in about 50% of human cancers (Coates *et al*., 2012; Eldar *et al*., 2013). Normally, p53 protein is present in an inactive form at very low levels, and it requires modification by other proteins to become active (Giaccia and Kastan; 1998). Functional inactivation of p53 protein by gene mutation has been associated with early relapse, poor survival and resistance to certain chemotherapies in breast cancer (Lacroix *et al*., 2006; Al-azawi *et al*., 2011; Davion *et al*., 2012; Eldar *et al*., 2013). The p53 mutation is present

in 18% to 25% of breast cancers and the p53 protein overexpression correlates with negative hormonal status, basal-like or HER2-like breast cancer subtypes (Friedrichs *et al*., 1993; Sorlie *et al*., 2001; Lacroix *et al*., 2006; Al-azawi *et al*., 2011; Jung *et al*., 2011). In addition, p53 positive tumours in luminal A and triple-negative subtypes show unfavourable prognosis (Jung *et al*., 2011). Some studies show p53 mutation to be an independent predictor of poor survival in breast cancer, but there are also contradictory results (Lai, 2004). So far guidelines do not recommend routine use of p53 immunohistochemistry, mainly because the sensitivity of p53 nuclear staining is only about 75% (Greenblatt *et al*., 1994).

Bcl-2

Bcl-2 is a cytoplasmic protein regulating apoptosis and programmed cell death (Dawson *et al*., 2010). In breast cancer, immunohistochemical bcl-2 expression correlates with ER- and PR- positivity, and with low grade and favourable survival (Nadler *et al*., 2008; Dawson *et al*., 2010; Subhawong *et al*., 2010). Although many previously reported studies have suggested bcl-2 to be an independent prognostic factor in breast cancer survival (Dawson *et al*., 2010; Hwang *et al*., 2012; Larsen *et al*., 2012), more studies are needed to support its routine use in clinical practice. A larger number of analyses is also required for the validation of immunohistochemical expression of bcl-2, because there is interlaboratory variation in results due to fixation, antigen retrieval and the used staining method. In addition, antibody types as well as cut-off values used to define immunohistochemical positivity differ in published reports (Dawson *et al*., 2010).

2.4 Molecular classification of breast cancer

Microarray-based gene expression profiling has confirmed breast cancer to be a heterogeneous group of molecularly, biologically and clinically distinct diseases suggesting the existence of multiple cells of origin (Rakha *et al*, 2009; Prat and Perou, 2009; Sotiriou and Pusztai, 2009; Reis-Filho and Pusztai, 2011; Toft and Cryns, 2011). The microarray-based gene expression profiling studies have shown that ER+ and ER– breast cancers are fundamentally different diseases at the molecular level (Perou *et al.*, 2000; Sørlie *et al*., 2001; Geyer *et al*., 2012). In 2000 and 2001, Perou and coworkers as well as Sørlie and coworkers identified in their studies that breast cancer could be divided into four types according to their gene expression profile: luminal-like, basal-like, normal-like and HER2–positive tumours (Perou *et al*., 2000; Sørlie *et al*., 2001). Other less common molecular subtypes have also been described including molecular apocrine, claudin-low and interferon-rich subtypes (Colombo *et al*., 2011) (Figure 3). Further studies have revealed that the main subtypes possess different clinical behaviour, sites of relapse, histological features, response to chemotherapy and prognosis that can be probed with molecular methods (Sotirou and Pusztai 2009; Reis-Filho and Pusztai 2011; Mackay *et al*., 2011).

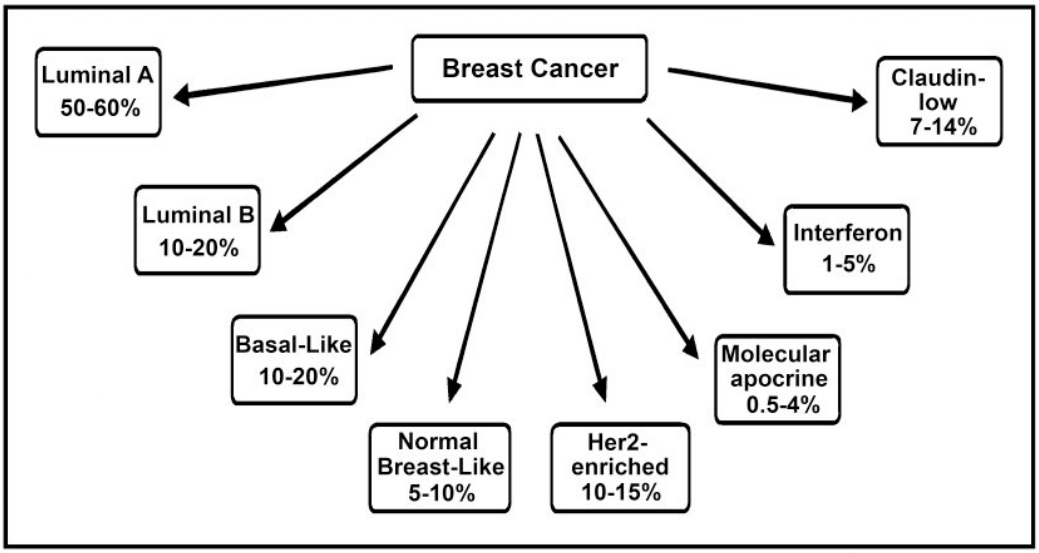

Figure 3. Molecular classification of breast cancer subtypes.

ER+ luminal breast cancers can be subdivided into at least two subgroups, ***luminal A and luminal B,*** each with a distinctive molecular genetic profile and different clinical outcome (Sørlie *et al*., 2001; Creighton, 2012). The luminal A and B subtypes represent the hormone receptor-positive (ER+/PR+) breast cancers, that are the most common subtypes representing at least 70% of breast cancers (Dunnwald *et al*., 2007). Luminal A tumours have high expression of ER-activated genes and low expression of proliferation-related genes and are thus considered to be histologically low grade tumours with a favourable clinical outcome (Perou *et al*., 2000; Sørlie *et al*., 2001; Loi, 2008; Sotirou and Pusztai, 2009).

Luminal B cancer is often a HER2–positive tumour expressing low levels of hormone receptors and is often a high grade tumour with high Ki-67 expression (Cheang *et al*., 2009; Sotiriou and Pusztai, 2009; Tran and Bedard, 2011). Luminal B subtype is resistant to standard therapies, generally carries a poor prognosis and appears to have a tendency to metastasize to bone and pleura (Strehl *et al*., 2011, Tran and Bedard, 2011). To separate highly heterogeneous ER+ luminal subgroups between luminal A and luminal B breast cancer, determination of Ki-67 expression levels may be useful (Strehl *et al*., 2011). Luminal B breast cancer represents a distinctive biology from that of hormone-sensitive luminal A cancer, rather than simply representing a more advanced or aggressive form of luminal A subtype (Creighton, 2012).

In gene expression studies three non-luminal breast cancer subtypes have been identified in the ER- branch of the cluster: basal-like, normal breast-like and HER2 (Weigelt and Dowsett, 2010).

A basal-like breast tumour (ER-, PR-, HER2-) is an aggressive breast cancer subtype overexpressing basal cytokeratins CK5/6, CK14 and CK17. Additional features used

to define basal-like breast tumours are high p53 and Ki-67 expression and poorly differentiated tumour grade (Kuroda *et al*., 2008; Loi, 2008; Tonini *et al*., 2008; Tang *et al*., 2009; Badve *et al*., 2011). The basal-like tumours represent about 15% to 20% of breast cancers and have a poorer prognosis compared to luminal tumours or triple-negative tumours lacking basal-like markers (Tonini *et al*., 2008; Voss *et al*., 2011; Toft and Cryns, 2011). The metastatic spread pattern of basal-like subtype is different compared with other subtypes. Basal-like subtype favours a haematogenous spread with a tendency to develop metastasis in the brain and lungs and is less likely to spread to lymph nodes, liver or bones (Badve *et al*., 2011; Sandhu *et al*., 2010). These breast tumours are common in young women and especially, if they carry *BRCA 1* mutation (Millikan *et al*., 2008; Toft and Cryns, 2011; Perou and Børresen-Dale, 2010; Lim *et al*., 2009). Because of its triple-negative receptor status, basal-like breast tumours have poor response to standard adjuvant therapies such as endocrine therapy or trastuzumab, leaving chemotherapy as the only therapeutic option (Kuroda *et al*., 2008). Epidermal growth factor receptor (EGFR) overexpression is found by immunohistochemistry in more than 50% of basal-like tumours (Cakir *et al*., 2012; Lavasani and Moinfair, 2012). Positive EGFR and CK5/6 immunohistochemical stainings have shown to identify patients with basal-like breast tumours, and EGFR targeted therapy could be useful in the treatment for this patient population (Sutton *et al*., 2010; Cakir *et al*., 2012). Although most of the basal-like tumours are high grade tumours, there are also low-grade breast carcinomas with a basal-like phenotype having an excellent prognosis, e.g. mammary adenoid cystic carcinoma, adenosquamous carcinoma and secretory carcinoma (Stolnicu, 2010; Kontos *et al*., 2011, Vasudev and Onuma, 2011, Badve *et al*., 2011, Wetterskog *et al*., 2012). Combining these factors, the expression of basal cell markers on its own does not affect the prognosis of breast cancer (Lavasani and Moinfar, 2012).

A less common molecular subtype is ***normal breast-like*** tumour that is rather poorly characterized. This tumour lacks ER and HER2 expression and can be CK5/6 positive (Bertolo *et al*., 2008; Weigelt *et al*., 2010). In gene expression analysis, normal breast-like tumour has been shown to cluster together with fibroadenomas and normal breast tissue and to express genes characteristic for adipose tissue and other non-epithelial cell types (Perou *et al*., 2000; Sørlie *et al*., 2001; Weigelt *et al*., 2010). Even though normal breast-like tumour has a basal-like triple-negative phenotype at the mRNA level (Perou *et al*., 2000; Finak *et al*., 2006), normal breast-like tumour has been shown to have a distinct response to neoadjuvant chemotherapy and better prognosis when compared to basal-like breast tumour (Kobayashi, 2008; Rakha *et al*., 2009; Yu *et al*., 2009; Weigelt *et al*., 2010; Toft and Cryns 2011). About 6% to 10% of all breast cancers are supposed to fall into the normal breast-like category. The clinical significance of the normal breast-like subtype is yet to be determined and it remains unclear whether this subgroup represents a true subtype or a contamination of samples with normal beast tissue (Foulkes *et al*., 2010; Weigelt *et al*., 2010).

The ***HER2-enriched subtype*** represents approximately 10% of all breast tumours and is characterized by high-grade tumours with metastases and in the absence of systemic

adjuvant and HER2 targeted therapies poor outcome (Tonini *et al*., 2008; Perou and Børresen-Dale 2010; Al-azawi *et al*., 2011). This subtype includes many, but not all, HER2 positive tumours or ones that overexpress HER2 gene product and are typically ER and PR negative (Allison, 2012). However, about 30% to 40% of the HER2-enriched tumours are ER+ leaving the majority as ER- (Perou, 2010). It should be noted that some of these tumours are clinically triple-negative, despite having the gene expression signatures of the HER2-enriched subtype (Perou, 2010). HER2-enriched subtype has to some degree sensitivity to chemotherapy and response to HER2-targeted therapy (Tonini *et al*., 2008). Despite trastuzumab-based therapy some patients with HER2 positive breast tumours do not benefit from this drug and some develop trastuzumab resistance during prolonged treatment (Barok *et al*., 2007; Prat and Perou, 2011).

In addition to these three ER- subtypes, at least three other molecular groups of ER- cancers have been identified including molecular apocrine, interferon-rich and claudin-low subgroups (Weigelt *et al*., 2010). To better understand the clinical and biological significance of these novel subtypes further studies are needed. ***Molecular apocrine*** tumours are characterized by the apocrine type epithelium, ER negativity and androgen receptor (AR) positivity, but with an expression profile resembling ER+ luminal breast cancer (Sanga *et al*., 2009; Robinson *et al*., 2011; Cha *et al*., 2012). Furthermore, a high frequency of HER2 overexpression accompanied by basal markers such as CK5/6 and EGFR are frequently found (Cha *et al*., 2012; Lehmann-Che *et al*., 2013). It is estimated that 0,5% to 4% of breast tumours have features of molecular apocrine breast cancer and they mostly occur at advanced ages (Lehmann-Che *et al*., 2013). In recent studies, molecular apocrine breast cancer patients have been shown to have poor survival (Farmer *et al*., 2005), but better prognosis than patients with basal subtype of tumours (Sanga *et al*., 2009).

The ***interferon-rich subtype*** is characterized by high expression of interferon regulated-genes and has a considerably better prognosis than other triple-negative breast cancers (Hu *et al*., 2006; Foulkes *et al*., 2010).

Recently identified ***claudin-low subtype*** shows a prevalence of 7% to 14% of breast cancers and is enriched for mesenchymal and stem cell markers (Foulkes *et al*., 2010; Prat *et al*., 2010). These tumours have been characterized by the lack of claudin proteins (Perou, 2010). Claudin proteins are important components of tight junctions that seal the potential space between adjacent epithelial cells controlling the cell polarity (Peddi *et al*., 2012; Blanchard *et al*., 2013). The claudin-low subgroup lacks cell-cell junction protein, including E-cadherin (Perou, 2010). The disruption of the tight junctions allows loss of cellular cohesion, aggressive growth and differentiation of cancer cells (Lu *et al.,* 2013). Claudin-low subtype has positivity for vimentin and low expression of epithelial markers (Gerhard *et al*., 2012). Most of these tumours lack lobular features (Perou, 2010). Claudin-low tumour, like basal-like, is clinically mostly ER-/PR-/HER2- (triple-negative tumour) with a high frequency of metaplastic and medullary features

with an intense immune cell infiltrate (Perou, 2010; Haughian *et al*., 2011; Gerhard *et al*., 2012). Claudin-low molecular subtype benefits from chemotherapy to some degree and it is characterized by short disease-free survival, high proliferation index and high tumour grade (Perou, 2010; Haughian *et al*., 2011). As primitive breast stem cells can be the cells of origin in claudin-low tumours, these tumours arise from more immature precursors than other breast cancers (Lim *et al*., 2009; Prat *et al*., 2010).

Although molecular and genetic testing for breast cancer is very attractive, having prognostic and predictive value, it is expensive and not yet widely used (Onitilo *et al*., 2009). Thus, the immunohistochemical classification of breast cancer as a clinical tool is still supported because it can be used at a reasonable cost and it has been shown to correlate well with gene expression categorization (Onitilo *et al*., 2009).

As cancer is a genetic disease driven by heritable or somatic mutations, new DNA sequencing technologies have revealed a number of novel cancer-related genes (Ulahannan *et al*., 2013). ***Next-generation sequencing*** **(NGS)** employs micro- and nanotechnologies to reduce the size of sample components and reagent costs and it allows simultaneous sequencing and analysis of millions of samples as well (Shokralla *et al.*, 2014). Using NGS hundreds to thousands genes involved in the development of a tumour can be sequenced all at once in a single test (Grada and Weinbrecht, 2013). A great strength of NGS system is that, they do not only recognize base substitutions but can simultaneously also find insertions, deletions, copy number alterations and translocations (Ross and Cronin, 2011). The application of NGS has identified extensive genetic variation between tumours (intertumour heterogeneity) and within tumours (intratumour heterogeneity) (Burrell *et al*., 2013; Hiley *et al*., 2014). While multiregional tumour sampling and molecular profiling of the tumour samples by NGS helps to understand cancer biology progresses and optimizes future cancer medicine for a patient, it is likely to have its limitations (Bedard *et al*., 2013). Although producing NGS data is today relatively straightforward, their analysis can be extremely difficult due to the increased complexity of results (Kim *et al*., 2014). Perhaps the greatest challenge now is to develop more efficient bioinformatics methods for organizing the information NGS provides.

2.5 Breast cancer treatment

Surgical and medical treatment of breast cancer has advanced dramatically in the past few decades. Nowadays, breast conserving therapy with segmental resection and sentinel lymph node biopsy followed by whole breast irradiation has become the preferred method of treatment for early-stage breast cancer. The sentinel lymph node technique is based upon the observation that tumour cells migrating from primary tumour metastasize to the first lymph node called sentinel lymph node before involving other lymph nodes of axilla (Hsueh and Giuliano, 1998). Presence or absence of lymph node metastases is one of the most important prognostic factors in women with early stage breast cancer (Lyman *et al.*, 2014). Patients with isolated tumour cells (metastasis size $\leq$ 0.2 mm)

and node-negative breast cancer appear to have similar prognosis, and patients with micrometastasis (metastasis size > 0.2 mm but not >2.0 mm) have a 38% higher risk of tumour recurrence than node negative cancer (Andersson *et al.*, 2010). The American Society of Clinical Oncology recommends no axillary lymph node dissection for women with early-stage breast cancer who have one to two sentinel lymph node metastases (found by sentinel node biopsy) and will undergo breast conserving surgery with whole-breast radiotherapy (Lyman *et al.*, 2014). Adjuvant radiation therapy is recommended to eliminate potential microscopic residual disease adjacent to the original tumour site after partial mastectomy (Hoover *et al*., 2011). Other adjuvant therapies include chemotherapy, endocrine therapy, targeted biologic therapy or a combination of treatments (Table 4). Adjuvant therapy is indicated after primary therapy for locally advanced breast cancer (Kataja *et al*., 2009) and for potential occult micrometastasis, which are thought to be responsible for distant metastasis (Owusu *et al*., 2012). Treatment is started if there is a clinically relevant reduction in risk of recurrence that can be reached with an acceptable level of treatment-related harmful side effects (Kataja *et al.*, 2009).

Table 4. Systemic treatment recommendations for early breast cancer.

Subtype	Recommended therapy	Note of therapy
Luminal A-like: ER and PR positive, HER2 negative, Ki-67 low	ET alone in the majority of cases	CT in some cases (e.g. high grade, >1cm tumour size, positive nodal status)
Luminal B-like (Her2-negative): ER positive, HER2 negative, Ki-67 high or PR low	ET and CT for the majority of cases	
Luminal B-like (HER2-positive): ER positive, HER2 positive, any Ki-67, any PR	CT, anti-HER2 and ET for all cases	
HER2-positive: HER2 positive, ER and PR negative	CT and anti-HER2	
Triple-negative (ductal): ER and PR negative, HER2 negative	CT	Triple-negative includes also medullary and adenoid cystic carcinoma with low risk of distant recurrence

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

2.6 Control of cell cycle

Cell proliferation is achieved through tightly controlled events called the cell cycle leading to replication of DNA and cell division (Caldon *et al*., 2006). The cell cycle is traditionally divided into four sequential phases known as G1, S, G2 and M (Sa and Das, 2008) (Figure 4). G1, S and G2 phases together are called the interphase that takes

place before cell division in mitosis (M phase). The G1 phase consists of cell growth and preparation of the chromosomes for replication. Once the required proteins and growth are complete, the cell enters the synthesis phase (S phase), which directs to the copying of the DNA. The second gap phase G2 lasts until the cell enters mitosis. The cell undergoes normal biosynthesis to ensure necessary cell functions. Near the end of G2 phase, the cytoplasmic organelles duplicate in preparation for cell division. Mitosis (M phase) is the last stage of the cell cycle where the cell divides into two cells (Harwood *et al*., 2007; Sa and Das, 2008; Caldon *et al*., 2006; Ross *et al*., 2003). Cells that have stopped dividing are in the resting phase (G0) or in the first gap phase (G1) of the cell cycle (Harwood *et al*., 2007). The cell cycle progression and activation of each phase is dependent on the proper completion of the previous one. Cancer cells often avoid normal control linked to the cell cycle progression that stops proliferation in the presence of damaged DNA or other physiological injuries (Deshpande *et al*., 2005; Malumbres and Barbacid, 2009).

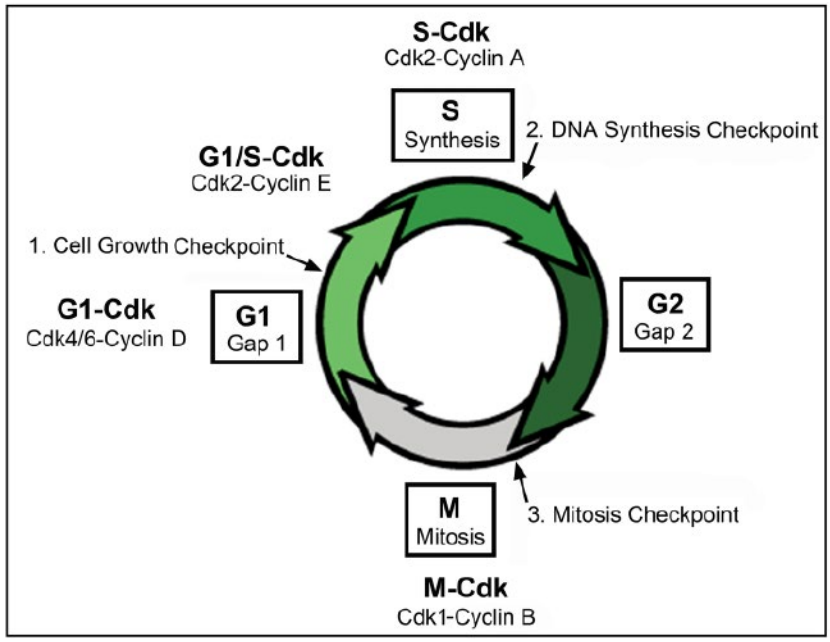

Figure 4. Cell cycle and cyclin-cyclin dependent kinase complex. (Modified from UCSF School of Medicine 2007).

The progression in cell cycle is regulated by activation and inactivation of different cellular proteins (Malumbres *et al*., 2009). The transition from one cell cycle phase to another is regulated by cyclin-dependent kinases (CDKs) and the activity of each CDK is controlled by cyclins, CDK inhibitors (CDKIs) and phosphorylating events (Malumbres *et al*., 2009; Vermeulen *et al*., 2003). In contrast to CDK protein levels, which remain stable during the cell cycle, cyclin levels raise and fall during the cell cycle (Figure 5).

In addition to cyclin binding, complete CDK activity is regulated by phosphorylation on conserved threonine and tyrosine residues by CDK activating kinases (CAKs) (Vermeulen *et al*., 2003). Of all CDKI, p27/Kip1 is one of the most important regulator which binds to complexes with CDK4, CDK6 or CDK2 and blocks the cell cycle at the G1/S phase checkpoint and is highly expressed in cells arrested at the G0 and G1 phases (Okabe *et al*., 2001). p27 level is frequently reduced in most common cancers and this is associated with a poor prognosis (*Chu et al*., 2008).

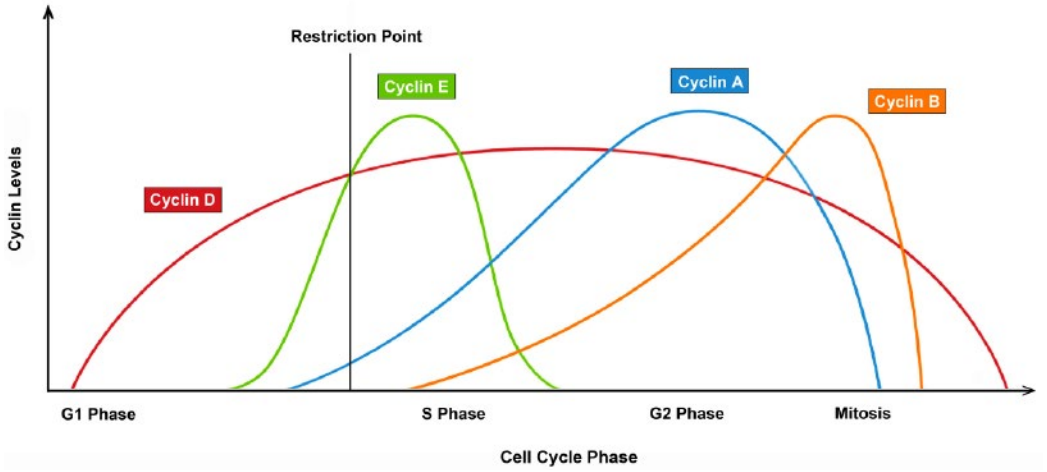

Figure 5. Expression levels of cyclins through the cell cycle. The restriction point is a G1 phase checkpoint at which a cell commits to division and no longer requires growth factors for the completion of the cell cycle. (Modified from Abeloff´s Clinical Oncology 2008).

CDK activity can be inhibited by CDKIs usually during the G1 phase or in response to signals from the environment or from damaged DNA (Clement *et al*., 2001). Selected proteins at particular cell cycle phase become phosphorylated by active cyclin-CDK complexes in the cell cycle progression (Vermeulen *et al*., 2003). Degradation of the M phase cyclin/CDK complex by ubiquitin-mediated proteosome pathway is required to cause the exit of mitosis and cytokinesis (Hoyt, 2000; Bowerman and Kurz, 2006). Although cyclin/CDKs are activated during cell cycle progression, it is their phosphorylation substrates that complete the ordered cellular changes leading to cell division (Deng *et al*., 2009).

2.7 Cyclins

Cyclins are nuclear proteins required for cell cycle progression (Husdal *et al*., 2005, Stamatakos *et al*., 2010). They are synthesized and destroyed at precise times during the cell cycle (Malumbres and Barbacid, 2009) so that the progression through the G1-S-G2-M cycle follows successive oscillations in the cyclin levels (Murray, 2004). As a result failure to degrade cyclins can trap cells in a particular cell cycle phase (White and Dalton, 2005).

D-type cyclins (D1, D2 and D3) act as links between extracellular signals, growth factors (e.g. insulin-like growth factor-1, transforming growth factor-α) and the cell cycle system (Sutherland *et al*., 1993; Yan *et al*., 1997; Roberti *et al*., 2009). ***Cyclin D1*** appears to be important in normal breast cell proliferation and differentiation during pregnancy (Neuman *et al*., 1997). The level of cyclin D1 does not show a typical fluctuation pattern displayed by other cyclins (Figure 5). Instead, its level is closely regulated by signalling from the extracellular matrix (ECM) including growth factor receptors and their downstream effectors (Yang K *et al*., 2006; Klein and Assoian, 2008; Roberti *et al*., 2009). High levels of cyclin D1 are observed in the G1 and G2 phases, and much lower levels in the S phase (Guo *et al*., 2005). Cyclin D1 binds to CDK4 or CDK6 thus regulating the passage of the cells from the G0 through the G1 into the S phase in the cell cycle (Kenny *et al*., 1999; Ahnström *et al*., 2005; Roy and Thompson, 2006). When it is time for a cell to enter the S phase, active cyclin D1-CDK4/CDK6 complex partially phosphorylates the retinoblastoma protein (pRb) inhibiting its activity (Weinberg, 1995). This growth inhibitory action of pRb serves as a gatekeeper of the G1 phase and allows the cell to pass through the restriction point and induces expression of some genes (e.g. cyclin E) important for S phase progression (Sherr and Roberts, 2004; Roberti *et al*., 2009). pRb is quite frequently mutated in cancers causing loss of cell cycle regulation (Kouraklis *et al*., 2006).

The transition from the G1 to the S phase is a key check point that prevents the cell from entering the S phase in the presence of the damaged DNA (Malumbres and Barbacid, 2009). Entrance into the S phase and initiation of DNA replication requires the activity of CDK2, which is activated by E-type cyclins (E1 and E2) (Bashir and Pagano, 2005). In normal cells, ***cyclin E*** is expressed when needed and then it is rapidly degraded (Roberti *et al*., 2009). Cyclin E interacts with CDK2 and this complex phosphorylates pRb and regulates the G1/S phase transition (Vermeulen *et al*., 2003). Strictly regulated, increased expression of cyclin E is thought to promote tumourigenesis through shortening the G1 phase and driving cells into the S phase more rapidly, thus increasing the rate of proliferation of the cells (Nanos-Webb *et al*., 2012; Mittendorf *et al*., 2010). In tumours, cyclin E undergoes proteolysis, which generates biochemically hyperactive low molecular weight (LMW) species of cyclin E (Porter *et al*., 2001). The nuclear localized LMW forms of cyclin E exhibit increased CDK2-associated kinase activity and resistance to inhibition by CDK2 inhibitors (Loeb and Chen, 2012). The LMW form of cyclin E phosphorylates substrates effectively and as a result tumour cells can bypass the restriction point (Porter *et al*., 2001). Furthermore, the overexpression of cyclin E induces genetic instability, a feature that leads tumour to a more aggressive state (Roberti *et al*., 2009).

A-type cyclins (A1 and A2) are expressed in the early S phase of the cell cycle (Fuchimoto *et al*., 2001). ***Cyclin A*** protein level increases during the cell cycle progression to the G2 phase and falls in mid of the M phase (Sherr, 1996; Pagano *et al.*, 1992). Therefore cyclin A is one of the most useful markers detecting proliferating cells (Jensen *et al*.,

2001; Poikonen *et al*., 2005). Cyclin A replaces cyclin E as a partner of CDK2, while later in S phase it leaves CDK2 to associate with CDK1 a bit earlier than cyclin B during the G2 phase (Yam *et al*., 2002; Bashir and Pagano, 2005; Roberti *et al*., 2009). Cyclin A2 is essential for the activation of DNA synthesis and it is overexpressed in a variety of human cancers when compared with normal cells and tissues (Dobashi *et al*., 1998; Traganos, 2004; Wang *et al.*, 2009; Sørby *et al*., 2012). The expression level of cyclin A2 seems to be a marker of tumour aggressiveness and to associate with reduced-free and overall survival (Poikonen *et al*., 2005; Aaltonen *et al.*, 2006; Wang *et al*., 2009; Liang *et al*., 2012). There are also data from different malignancies indicating that a high proliferation rate correlates with a better chemotherapy response indicating that tumours with high cyclin A2 expression are more suitable for chemotherapy (Li *et al*., 2002; Poikonen *et al*., 2005; Wang *et al*., 2009).

Cyclin B1, like cyclin A, is classified as a mitotic cyclin and it functions as a key cell cycle regulator of the G2/M checkpoint (Ho *et al*., 2002). The onset of cyclin B1 synthesis starts when the cell exits the S phase, reaches the maximal level when the cell enters mitosis and persists to mid-mitosis (Pines and Hunter, 1991; Ho *et al*., 2002). Overexpression of cyclin B1 is reported to enable cells to override the G2 DNA damage checkpoint (Androic *et al*., 2008). Overexpression of cyclin B1 is demonstrated in several malignancies including breast cancer, gastric cancer, cervical cancer, hepatocellular cancer, oesophageal squamous cell cancers and non-small cell cancers (Androic *et al*., 2008; Aaltonen *et al*., 2009a; Weng *et al.*, 2012).

2.7.1 Cyclin D1 and breast cancer

Cyclin D1 is overexpressed at the mRNA and protein level in approximately 50% of primary breast cancer cases being one of the most commonly overexpressed proteins in breast cancer (Roy and Thompson 2006; Rudas *et al*., 2008; Quintayo *et al.*, 2012). Overexpression of cyclin D1 may occur with or without *CCND1* (cyclin D1 gene) gene amplification, which is observed in about 15% of all breast cancers (Roy and Thompson, 2006; Weigel and Dowsett, 2010; Tobin and Bergh, 2012). Gene amplification of *CCND1* has been linked to an aggressive disease, whereas overexpression of the cyclin D1 protein has been linked to both poor and good clinical outcome as well as to endocrine therapy resistance (Reis-Filho *et al*., 2006; Tobin *et al*., 2011; Lundgren *et al*., 2012; Tobin and Bergh, 2012).

Uncontrolled production of cyclin D1 affects the amount of cyclin D-CDK4/CDK6 complex being formed, even when the growth factors are not present (Yang *et al*., 2006). Overexpression of cyclin D1 and/or CDK4/6 is considered to be the major mechanism behind the oncogenic function of cyclin D1 (Sun *et al*., 2011). Cyclin D1 overexpression has been shown to associate with ER and PR positivity and to have a strong inverse correlation with the expression of the basal-like markers such as EGFR, CK14, CK5/6 and CK17 (Reis-Filho *et al*., 2006; Peurala *et al*., 2013), that is, breast cancers with a good prognosis. While estrogen can induce cyclin D1 expression, anti-estrogen therapy

has an inhibitory effect on cyclin D1 (Liang and Shang, 2013). Inhibition of cyclin D1 activity decreases estrogen-stimulated breast cancer cell proliferation, whereas induction of cyclin D1 mimics the effect of estrogen and starts cell cycle progression in anti-estrogen arrested cells (Butt *et al*., 2005; Liang and Shang, 2013). There are studies suggesting that amplification as well as overexpression of cyclin D1 are linked to early relapse, resistance to endocrine therapy and poor prognosis in ER+ and PR+ breast cancers (Aaltonen *et al.,* 2009b; Taneja *et al*., 2010; Yang *et al.*, 2010; Tobin and Bergh, 2012). It has been suggested that cyclin D1 activates ERs in the absence of estrogen and independent of complex formation to a CDK partner and this activation is not inhibited by anti-estrogens (Zwijsen *et al*., 1997). In several clinical studies, early relapse and poor survival were observed in cyclin D1 positive breast cancer patients who received tamoxifen therapy (Musgrove *et al.*, 1993; Rudas *et al*., 2008). It seems that a large group of breast cancer patients receiving anti-estrogen tamoxifen therapy develop resistance to hormonal treatment or do not benefit from it, because overexpression of cyclin D1 reverses the growth-inhibitory effects of anti-estrogens (Stendahl *et al*., 2004; Fouladdel *et al*., 2005; Berglund *et al*., 2008).

Conflicting data on the association between cyclin D1 protein level and clinicopathological parameters have been reported (Lehn *et al*., 2010). The previous studies have shown association between overexpression of cyclin D1 and breast cancers that are hormone receptor positive, low histologic grade and non-triple negative subtype (Peurala *et al*., 2013; Chung *et al*., 2014). However, cyclin D1 overexpression is found to correlate with high Ki-67 expression among ER positive breast cancer, but with low proliferation among ER negative breast cancer (Aaltonen *et al*., 2009b). High *CCND1* amplification is shown to associate with high tumour grade, poor prognosis and it may be indication for additional chemotherapeutic treatment in women with ER positive breast cancer (Roy *et al.,* 2010). Cyclin D1 is the product of *CCND1* and the overexpression of cyclin D1 is associated with longer disease specific survival (Chung *et al*., 2014) and decreased recurrence rate (Lundgren *et al.,* 2012). If cyclin D1 is used as a prognostic or treatment predictive biomarker in breast cancer, patients' tumour samples should first be separated by *CCND1* copy number to find out the true clinical value of cyclin D1. *CCND1* amplified cases should be removed before conducting cyclin D1 protein expression of remaining tumour samples and before relating this expression to other clinicopathological data, e.g., ER, PR, HER2 and Ki-67 expression (Tobin and Bergh, 2012).

2.7.2 Cyclin E and breast cancer

Cyclin E is overexpressed in about 25% of breast tumours and it is consistently linked to poor prognosis with a strong association to ER negativity and a high histological grade (Cooley *et al*., 2010; Keyomarsi *et al*., 2002; Agarwal *et al*., 2009; Roberti *et al*., 2009). In tumour, cyclin E undergoes proteolytic processing by the serine protease, elastase, generating low molecular weight cyclin E (LMW-E) that exhibit increased kinase activity and resistance to inhibition by cyclin kinase inhibitors p21/p27 (Keyomarsi *et*

al., 2002; Loeb and Chen 2012; Nanos-Webb *et al*., 2012). This LMW-E is not found in normal tissue and breast cancer patients with tumours expressing LMW-E have a poor prognosis (Nanos-Webb *et al*., 2012). It has been suggested that the generation of the LMW forms of cyclin E in tumours may be due to an increase in elastase activity (Roberti *et al*., 2009). Targeting LMW-E in metastatic breast cancer could have important therapeutic implications, because there is no toxity to normally proliferating cells in the body (Roberti *et al*., 2009; Loeb and Chen, 2012). Furthermore**,** high expression or amplification of cyclin E and corresponding CDK2 activity have been shown to predict the failure of endocrine therapy as well as the resistance to trastuzumab in HER2 patients (Span *et al*., 2003; Cooley *et al*., 2010; Mittendorff *et al*., 2010; Scaltriti *et al*., 2011). Co-treatment of trastuzumab with CDK2 inhibitors may be a valid strategy for patients whose tumours display cyclin E amplification/overexpression (Scaltriti *et a*l., 2011). The contribution of cyclin E to tamoxifen resistance is unclear, but a link between cyclin E overexpression and diminished response to tamoxifen has been suggested (Dhillon and Mudryj, 2002).

2.7.3 Cyclin A and breast cancer

There are two subtypes of cyclin A, cyclins A1 and A2, of which cyclin A2 is proposed to be a prognostic factor in breast cancer (Fuchimoto *et al*., 2001; Gong and Ferrell, 2010; Li *et al*., 2010b). Overexpression of cyclin A2 has been associated with a poor outcome in breast cancer patients in several studies (Michalides *et al*., 2002; Kühling *et al*., 2003; Poikonen *et al*., 2005; Ahlin *et al*., 2009; Nilsson *et al*., 2013). In addition, upregulation of cyclin A has been reported to correlate with increased risk of recurrence among patients with node-negative breast cancer (Baldini *et al*., 2006). Furthermore, decreased level of cyclin A has been shown to correlate with decreased level of cells in the S and G2 cell cycle phases (Wang *et al*., 2013). Interestingly, metastatic cancer cells have, however, been reported to show less cyclin A2 expression than nonspreading tumour cells (Casimiro *et al*., 2012).

2.7.4 Cyclin B1 and breast cancer

Cyclin B1 is not so extensively studied in breast cancer as cyclins D1 and E, but overexpression of cyclin B1 has been shown to associate with aggressive tumour phenotype and to be an independent predictor of poor overall and metastasis-free survival in breast cancer patients (Suzuki *et al*., 2007; Aaltonen *et al*., 2009a; Agarwal *et al*., 2009). High cyclin B1 expression occurs in luminal B and basal-like breast tumours and overexpression of cyclin B1 is associated with an aggressive behaviour in hormone receptor-positive breast cancers (Agarwal *et al*., 2009). Nuclear cyclin B1-positive breast cancers have been shown to be resistant to adjuvant chemotherapy and tamoxifen therapy (Suzuki *et al*., 2007). Thus, inhibiting cyclin B1 function in combination with chemotherapeutic drugs can strengthen the antiproliferative effect of chemotherapy, and this way cyclin B1 has been proposed to be a potential target for anticancer therapy (Androic *et al*., 2008).

2.8 Extracellular matrix

Extracellular matrix (ECM) is essential for tissue structure and influences many biological processes including cell differentiation and proliferation (Velleman *et al*., 2012). The ECM is prescribed as a dynamic structure that consists of a varying collection of proteins and sugars and generates signals through feedback loops to control the behaviour of cells (Järveläinen *et al*., 2009; Cox and Erler, 2011). For these processes, the ECM must be degraded to let cell migration, cellular signal transmission, modulation of growth factor activity and deposition of new matrix to occur (Kim *et al*., 2011). Studies emphasize that ECM alterations have biological importance also in tumour invasion and metastasis as well as in response to pharmacotherapy (Järveläinen *et al*., 2009; Polyak and Kalluri, 2010; Bremnes *et al*., 2011; Desmedt *et al*., 2012; Velleman *et al*., 2012).

Mammary epithelium is bilayered, with milk-producing luminal cells and a basal layer of contractile myoepithelial cells (Deugnier *et al*., 2002), which produce the basement membrane that separates the epithelium from the ECM (Guo *et al*., 2012). The normal tissue stroma is composed of both cellular (fibroblasts, endothelial cells, smooth muscle cells, adipocytes, inflammatory cells, nerve cells) and non-cellular components (e.g. proteins, proteases, cytokines, growth factors) (Cirri and Chiarugi, 2012). During early stages of tumour progression, cancer cells attach and invade through the basement membrane to become into direct contact with the activated stroma that has long time been considered to be a passive responder in the tumourigenesis (Kalluri and Zeisberg, 2006). The tumoural stroma has been observed to undergo extensive gene expression changes even at the pre-invasive stage of DCIS, supporting the view that stromal mechanisms play an important role in modulating anti-tumourigenic properties (Buraschi *et al*., 2012).

Changes in the ECM surrounding the tumour are largely due to the actions of carcinoma-associated fibroblasts (CAFs) (Eck *et al*., 2009). Tumour cells induce and maintain fibroblasts in an activated CAF phenotype which, in turn, produce growth factors and cytokines that maintain tumour progression (Bremnes *et al*., 2011; Cirri and Chiarugi *et al*., 2011; Conklin and Keely, 2012, Koontongkaew, 2013). CAFs have been shown to originate from various sources, such as nearby tissue or bone marrow via circulation, indicating their diverse cellular origin (Togo *et al*., 2013). At the very early stages of breast cancer, CAFs inhibit invasion mainly through the formation of gap junctions between activated fibroblasts (Cirri and Chiarugi *et al*., 2011). Later on CAFs are stimulated by several tumour-secreted factors, and activated CAFs with abnormal ECM interrupt tissue polarity, architecture and promote epithelial cell transformations leading to tumour-promoting angiogenesis, inflammation by endothelial and immune cells, and invasion (Järveläinen *et al*., 2009; Cirri and Chiarugi, 2011; Khamis *et al*., 2012; Lu *et al*., 2012). Once the basement membrane is degraded, CAFs accumulate causing the expansion of tumour stroma. This stromal reaction is called desmoplasia and it is observed also in metastatic sites (Cirri and Chiarugi, 2011; Khamis *et al*., 2012). CAFs are abundant providers of various types of ECM components, which are

critical regulators of desmoplasia and tissue stiffening in tissue fibrosis (Levental *et al*., 2009).

Activated stromal cells, together with tumour cells, have an increased ability to secrete a wide variety of growth factors, chemokines, hormones and to synthesize collagens and other ECM macromolecules such as proteoglycans (PGs) as well as several ECM-modifying enzymes, including MMPs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Schauer *et a*l., 2011; Cirri and Chiarugi, 2012). Although it is unlikely that a single proteinase or a single cascade of proteinase activation is involved in all tumours, the MMPs represent the most prominent group of proteinases associated with tumour progression (Kessenbrock *et al*., 2010). All cellular processes that involve molecular interactions at the cell surface involve PGs, because these molecules bind proteins and are abundant in the ECM (Perrimon and Bernfield 2001). PGs fill the majority of the extracellular interstitial space within the tissue (Järveläinen *et al*., 2009). A matrix PG that maintain the structural and functional integrity of the interstitial ECM is decorin, a small extracellular matrix proteoglycan that importantly affects the biology of various types of cancer (Iozzo and Sanderson, 2011; El Behi *et al*., 2013).

2.9 Matrix Metalloproteinases

The MMP family includes connective tissue degrading zinc-dependent endopeptidases (Conklin and Keely, 2012). Degradation of the ECM by MMPs is essential in many normal biological processes including development, growth and angiogenesis, as well as in pathological processes including wound healing and tumour invasion (Yan and Boyd, 2007). MMP family currently consists of 24 proteins that collectively are able to cleave all components of the ECM in mammals (Lenglet *et al*., 2013; Logan *et al*., 2012). Traditionally, the MMPs have been divided into collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs on the basis of their specificity for ECM components and subcellular localization (Jackson *et al*., 2009; Mannello and Medda 2012; Micheal *et al*., 2013).

MMP synthesis. Because MMPs are synthesized and secreted as inactive zymogens, they need extracellular activation by the MMP itself, another MMP or a proteinase of an other group (Egeblad and Werb, 2002). In the latent pro-MMP form the prodomain is thought to fold over and cover the catalytic site. This folded conformation is necessary to keep the MMP in its inactive proform. The latent pro-MMP structure includes signal peptide, propeptide, catalytic domain, hemopexin domain and a small hinge region (Nelson and Melendez, 2004). During the synthesis the signal peptide guides the enzyme into the endoplasmic reticulum. The catalytic domain contains the zinc-binding region bound by a cysteine residue within the propeptide domain. The hemopexin domain mediates protein-protein interactions. The disruption of the zinc-cysteine bond is thought to represent a critical step in initiating the MMP autoactivation (Figure 6). This uncontrolled proteolysis leads to an imbalance in the expression or activities of MMPs

and this has been implicated to take place in many diseases including cancer growth, cancer cell invasion and metastasis (Nelson and Melendez, 2004; Page-McCaw *et al*., 2007; Mannello, 2011).

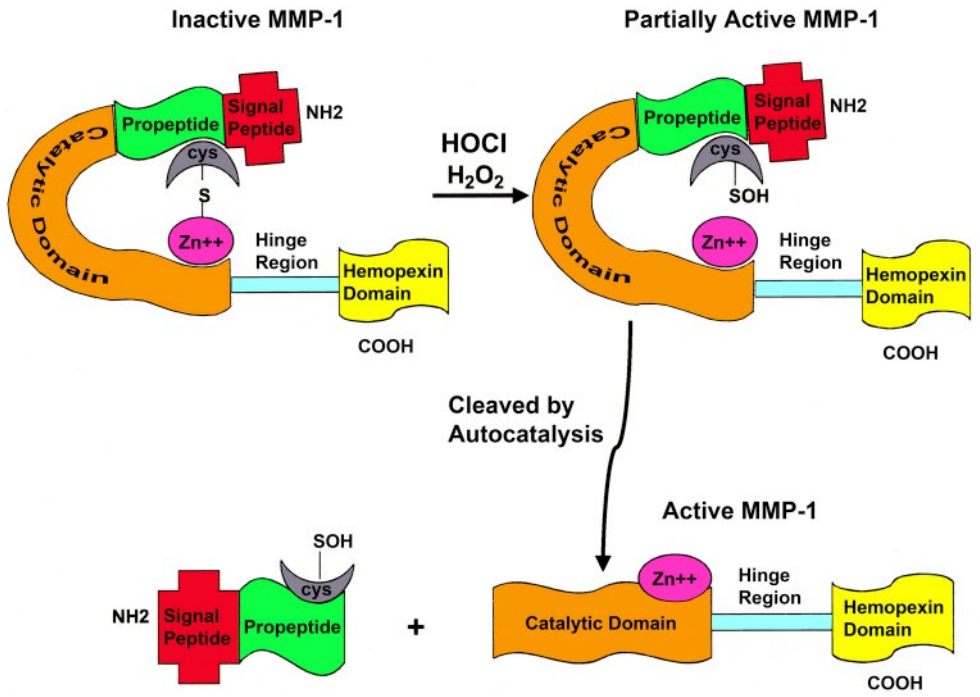

Figure 6. Structure of MMP-1. MMP-1 is initially secreted as inactive zymogens with a propeptide domain that must be removed before the enzyme is active. The propeptide domain contains a conserved cysteine, which is ligated with the active site Zn++. Activation of pro-MMP-1 occurs when the prodomain is cleaved by other proteases or when the cysteine switch is disrupted. Oxidation activation may regulate many MMPs. Active MMP-1 has an open configuration. (Modified from Nelson and Melendez 2004).

MMP activity and function. The expression and proteolytic activity of MMP is regulated at different levels by gene expression, compartmentalization (i.e., pericellular accumulation of the enzyme), conversion from zymogen into active proteolytic enzyme or by the presence of specific inhibitors, TIMPs (Kessenbrock *et al*., 2010). The majority of MMPs are proteolytically activated in the extracellular space (Hidalgo and Eckhardt, 2001). In addition to the components of the ECM, MMPs can cleave also many of the bioactive molecules at or around the cell surface (Murphy and Nagase, 2011).

MMPs are capable of degrading almost all ECM components including a variety of proteins and growth factors (Mannello, 2011). However, ECM components are sensitive to proteolysis by different MMPs and not all ECM components are cleaved by each MMP (Löffek *et al*., 2011). The proteolytic activity of MMPs has an essential role in forming space for cells to migrate to organize tissue architecture through effects on the

ECM and intercellular junctions. Furthermore, the proteolytic activity of MMPs can produce specific substrate-cleavage fragments with different biological activities, and activate or deactivate signalling molecules (Page-McCaw *et al*., 2007). Most MMPs function in the extracellular environment, but recent studies have found MMP function also in the cytoplasm and nucleus (Mannello and Medda, 2012).

MMPs play an important role in tissue destruction and fibrosis as well as in weakening the ECM (Mannello and Medda, 2012). Althought the activity of MMPs has been shown to be essential in many biological processes, the uncontrolled MMP activity is an emerging area of interest. The central role for MMPs in cancer is increased tissue remodelling as well as degradation (Nagase *et al*., 2006; Mannello and Medda, 2012). This biological activity degrades ECM to make a path for cells to migrate and to invade to peripheral tissue and metastasize (Kessenbrock *et al*., 2010). Thus, in a wide range of human malignancies, the MMP expression is increased and correlates with tumour growth, increased invasiveness and poor survival (Eiseler *et al*., 2009; Köhrmann *et al*., 2009, Kaimal *et al*., 2013).

2.9.1 MMP-1 and breast cancer

Among the MMPs, the collagenolytic protease MMP-1 is the most highly expressed interstitial collagenase that degrades different types of fibrillar collagens (Sauter *et al*., 2008). Several studies indicate the important role of MMP-1 in the proteolysis of the ECM in normal tissue remodelling and growth (Zhang *et al*., 2013). MMP-1 is widely associated with many pathological conditions including cancer progression during invasion and metastasis, e.g. in melanoma, lung, colorectal and breast cancers (Fanjul-Fernandez *et al*., 2013). High MMP-1 expression is responsible for loosening cell adhesion enhancing both the invasion and migration of cancer cells during the metastatic process (Pulukuri and Rao, 2008; Sauter *et al*., 2008). In addition, MMP-1 activates other MMPs, such as MMP-2 and -9 (Mannello, 2011). Overexpression of MMP-1 is a high risk factor that associates with the overall survival in breast cancer patients (Feng *et al*., 2011; Liu *et al*., 2011). MMP-1 is expressed by the cancer cells themselves or by the surrounding stromal cells adjacent to invasive tumour (Eck *et al*., 2009; Köhrmann *et al*., 2009). By immunohistochemistry MMP-1 expression has been localized to the stromal cells of neoplastic tissue (Shiozawa *et al*., 2000) as well as to the cytoplasm of tumour epithelial cells (Sunami *et al*., 2000; Jordan *et al*., 2004), while in normal breast tissue no positive MMP-1 has been found (Köhrmann *et al*., 2009; Mannello and Medda, 2012). In situ hybridization studies have detected MMP-1 mRNA expression in stromal cells as well as in carcinoma cells (Shiozawa *et al*., 2000). In one report, MMP-1 immunostaining was restricted to the nuclei of tumour epithelial cells, which was unexpected, because MMPs are considered to be cytoplasmic or membrane-bound proteins (Köhrmann *et al*., 2009). Understanding the role of nuclear MMP-1 in various pathological events can reveal new MMP functions (Mannello and Medda, 2012).

Recent studies have shown that members of the MMP family have distinct roles at different stages during cancer progression and in different breast cancer subtypes (McGowan and Duffy, 2008; Gialeli *et al*., 2011). In breast cancer, MMP-1 expression has been shown to be up-regulated in basal-type cancers compared to non-basal type cancers (McGowan and Duffy, 2008). Because basal-type cancers have triple-negative receptor status, in addition to surgery, the only systemic therapeutic option is chemotherapy. Since MMP-1 is preferentially elevated in basal-type tumours, blocking the activity of MMP-1 should theoretically prove to be beneficial in the treatment of this subtype of breast cancer (McGowan and Duffy, 2008; Liu *et al*., 2012).

Latest studies report that tumour-produced MMP-1 regulates the permeability and breakdown of the endothelial barrier and makes it more penetrable for tumour cell invasion into the ECM (Juncker-Jensen *et al*., 2013). Stromal-derived MMP-1 is able to cleave and activate a G-protein-coupled receptor, namely protease-activated receptor-1 (PAR-1), which is a membrane protein and an oncogene (Mercuri and Thompson, 2005). Endothelial PAR-1 activation by MMP-1 leads to the activation of intracellular signal promoting cell migration towards the stroma (Ho *et al*., 2009). Blocking the action of either MMP-1 or PAR-1 should induce apoptosis and theoretically might serve as an ideal drug target in the treatment of invasive and metastatic breast cancer (Yang *et al*., 2009).

2.10 Decorin

Structure of decorin. Decorin, a small condroitin/dermatan sulfate PG produced by fibroblasts, has been cloned and sequenced from human sources in 1980s (Krusius and Ruoslahti, 1986). PGs are proteins that consist of one or more glycosaminoglycan side chains linked to a core protein and they are a significant component in the ECM participating in signalling events (Ruoslahti, 1989; Järveläinen *et al*., 1991; Kim *et al*., 2011). Decorin binds to the surface of the collagen fibrils through their core protein and project their side chains into the interfibrillar space (Orgel *et al*., 2009).

Decorin is a small leucine-rich extracellular PG, built up of four domains. The first domain encompasses a signal peptide followed by the propeptide. Both signal peptide and propeptide are proteolytically processed before the secretion of the mature decorin molecule into the extracellular space (Ameye and Young, 2002). The second domain has a single chondroitin or dermatan sulphate glycosaminoglycan (GAG) side chain and a cysteine-rich region. The third and central region of the decorin core protein is composed of ten leucine-rich repeats that take part in biological functions of decorin with other proteins. This domain contains also three potential sites for N-linked oligosaccharine substitution. The fourth domain of decorin contains two cysteine rich recidues (Reed and Iozzo, 2003; Zhao *et al*., 2013) (Figure 7). While much of decorin´s activity is associated with the core protein, a single GAG chain also plays a role in decorin´s function and the structure of protein-free GAG chain varies depending on the tissue in which it is biosynthesized (Zhao *et al*., 2013).

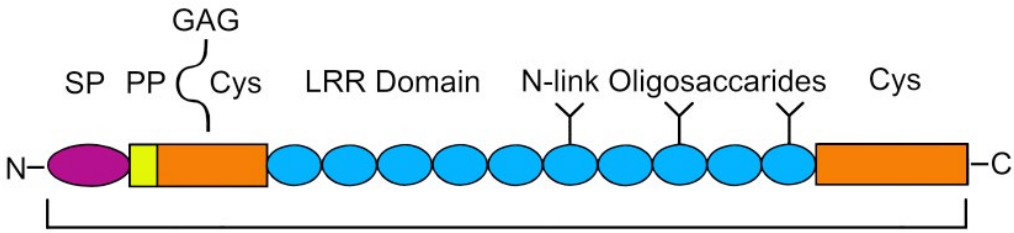

Figure 7. Structure of decorin. Decorin consists of a protein core containing ten leucine-rich repeats (LRR) flanked by cysteine-rich domains and a single chondroitin sulphate or dermatan sulphate glycosaminoglycan (GAG) chain, which is located near its N-terminus. Three N-linked oligosaccharides are located on the same side of the molecule. SP (signal peptide), PP (propeptide). (Modified from Chen and Birk 2011).

Regulation of collagen fibrillogenesis. Because decorin is widely expressed in human tissues and it is particularly abundant in type I and II collagen fibril-rich connective tissues, e.g. in skin, tendon, cornea and cartilage, it is thought to play a part in the control of fibrillogenesis (Vogel *et al.,* 1984; Danielson *et al.,* 1997; Reese *et al*., 2013). Decorin is primarily synthesized by fibroblasts and myofibroblasts (Goldoni and Iozzo, 2008), as well as by vascular smooth muscle cells (Järveläinen *et al*., 1991; Dugan *et al*., 2006). Decorin promotes the formation of fibers with variations in the stability and solubility of also other collagens including types III, V, VI, XII and XIV (Iozzo, 1999; Neill *et al*., 2012b). Decorin may bind to collagen with its core protein through leucine-rich repeat regions and to another decorin through the interaction with their GAG chains (Liu *et al*., 2005). In addition to regulating collagen fibril formation, decorin has a role in cell proliferation, differentiation, migration, adhesion, angiogenesis and in apoptosis (Schaefer *et al*., 2002; Reed and Iozzo, 2003; Tufvesson and Westergren-Thorson, 2003; Järveläinen *et al*., 2006; Fiedler and Eble, 2009; Bi *et al*., 2012; Buraschi *et al*., 2012; Jungman *et al*., 2012; Bi and Yang, 2013; Sainio and Järveläinen, 2013; Yu *et al*., 2014).

Regulation of growth factor activity. In normal adult tissue, decorin seems to play an important role in maintaining fibroblasts within an inactive state (Tran *et al*., 2003). In fact, decorin is seldom expressed by actively proliferating or transformed tissues (Tralhão *et al*., 2003). Through a specific region within the third domain of decorin core protein it forms a complex with TGF-β1, which is a powerful pro-fibrotic cytokine and an inhibitor of cell cycle progression of normal mammary epithelial and endothelial cells (Reiss and Barcellos-Hoff, 1997; Kolb *et al*., 2001; Seoane, 2006; Hu *et al*., 2009; Baghy *et al*., 2012). In the ECM, by directly binding to TGF-β1, decorin core protein has been shown to modulate a feedback system that regulates cell growth and prevents the fibrotic activity of TGF- β1 (Yamaguchi and Ruoslahti, 1988; Akman *et al*., 2013). TGF-β1 acts as an antiproliferative factor in normal epithelial cells and becomes a positive mediator of tumour progression later in tumour development (Reiss and Barcellos-Hoff, 1997; Bierie and Moses, 2006). The antifibriotic activity of decorin is related to the fact that it directly interacts with TGF-β1 as well as with collagen (Akman *et al*., 2013, Honardoust *et al*.,

2013). TGF-β1 induces fibroblast proliferation and transformation into myofibroblasts, stimulates the synthesis of ECM proteins and glycoproteins as well as activates growth factor activity (Maroni and Davis, 2012, Botfield *et al*., 2013). Decorin has the ability to bind and store free TGF-β temporarily in the ECM and to prevent TGF-β from binding to its receptor (Bi and Yang, 2013). TGF-β is released from decorin-complex by MMPs to carry out its biological function (Boivin *et al*., 2012).

Decorin is reported to be a bi-functional proteoglycan being a structural ECM component and also having the ability to act as a signalling molecule on the cell surface (Goldoni and Iozzo, 2008; Baghy *et al*., 2012; Frey *et al*., 2013). One of the main targets of decorin is to affect the biology of several receptor tyrosine kinases by triggering receptor internalization and degradation (Buraschi *et al*., 2013). Decorin binds to and modulates the signalling of epidermal growth factor receptor (EGFR) and other members of the ErbB family of receptor tyrosine kinases, such as hepatocyte growth factor receptor (Met), and tyrosine kinase type I insulin-like growth factor receptor (IGF-IR) (Zhu *et al*., 2005; Goldoni and Iozzo, 2008; Buraschi *et al*., 2010). Unlike the other EGF family of growth factors, the activation of EGFR by decorin induces growth suppression of many cell types such as fibroblasts and endothelial cells (Tran *et al*., 2003, Howe and Brown, 2011).

Decorin binds to multiple receptors with diverse affinity and especially with EGFR decorin has a high affinity binding (Iozzo and Schaefer, 2010). The core protein of decorin activates EGFR by rapid phosphorylation, which leads to the stimulation of mitogen-activated protein kinase (MAPK) and to elevation of endogenous p21, a cyclin-dependent kinase inhibitor, arresting the cells in the G1 phase of the cell cycle (Csordás *et al*., 2000; Bi *et al*., 2008; Goldoni and Iozzo, 2008; Iozzo and Schaefer, 2010; Neill *et al*., 2012b, Feugaing *et al*., 2013). In addition to EGFR, decorin has been shown to suppress cell growth by binding and modulating the signalling of other ErbB members such as ErbB2 and ErbB4 (Zhu *et al*., 2005; Goldoni and Iozzo, 2008; Bi and Yang, 2013).

Decorin induces tyrosine phosphorylation of Met, the receptor of hepatocyte growth factor (HGF), leading to a wide range of biological effects on mesenchymal and epithelial cells (Goldoni *et al*., 2009). Met is normally expressed by epithelial cells, while expression of HGF is restricted to cells of mesenchymal origin (Gentile *et al*., 2008). Met signalling promotes multiple biological activities, including proliferation, angiogenesis and motility of epithelial cells (Trusolino *et al*., 2010; Lefebvre *et al*., 2012). Decorin induces suppression of intracellular β-catenin via down-regulation of Met (Goldoni *et al*., 2009). β-catenin level and localization throughout the cell is controlled by E-cadherin, a protein that binds β-catenin and links the complex to the actin cytoskeleton maintaining epithelial integrity and epithelial barrier (George and Dwivedi, 2004). Thereby, the antimigratory action of decorin is proposed to happen through the interaction with E-cadherin (Bi *et al*., 2012; Feugaing *et al*., 2013).

Decorin has also been shown to act as a regulator of programmed cell death (Merline *et al*., 2009). Decorin prevents apoptosis by binding to IGF-IR (Hermanto *et al*., 2002; Galvan *et al*., 2003) and signalling via the P13K/Akt pathway (Merline *et al*., 2009; Iozzo *et al*., 2011). Decorin can positively regulate the IGF-IR system in endothelial cells leading to its phosphorylation (Schönherr *et al*., 2005; Iozzo *et al*., 2011). This interaction modulates endothelial cell motility and capillary morphogenesis (Fiedler *et al*., 2008). IGF-1 leads to the activation of ER protein via receptor-associated intracellular signalling and they act in a synergistic manner promoting cell proliferation in breast epithelial cells (Sarfstein *et al*., 2012; Skandalis *et al.,* 2013; Tsonis *et al*., 2013). Lack of decorin causes a significant increase in IGF-IR levels and promotes apoptosis, inflammation and fibrosis in renal tubular cells (Iozzo and Schaefer, 2010).

Role of decorin in angiogenesis. Angiogenesis, the formation of new blood vessels from existing vasculature, involves degradation of the ECM, migration, proliferation, and capillary tube formation of endothelial cells, followed by matrix remodelling (Grant *et al*., 2001; Salomäki *et al*., 2008; Pozzi and Zent, 2009). The involvement of decorin in angiogenesis is somewhat controversial (Neill *et al*., 2012b). Decorin is upregulated during angiogenesis (Järveläinen *et al*., 1992; Fiedler and Eble, 2009) and in the absence of decorin angiogenesis is dysregulated (Fiedler *et al*., 2008). While vascular endothelial cells normally do not express decorin (Järveläinen *et al*., 1991), it is thought that decorin controls endothelial cell-matrix interactions by signalling through IGF-IR and influencing integrin (Fiedler and Eble, 2009; Bi and Yang, 2013). Capillary endothelial cells synthesize decorin only during angiogenesis associated with profound inflammatory processes (Nelimarkka *et al*., 2001). On the other hand, in an experimental mouse model decorin has been shown to be primarily an inhibitor of angiogenesis (Järveläinen *et al*., 2006, Neill *et al*., 2012b). Later stages of angiogenesis, such as lumen formation and maturation, are probably also regulated by decorin (Fiedler and Eble, 2009). Direct interactions of decorin with cell surface receptors could play an important role in this process (Fiedler *et al*., 2008). The different biological activity of decorin may be explained by the different length or sulfation patterns of GAG chain as well as signals from the local environment (Fiedler and Eble, 2009).

Role of decorin in inflammation. In addition to the above mentioned functions, decorin is involved in the complex signalling during inflammation (Frey *et al*., 2013). Inflammation is not only a protective mechanism against microbial invasion, but inflammation may occur during tissue injury under sterile conditions (Moreth *et al*., 2012). In the latest studies decorin has been reported to act as an endogenous ligand of the toll-like receptors (TLR), a group of central receptors functioning in innate immune responses (non-specific immune system) (Merline *et al*., 2011; Neill *et al*., 2012a). By signalling through TLR2 and TLR4 pathways decorin gives rise to proinflammatory signalling of various chemo- and cytokines, including programmed cell death 4 (PDCD 4), and triggers an acute inflammatory response (Frey *et al*., 2013). In the ECM, decorin blocks the binding of TGF-β1 to its receptor, thereby suppressing the maturation of

oncogene miR-21, a post-transcriptional inhibitor of PDCD 4 (Merline *et al*., 2011). Overexpression of decorin increases TLR2/4 driven synthesis of PDCD 4, which reduces the production of the anti-inflammatory cytokines such as interleukin-10. As a result, the decorin-evoked signalling drives the immune reaction toward a more apoptotic and inflammatory response (Merline *et al*., 2011; Moreth *et al*., 2012; Frey *et al*., 2013). In sepsis, decorin is an early response gene evoked by inflammation, and treatments that decrease decorin abundance could be used to calm inflammation (Moreth *et al*., 2012; Merline *et al*., 2011).

2.10.1 Decorin and cancer

During tumourigenesis, cancer cells secrete growth factors, chemokines and matrix-degrading enzymes that stimulate cell growth, angiogenesis and activate stromal cells to release mitogenic substances which in turn further stimulate tumour cell growth (Wels *et al*., 2008; Theocharis *et al*., 2010). The role of decorin in cancer progression and the possible therapeutic potential of decorin as a tumour suppressing antimetastatic agent have been recognized in numerous studies (Iozzo and Sanderson, 2011; Theocharis *et al*., 2010; Neill *et al*., 2012b; Sainio and Järveläinen, 2013). Earlier studies have shown that lack of decorin does not lead to the development of spontaneous tumours (Danielson *et al*., 1997), but it assists tumourigenesis (Iozzo *et al*., 1999).

High level of decorin mRNA expression has been reported during early stages of tumourigenesis, while low expression levels has been found in advanced disease stage, e.g in lymphoma and prostate cancer (Feugaing *et al*., 2013; Suhovskih *et al*., 2013). Decorin expression is down-regulated both at protein and mRNA level in a variety of epithelial tumours such as in breast (Troup *et al*., 2003), colorectal (Bi *et al*., 2012), ovarian (Nash *et al*., 2002), lung (Campioni *et al*., 2008) and prostate (Henke *et al*., 2012) cancers, while decorin expression is preserved in the surrounding ECM (Köninger *et al*., 2004, Feugaing *et al*., 2013). In addition, cancers such as pancreatic cancer that is characterized by a strong desmoplastic reaction, express abundant decorin (Goldoni and Iozzo, 2008). Loss of decorin favours a clear decrease in cell differentiation, an increase in cell proliferation and metastatic spreading (Bi *et al.*, 2008; Goldoni and Iozzo, 2008). Breast and lung cancers expressing low levels of decorin and high levels of EGFR have been reported to be associated with a short progression time and a poor outcome (Troup *et al*., 2003; Goldoni and Iozzo, 2008; Araki *et al*., 2009; Biaoxue *et al*., 2011). While in most studies decorin has been shown to have an antioncogenic role, it has been found to have also an opposite role on tumour progression (Cawthorn *et al*., 2012). There are studies suggesting a pro-invasive effect for decorin and decorin overexpression is correlated with increased tumour invasion and metastasis, e.g. in prostate, oral squamous cell and bladder cancers (Dil and Banerjee, 2011; Neill *et al*., 2012a; El Behi *et al*., 2013; Suhovskih *et al*., 2013). Muscle-invasive bladder cancers have shown decorin overexpression compared to non-invasive cancer (El Behi *et al*., 2013).

Possible variations in decorin expression by immunohistochemistry may be explained by intratumour heterogeneity or even by inappropriate analysis tools (Michor and Polyak, 2010). Immunohistochemical analysis can be influenced by length and method of specimen fixation and processing, pretreatment protocols, reagents and substrates used, specificity and sensitivity of detection system and choice of control material (Dacic *et al*., 2006). Also the controversial results can be explained by individual variation of decorin protein in different types and stages of carcinomas (Niedworok *et al*., 2013; Suhovskih *et al.,* 2013).

Anti-oncogenic properties of decorin. Decorin binds a large number of structural components within the ECM and affects the biology of various types of cancer by inhibiting the activity of several receptors that are overexpressed, mutated or involved in tumour progression (Cabello-Verrugio and Brandan, 2007; Buraschi *et al*., 2012; Bi and Yang, 2013). Decorin has been proposed to be a "pan-ErbB receptor inhibitor" that can antagonistically interact with multiple signalling pathways causing a reduction in primary tumour growth and metastatic spreading by slowing cell motility and weakening cell invasion through the ECM (Goldoni *et al*., 2008; Neill *et al*., 2012b). Studies on prostate cancer have identified decorin induced protein mitostatin, which is expressed in most normal human tissues, but markedly down-regulated in advanced stages of bladder and breast tumours (Fassan *et al*., 2011). The role of mitostatin is thought to be in the control of cell growth and apoptosis (Vecchione *et al*., 2009).

By inducing proinflammatory signalling, decorin links inflammation, immunosuppression and tumour growth together (Merline *et al*., 2011; Neill *et al*., 2012b). During cancer progression immune cells including tumour associated macrophages polarize differently and do not exert their immune function but rather favour tumour growth and angiogenesis at later stages to promote cancer (Lamagna *et al*., 2006). Decorin is a natural biological non-toxic product and therefore it may not be immunogenic by itself (Reed *et al*., 2002; Feugaing *et al*., 2013). In cancer, decorin has been identified to reduce the abundance of anti-inflammatory molecules (e.g. interleukin-10) and to induce the synthesis of pro-inflammatory modulators (e.g. tumour necrosis factor-α, interleukin-12b) (Merline *et al*., 2011; Neill *et al*., 2012b). Decorin mediated regulation of inflammatory processes can shift the immune reaction to the suppression of tumourigenic growth (Merline *et al*., 2011; Moreth *et al*., 2012; Neill *et al*., 2012b; Frey *et al*., 2013).

Localization of decorin expression in cancer cells. Although several studies have been performed to examine decorin expression in different cancers, some uncertainty has remained whether cancer cells express decorin or not. Particularly, the localization of decorin expression at the cellular level in cancer in vivo has remained open. In spite of the fact that most epithelial cells do not produce decorin, ectopic expression of decorin induced by adenoviral-mediated delivery has been shown to change the expression of several hundred stromal genes producing an unfavourable tumour microenvironment for tumour progression and metastasis (Santra *et al*., 1997; Köninger *et al*., 2004; Sainio

and Järveläinen, 2013). Overexpression of decorin or its addition to culture media has been shown to cause profound cytostatic effects on many cancer cells independent of their histogenetic backgrounds (Santra *et al*., 1997). The mechanisms by which decorin expression is generally blocked and modified in cancer patients are not known (Goldoni and Iozzo, 2008). As can be expected, the potential use of decorin as an anticancer agent is a promising target for cancer research (Neill *et al*., 2012a; Pucci-Minafra *et al*., 2008; Järveläinen *et al*., 2009; Theocharis *et al*., 2010; Sainio and Järveläinen, 2013).

3. AIMS OF THE STUDY

Because of the complexity of breast cancer, this study aimed to bring up new insights to predict the outcome of breast cancer patients and to find out new prognostic factors. The purpose of this research was to study cell cycle regulators, cyclins, and matrix metalloproteinase MMP-1 in breast cancer. Furthermore, special emphasis was made to explore the antitumour effect of the ECM proteoglycan, decorin, in human breast cancer. The specific aims of this study were:

1. To study the prognostic value of combined expression of cyclins A, B1, D1 and E in different breast cancer subtypes in combination with traditional prognostic factors

2. To examine the immunohistochemical expression of MMP-1 and its localization in correlation with well-known traditional prognostic factors in breast cancer epithelial cells and cancer associated stromal cells

3. To localize decorin expression in healthy human breast tissue and in human breast cancers. In addition, the effect of decorin transduction on the behaviour of cultured human breast cancer MCF7 cells was examined

4. MATERIALS AND METHODS

4.1 Patients and tumour samples

Studies I-III comprised tissue samples from breast cancer patients who were operated and treated at Turku University Hospital during the years 1985-2008. The clinical characteristics of the patients in the studies are described in Table 5. The patients were selected from the routine files of radical mastectomies to have enough material for the examination. Only tumours derived from epithelial cells were included in the studies I-III. All patients had over 10 mm invasive breast cancer and the age of the patients ranged from 30 to 94 years at the time of diagnosis.

Samples for immunohistochemical stainings and for mRNA analyses in the studies I and III were excised from the invasive border of the tumour after the surgical removal of the breast. The fresh tumour specimens were cut in two pieces. One half was frozen in liquid nitrogen and stored at -72 °C for mRNA analysis with real-time quantitative polymerase chain reaction (RT-qPCR), while the other half was fixed in 10% phosphate buffered formaldehyde and embedded in paraffin.

In the study II, follow-up information on life status was collected for each case (Central Statistical Office of Finland). Patients´ clinical history and tumour characteristics including tumour size and lymph node status were obtained from the pathology database (Table 5). None of the patients received radiation- or chemotherapy before the operation. Slides were reviewed to confirm the diagnosis of breast carcinoma. In situ carcinomas were not included in this study.

In the study III normal human female breast tissue (three samples) and intraductal papillomas (three samples) were obtained through reduction mammoplasty of the healthy side.

Table 5. Patients and tumour characteristics in the studies I-III.

Characteristics	Number of patients (%) in studies I-III		
	I	II	III
Number of the patients	53	125	69
Age	40-94 (mean 67)	30-90 (mean 57.5)	40-94 (mean 65.4)
Grade			
I	7 (13.2%)	10 (8%)	8 (11.6%)
II	24 (45.3%)	66 (52.8%)	36 (52.2%)
III	18 (34%)	49 (39.2%)	25 (36.2%)
in situ II	1 (1.9%)		
in situ III	3 (5.7%)		
Axillary nodal status			
N0	25 (47.2%)	64 (51.2%)	33 (47.8%)
N≥1	26 (49%)	50 (40%)	33 (47.8%)
Unknown (axillary evacuation done 1993 and 1994)	2 (3.8%)	11 (8.8%)	3 (4.3%)
Estrogen receptor (ER) status [1)]			
Positive	35 (66%)	80 (64%)	56 (81.2%)
Negative	14 (26.4%)	45 (36%)	13 (18.8%)
Positive in DCIS	3 (5.7%)		
Negative in DCIS	1 (1.9%)		
Progesterone receptor (PR) status [1)]			
Positive	36 (68%)	82 (65.6%)	53 (76.8%)
Negative	13 (24.5%)	43 (34.4%)	16 (23.2%)
Positive in DCIS	3 (5.7%)		
Negative in DCIS	1 (1.9%)		
Ki-67 status [2)]			
low ≤ 15%	17 (32%)	63 (50.4%)	23 (33.3%)
intermediate 16-30%	19 (35.8%)	41 (32.8%)	26 (37.7%)
high > 30%	17 (32%)	20 (16%)	20 (29%)
one value missing	1 (0.8%)		
Histologic type			
Ductal	37 (69.8%)	110 (88%)	49 (71.0%)
Lobular	8 (15.1%)	10 (8%)	11 (15.9%)
Subtypes	4 (7.5%)	5 (4%)	9 (13.0%)
Ductal carcinoma in situ	4 (7.5%)		
HER2 [3)]			
IHC positive (2+ and 3+)	20 (37.7%)	25 (20%)	27 (39.1%)
IHC negative (0 and 1+)	29 (54.7%)	100 (80%)	4 (60.9%)
IHC positive in DCIS	2 (3.8%)		
IHC negative in DCIS	2 (3.8%)		
CISH positive	10 (18.9%)		11 (15.9%)
CISH positive in DCIS	2 (3.8%)		
CK 5/6 [4)]			
Triple-negative (ER-, PR-, HER2-)	11 (20.8%)	35 (28%)	7 (10.1%)
Basal-like carcinoma (ER-, PR-, HER2-, CK5/6+)	8 (15.1%)	20 (16%)	4 (5.8%)

[1,4)] Cut off point used for ER and PR immunohistochemistry is nuclear positivity in 10% of tumour cells and 10% cytoplasmic staining in tumour cells for CK5/6.

[2)] Proliferation index according to St Gallen Consensus (Goldhirsch *et al*., 2009).

[3)] All IHC HER2+ and 3+ cases were retested by CISH. Scoring of HER2 immunohistochemistry: Score 0: no staining is observed or cell membrane staining is observed in less than 10% of tumour cells. Score 1+: a faint perceptible membrane staining can be detected in more than 10% of the tumour cells or cells are only stained in part of their membrane. Score 2+: a weak to moderate complete membrane staining is observed in more than 10% of the tumour cells. Score 3+: a strong complete membrane staining is observed in more than 10% of the tumour cells.

4.2 Methods

4.2.1 Histology and immunohistochemistry (I, II, III)

In the studies I-III, four µm thick serial paraffin sections were cut from tumour tissue and stained with haematoxylin and eosin (HE). The tumour histology in the studies I-III was assessed according to the WHO classification (Ellis *et al*., 2003) and tumour grading was based on the recommendations made by Elston and Ellis in 1991 (Elston and Ellis, 1991). Tumour size and axillary lymph node status were also analysed. In the study I immunohistochemical staining for cyclins A, B1, D1 and E, ER and PR, Ki-67, CK5/6 and HER2 were performed from subsequent sections. After reviewing the HE sections, two breast carcinoma areas per patient were selected for tissue microarray technique (TMA). Four µm thick sections were cut from array blocks and transferred to glass slides. Immunohistochemical staining for cyclins A, B1, D1 and E were performed on TMA-slides. Mitotic figures were counted on light microscope at the most cellular area of the tumour periphery from 10 high-power fields (40x objective, area of a single field 0,23 mm^2, field diameter 0,54 mm) (Meyer *et al*., 2009). From these areas, the standardized mitotic index (SMI), which gives the number of observed mitoses per square millimetre of malignant epithelium, was determined.

In the study II immunohistochemical staining for MMP-1, ER, PR, Ki-67, HER2, bcl-2, p53 and CK5/6 were performed.

In the study III the immunohistochemical profile of different human breast cancers was characterized by ER and PR, HER2, Ki-67 and p63 stainings, of which p63 was done to identify myoepithelial cells in some cases. Immunohistochemical stainings for cyclins A, B1, D1 and E, MMP-1, bcl-2, ER and PR receptors, Ki-67, CK5/6 and HER2 were carried out in the studies I-II with Tech-Mate 500+ immunostainer using monoclonal antibodies and a peroxidase/diaminobenzidine LSAB+ or EnVision detection kit (Dako, Hamburg, Germany). The LSAB method is based on a modified labeled avidin-biotin (LAB) technique, in which a biotinylated secondary antibody forms a complex with peroxidise-conjugated streptavidin molecules. In EnVision technique the primary antibody is followed by a polymeric conjugate in sequential steps (Sabattini *et al*., 1998). The used antibodies, dilutions and pre-treatments in the studies I-II are presented in Tables 6-7.

Table 6. Immunohistochemical staining protocol used in the studies I and II. Immunohistochemical stainings for cyclins A, B1, D1 and E as well as ER, PR, Ki-67, HER2, MMP-1, p53, bcl-2 and CK5/6 were performed from formalin fixed, paraffin embedded specimens with TechMate 500+ immunostainer using monoclonal antibodies and a peroxidase/ diaminobenzidine LSAB+ detection kit (DAKO, K5001).

Antibody	Source (clone)	Dilution	Pre-treatment in microwave oven, 2 x 7 min, 850W	Study
ER	DAKO M7047 **(1D5)**	1:40	10 mM TRIS-HCl 1mM EDTA (pH 9)	I, II
PR	Novocastra NCL-PGR) **(1A6)**	1:20	10 mM TRIS-HCl 1mM EDTA (pH 9)	I, II
Ki-67	DAKO M7240 **(MIB1)**	1:10	10 mM sodium citrate buffer (pH 6)	I, II
HER2	Novocastra NCL-CB1 **(CB11)**	1:50	10 mM sodium citrate buffer (pH 6)	I, II
Cyclin A	Novocastra NCL-CYCLIN A **(6E6)**	1:60	10 mM sodium citrate buffer (pH 6)	I
Cyclin B1	Novocastra NCL-CYCLIN B **(7A9)**	1:10	10 mM sodium citrate buffer (pH 6)	I
Cyclin D1	LabVision RM-9104-S **(SP4)**	1:50	10 mM TRIS-HCl 1mM EDTA (pH 9)	I
Cyclin E	Zymed **(HE12)**	1:50	10 mM TRIS-HCl 1mM EDTA (pH 9)	I
Cytokeratin5/6	DAKO M7237 (**D5/16B4)**	1:50	10 mM TRIS-HCl 1mM EDTA (pH9)	I, II
MMP-1	Oncogene **(41-1E5)**	1:100	10 mM TRIS-HCl 1mM EDTA (pH9)	II
p53	DAKO M7001 **(DO-7)**	1:200	10 mM TRIS-HCl 1mM EDTA (pH9)	II
bcl-2	DAKO M0887 **(124)**	1:200	10 mM TRIS-HCl 1mM EDTA (pH9)	II

Table 7. Immunohistochemical staining protocol used in the studies I and III. Five different ready-to-use mouse or rabbit monoclonal antibodies and cyclins for tissue microarray study were used with Benchmark XT immunostainer and *ultra*View Universal DAB Detection Kit (Ventana/ Roche, Tucson, Arizona, USA).

Antibody	Source (clone)	Dilution	Program	Study
ER	Roche **(SP1)**	ready to use	mild CC1 + 24 min antibody incubation	III
PR	Roche **(1E2)**	ready to use	mild CC1 + 28 min antibody incub. + ampl.kit	III
HER2	Roche **(4B5)**	ready to use	mild CC1 + 24 min antibody incubation	III
Ki-67	Roche **(30-9)**	ready to use	mild CC1 + 12 min antibody incubation	III
p63	Roche **(4A4)**	ready to use	standard CC1 + 32 min antibody incubation	III
Cyclin A	Novocastra **(6E6)**	1:5	standard CC2 + 44 min antibody incubation	I
Cyclin B1	Novocastra **(7A9)**	1:5	standard CC2 + 32 min antibody incubation	I
Cyclin D1	NeoMarkers **(SP4)**	1:20	standard CC1 + 44 min antibody incubation	I
Cyclin E	Zymed **(HE12)**	1:10	standard CC1 + 32 min antibody incubation	I

In the study III five different ready-to-use mouse or rabbit monoclonal antibodies for ER, PR, HER2, Ki-67 and p63 (Ventana Medical Systems/Roche Diagnostics, Tucson, Arizona, USA) and in study I for cyclins A, B1, D1 and E for TMA were used with

Ventana BenchMark XT immunostainer and *ultra*View Universal DAB Detection Kit (Ventana/Roche, Table 7). *Ultra*View Universal DAB is a multimer-technology based detection system intended for the specific and sensitive detection of mouse and rabbit primary antibodies. The kit is biotin-free and thus it eliminates non-specific staining resulting from endogenous biotin. The cyclin staining's of TMA were comparable with the staining performed with TechMate 500+ immunostainer and LSAB+ detection kit.

4.2.2 Evaluation of immunohistochemical stainings (I, II, III)

The nuclear staining of cyclins A, B1, D1 and E, p53, ER, PR and Ki-67 was evaluated from cells at the border of the most cellular part of the carcinoma. The immunohistochemical staining for MMP-1 in breast cancer cells was observed both in nuclei and in the cytoplasm. HER2 expression was evaluated as membrane staining of invasive tumour cells and scored to four classes (0/1+/2+/3+). The areas showing necrosis or inflammation were excluded from the analysis. The cell counting analysis was performed for cyclins, Ki-67, MMP-1, p53 and hormone receptors independently by two observers without any knowledge of the clinical data. Using the high-power (40x) objective the number of immunopositive cancer cells per 100 malignant cells (0% to 100%) from three separate cell rich areas was counted and the mean value of immunopositive areas was recorded. In addition to tumour cells MMP-1 staining in stromal fibroblasts near the tumour was evaluated in the same way. For bcl-2, cytoplasmic staining was scored and the percentage of positive tumour cells was recorded. Carcinomas were divided by their immunohistochemical profiles into luminal A and B, HER2 overexpressing, normal breast-like and triple-negative carcinomas. Triple-negative cases (ER-, PR- and HER2-) were stained with basal cytokeratin CK5/6, and the expression was considered positive if at least 10% of the cancer cells showed cytoplasmic and/or membranous staining (Rakha and Ellis, 2009).

4.2.3 Construction of tissue microarrays (I)

Two carcinoma areas from each breast tumour were selected on HE-stained sections and two 1,2 mm diameter cores from the donor block were transferred to the recipient block with a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Four μm thick sections were cut from the recipient TMA block on electrostatically charged glass slides (SuperFrost +) for immunohistochemical staining. The interpretation of results was performed as described above. The immunohistochemical staining data of TMA for cyclins A, B1, D1 and E were compared with the results of the traditional large histological slides.

4.2.4 HER2 chromogen in situ hybridization (I,III)

Chromogen in situ hybridization (CISH) of breast cancer samples was performed in the studies I and III in order to confirm the immunohistochemical 2+ - 3+ cases of HER2 protein expression as well as the ambiguous results. The test was performed on 4 μm

thick paraffin sections using the SPoT-Light HER2 CISH-kit, in which the gene specific HER2 probe was labelled with digoxigenin (Zymed, South San Francisco, CA, USA). The target gene was localized with a HRP/DAB-detection system. The gene expression of HER2 was interpreted as positive if 6 or more gene copies and/or clusters were observed in at least 10% of the tumour cells (Zhao *et al*., 2002).

4.2.5 Reverse transcription quantitative polymerase chain reaction (I, III)

Cyclins A, B1, D1 and E mRNA levels in the study I were analysed from 12 tumour samples (one ductal carcinoma grade II and lobular carcinoma grade II, two ductal carcinomas grade I and lobular carcinomas grade I, three ductal carcinomas grade III and ductal carcinoma in situ grade III) using normal human breast tissue as a reference. Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). For each sample, 1µg of RNA was treated with RQ1 DNase (Promega, Madison, WI, USA) and reverse transcribed using Moloney murine leukaemia virus RNase H (Promega, Madison, WI, USA). Primers and probes for the cyclin genes were chosen using the ProbeFinder software program (Roche Applied Science, Basel, Switzerland) and for the *GAPDH (*glyceraldehyde 3-phosphate dehydrogenase*)* the Primer Express 2.0 software program (Roche, Applied Biosystems, Mannheim, Germany). Sequences of primers used in RT-qPCR studies I and III are presented in Table 8. ABsolute QPCR ROX Mix (Abgene, Epsom, United Kingdom) was used to prepare the reaction mixes, and the PCR was performed with ABI Prism 7700 Sequence Detection system (Roche, Applied Biosystems, Mannheim, Germany). Relative quantities of cyclin mRNAs were normalized against *GAPDH* and relative gene expression was calculated using the $2^{-\delta\delta ct}$ method with cDNA from normal breast tissue as a reference (Livak, 2001).

Table 8. Primer and probe sequences used in real-time quantitative polymerase chain reaction (Studies I and III).

Gene	Primer sequence (5´-3´)	
Cyclin A	Sense	CCATACCTCAAGTATTTGCCATC
	Antisense	TCCAGTCTTTCGTATTAATGATTCAG
Cyclin B1	Sense	CATGGTGCACTTTCCTCCTT
	Antisense	AGGTAATGTTGTAGAGTTGGTGTCC
Cyclin D1	Sense	TCCAGAGTGATCAAGTGTGACC
	Antisense	TGGGGTCCATGTTCTGCT
Cyclin E	Sense	ACAGCTTGGATTTGCTGGA
	Antisense	TCTGCTTCTTACCGCTCTGTG
GAPDH	Sense	ACCCACTCCTCCACCTTTGA ACCCACTCCTCCACCTTTGA
	Antisense	TTGCTGTAGCCAAATTCGTTGT
Decorin	Sense	GGACCGTTTCAACAGAGAGG
	Antisense	GAGTTGTGTCAGG GGGAAGA
GNB2L1	Sense	GAGTGTGGCCTTCTCCTCTG
	Antisense	GCTTG CAGTTAGCCAGGTTC

In the study III RNA concentration from the extractions was determined using a NanoDrop spectrophotometer (ThermoScientific, Waltham, MA, USA) and the integrity of the RNA was confirmed with agarose gel electrophoresis. One µg of RNA was DNase treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) and reverse transcribed into cDNA using M-MLV reverse transcriptase and Oligo(dT)15 primer (Promega, Madison, WI, USA) according to manufacturer's instructions. RT-qPCR was performed using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) with 100 nM primer concentrations and final volume of 10 µL according to manufacturer's protocol. GNB2L1 was chosen as a reference gene (Zhang *et al*., 2005). Reactions were run on an Applied Biosystems 7900HT machine (Roche, Applied Biosystems). The qPCR protocol consisted of initial denaturation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 40 seconds and extension at 60°C for 45 seconds. The specificity of the reactions was confirmed by melt-curve and agarose gel analysis. Triplicate CT values were analysed using the comparative CT ($2^{-\Delta\Delta CT}$) method**.**

4.2.6 Decorin in situ hybridization (III)

Decorin in situ hybridization (ISH) in the study III was performed on 4 µm breast tissue sections by probing with human decorin antisense and sense single-stranded RNA riboprobes. A 533 bp fragment containing human decorin cDNA was cloned into the Eco RI/Hind III site of pGEM-4Z transcription vector (kindly provided by Dr. Liliana Schaefer, University of Frankfurt, Frankfurt am Main, Germany). Linearized plasmid DNA was purified with QIAquick PCR Purification Kit (Qiagen) and digoxigenin (DIG)-labeled sense and antisense RNA probes were synthesized by in vitro transcription with SP6 and T7 polymerases, respectively, by using a DIG RNA Labeling Kit (Roche, Applied Science, Mannheim, Germany). Probe quantification was carried out with a DIG Nucleic Acid Detection Kit (Roche, Applied Science, Mannheim, Germany) and ISH was performed as described (Salomäki *et al*., 2008).

4.2.7 Adenoviral vectors (III)

For transduction experiments in the study III, a recombinant replication-deficient adenoviral vector dcn-pxc1c-1 was used. This vector harbors the human decorin (dcn) cDNA under the control of cytomegalovirus (CMV) promoter. For the preparation of the vector, full length human decorin cDNA (Fisher *et al*., 1989) in pGEM plasmids was cloned and inserted into shuttle plasmid pxcJL-1. The viruses were prepared by cotransfecting HEK293 –cells with back bone plasmid pBHG10. As a control vector RAdlacZ, which harbors the E. coli ß-galactosidase gene (lacZ) under the control of CMV IE promoter was used (Wilkinson, 1992). This vector was purchased from the Virus Vector Facility, Centre for Biotechnology, University of Turku, Turku, Finland.

4.2.8 Decorin transduction (III)

Human breast adenocarcinoma cell line MCF-7 was used in the study III for transduction with a recombinant replication-deficient adenoviral vector dcn-pxc1c-1. MCF-7 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 25 μM insulin, 1 nM β-estradiol, 2 mM L-glutamine, penicillin (100 IU/mL) and streptomycin (100 μg/mL), and grown at 37 °C with 5% CO_2. The cells were plated on a 24-well plate (Greiner Bio-One, Kremsmuenster, Austria), 30 000 per plate. The next day, cells were transduced with 0, 3, 30, 100, 300 and 1000 pfu/cell of dcn-pxc1c-1 or RAdlacZ in reduced medium containing no FBS. Four parallels were made of each vector concentration. After 24 h incubation, the cells were washed twice with reduced medium and incubated in this medium for another 24 h. The cells were trypsinized, pooled, and the RNA was extracted using NucleoSpin RNA II –kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

4.2.9 GeneSapiens database (III)

The GeneSapiens database was used to compare decorin gene expression levels between healthy human breast tissue and selected types of human breast cancer (Kilpinen *et al*., 2008). This database (http://www.genesapiens.org/) covers the relative gene expression patterns for 17330 genes across all the 9783 annotated healthy and pathological human tissue samples from publicly available Affymetrix microarray experiments. The database contains 15 healthy breast tissue samples and 1504 different human breast cancer samples.

4.2.10 Statistical analyses (I, II, III)

The association between tumour grade and the immunohistochemical expression of different cyclins in the study I was tested with Kruskal-Wallis and pairwise comparisons with Mann-Whitney U–tests. In pair wise comparison Bonferroni-adjustment was applied. Correlations were calculated using Spearman rank-order correlation coefficients. Non-parametric tests were applied because of non-normal distribution of variables. Unpaired Student´s *t*-test was used in statistical analyses in study III. All p values <0.05 were considered statistically significant.

Breast cancer-specific survival time in the study II was determined from time of diagnosis until death from breast cancer. Patients who were alive at the end of the follow-up on October 2009 or died of other causes were used as censored values in survival analyses. Kaplan-Meier survival analyses were carried out to compare breast cancer-specific survival curves. Univariate Cox regression model was used to examine prognostic factors for breast cancer-specific survival. Factors significantly associated with disease-specific survival in univariate models were included in multivariate stepwise Cox regression model (inclusion and exclusion criteria p=0.05). Results were expressed using hazard ratios (HR) with 95% confidence intervals (CI) and P-values less

than 0.05 were considered as significant. The statistical analyses were carried out using SAS/STAT(r) software, Version 9.1.3 SP4 of the SAS System for Windows.

4.3 Ethics

Ethical approval for the use in clinical material of the studies was given by Turku University Hospital Ethics committee (no 241/2005) and the Finnish National Authority for Medicolegal Affairs (no 4424/32/300/02).

5. RESULTS

5.1 Cyclins A, B1, D1 and E in breast cancer (I)

5.1.1 Comparison between immunohistochemical expression of cyclins A, B1, D1, E and traditional prognostic factors (I)

In the study I the aim was to collectively analyse the expression of cyclins A, B1, D1 and E in 53 breast cancer cases and to determine the correlation with tumour grade, immunohistochemical analysis of ER, PR, HER2 and Ki-67. Based on the expression of ER, PR, HER2 and CK5/6, tumours were divided into subgroups such as triple-negative (ER-, PR-, HER2-, CK5/6-) and basal-like (ER-, PR-, HER2-, CK5/6+) breast carcinomas and the combined analysis of cyclin expression was correlated with these subtypes.

The mean standardized mitotic index (mitoses/mm²) was in invasive breast carcinomas 8.6 (range 0-72.5) and in ductal carcinomas in situ 16.3 (range 6.8-29.3). A significant positive correlation was found between tumour grade and Ki-67 status ($r = 0.62$, $p < 0.0001$) as well as HER2 expression ($r = 0.35$, $p = 0.0104$). In addition, tumour grade showed a significant positive correlation with standardized mitotic index ($r = 0.60$, $p < 0.0001$).

The expression of cyclin A ranged from 0% to 59% and cyclin B1 from 0% to 30% in breast cancer samples. The expression of cyclin E was higher as compared to cyclins A and B1 ranging from 1% to 76%. The widest range between 3% to 90% was in the expression of cyclin D. The immunohistochemical expression of cyclins A, B1 and E showed significant association with tumour grade ($p = 0.0011$ for cyclin A, $p = 0.0047$ for cyclin B1, $p = 0.0005$ for cyclin E), while cyclin D1 showed no significant correlation (Figure 8).

When the traditional prognostic factors were studied, cyclin A, B1 and E expression showed positive correlation with Ki-67 expression ($r = 0.71$, $p < 0.0001$ for cyclin A, $r = 0.57$, $p < 0.0001$ for cyclin B1 and $r = 0.60$, $p < 0.0001$ for cyclin E) and no significant correlation was found between cyclin D1 staining and Ki-67 expression. Cyclins A and B1 did not show any correlation with hormone receptors ER or PR. However, a significant negative correlation came up between cyclin E and ER ($r = -0.37$, $p = 0.0100$) and PR ($r = -0.35$, $p = 0.0153$), while cyclin D1 had a significant positive correlation with the expression of ER ($r = 0.37$, $p = 0.0088$) and PR ($r = 0.33$, $p = 0.0233$). In addition, cyclin A, B1 and E expression showed positive correlation with HER2 ($r = 0.32$, $p = 0.0264$ for cyclin A, $r = 0.43$, $p = 0.0026$ for cyclin B1, $r = 0.34$, $p = 0.0199$ for cyclin E), while cyclin D1 showed no significant correlation with HER2.

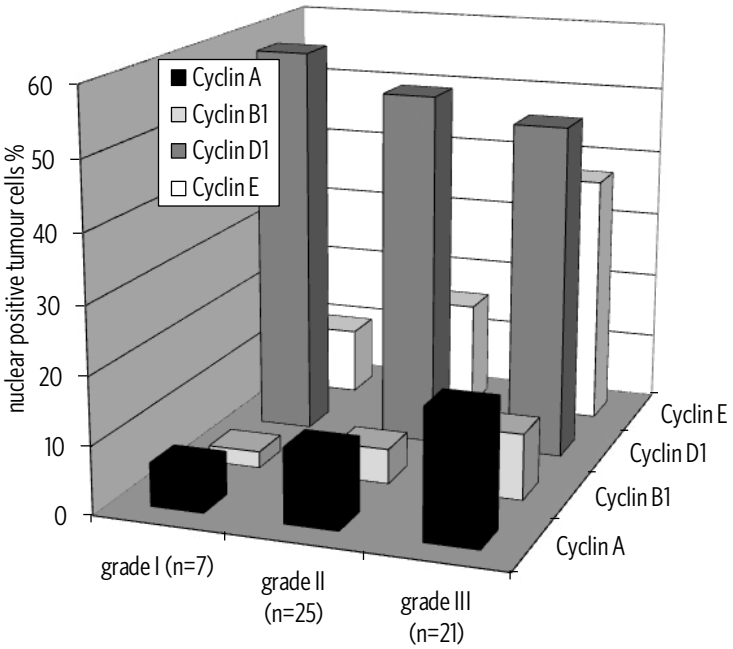

Figure 8. The association of histological grade of the breast cancer with the immunohistochemical expression of cyclin A, B1, D1 and E. P-values for cyclin A: overall p=0.0025, grade I vs. II P=0.3774, grade I vs III p=0.0069, grade II vs III p=0.0321. P-values for cyclin B1: overall p=0.0058, grade I vs II p=0.5466, grade I vs III p=0.015, gradus II vs III p=0.048. Cyclin D1 did not show any correlation with the grades. P-values for cyclin E: overall p<0.0001, grade I vs II p=0.294, grade I vs III p=0.0063, grade II vs III p<0.0003.

Cyclin A, B1 and E expression showed also positive correlation with standardized mitotic index ($r = 0.71$, $p < 0.0001$ for cyclin A, $r = 0.44$, $p = 0.0019$ for cyclin B1 and $r = 0.54$, $p < 0.0001$ for cyclin E), while cyclin D1 showed no significant correlation. Furthermore, cyclin E expression correlated with triple-negative cancers ($p = 0.0474$), while no correlation was found with basal-like carcinomas. Cyclin D1 expression correlated with non-triple negative ($p = 0.0156$) and non-basal-like ($p = 0.0279$) carcinomas. Cyclin A did not show any correlation with triple-negative or basal-like breast cancers, nor did cyclin B1.

The immunohistochemically detected expression of cyclins A, B1 and E strongly correlated with each other, specially cyclins A and B1 ($r = 0.60$, $p < 0.0001$), which also had a significant positive correlation with cyclin E ($r = 0.49$, $p = 0.0004$ for cyclin A, $r = 0.52$, $p = 0.0001$ for cyclin B1), while the expression of cyclin D1 correlated with none of the other cyclins.

5.1.2 Results of tissue microarray (I)

In the present study I the results of the conventional large section slides were similar with the staining results of tissue microarray slides (Figure 9), which also showed a

positive correlation with mitotic index (r=0.48, p=0.0006 for cyclin A, r=0.28, p=0.0530 for cyclin B1, r=0.36, p=0.0137 for cyclin E), as well as with Ki-67 expression (r=0.53, p=0.0001 for cyclin A, r=0.41, p=0.0041 for cyclin B1, r=0.41, p=0.0039 for cyclin E). In summary, the immunohistochemical staining of tissue sections and tissue microarrays were quite well comparable with each other. Intraclass correlation coefficients (ICC) between the immunohistochemical stainings of tissue sections and tissue arrays were good (for cyclin A ICC=0.69, for cyclin D1 ICC=0.80) or moderate (for cyclin B1 ICC=0.49, and for cyclin E ICC=0.49).

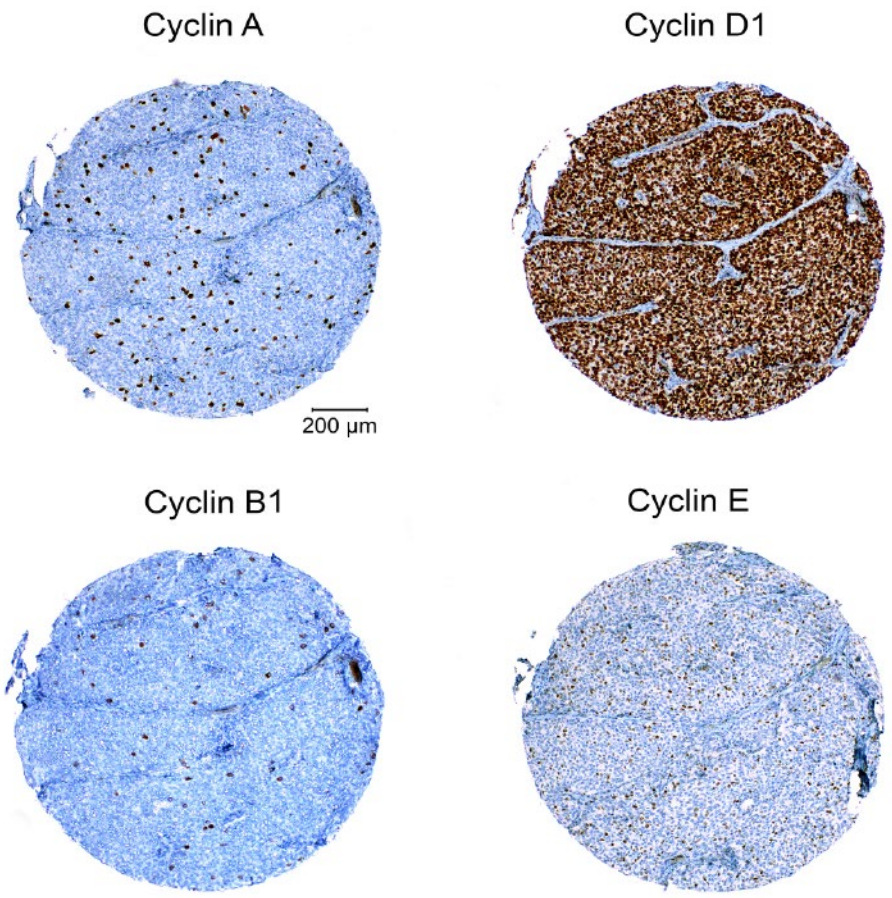

Figure 9. Immunohistochemical staining for cyclins A, B1, D1 and E performed on breast cancer tissue microarray (invasive ductal carcinoma GIII). The scale bar represents 200 µm for all images.

5.1.3 Gene expression levels of cyclins A, B1, D1 and E (I)

The mRNA expression of cyclins A, B1, E and D1 was analysed by quantitative PCR in 12 breast cancer cases to find out whether the cyclin mRNA expression correlated with

the cyclin protein levels. The expression was compared to normal breast tissue, which was used as a reference when calculating the relative gene expression of cyclins.

An increased cyclin gene expression was observed in most of the analysed breast cancer samples. Cyclin A mRNA expression was increased in 9/12 tumours, cyclin B1 in 9/12 tumours, cyclin D1 in 7/12 tumours and cyclin E in all 12 samples analysed. Although differences were seen between the mRNA expressions, a number of similarities were also found, for example higher expression of cyclins A, B1 and E in poorly differentiated ductal carcinomas, grade III. In addition, two ductal carcinomas in situ GIII had higher cyclin A gene expression than other breast cancers. Furthermore, ductal carcinoma in situ GIII patients were found to have a moderate cyclin B1 and E mRNA expression. In our tumour samples, cyclin D1 mRNA expression was variable with the highest expression in grade III tumours.

Comparison between the immunohistochemical staining of cyclins and the results of quantitative PCR showed some variation, e.g expression level of cyclin D1 protein as higher than mRNA level. Further consideration of the statistical correlation between the gene expression and immunohistochemical staining is not possible due to the limited number of samples (12) analysed.

5.2 Identification of MMP-1 in breast cancer (II)

5.2.1 MMP-1 expression in breast cancer (II)

MMP-1 expression was observed in all analysed breast tumours (Figure 10). In the study II original tissue sections were used instead of TMA, because the tissue fixation and tumour heterogeneity could have caused variability in results. The MMP-1 antibody used recognises both the latent and active form of MMP-1 protein. MMP-1 expression was higher in tumour cells than in tumour associated stromal cells and the MMP-1 expression ranged from 10% to 95% in tumour cells. 7.2% of the cases showed MMP-1 expression in tumour epithelial cells with value $\leq$ 30%, 8% with value $\leq$ 50%, 31.2% with value $\leq$ 70% and 53.6% with value over 70%. In stromal cells the MMP-1 expression ranged from 5% to 80% and 47,6% of cases were positive with value $\leq$ 30%, 14,5% were positive with value $\leq$ 50%, 30,6% were positive with value $\leq$ 70% and 7,3% were positive with value over 70%. A significant correlation was seen between tumour grade and MMP-1 expression in tumour epithelial cells ($r=0.23$, $p=0.0101$) as well as in stromal cells ($r=0.21$, $p=0.0170$), the higher grade tumours showing the strongest MMP-1 expression (Figure 11). HER2 immunohistochemical staining correlated with MMP-1 expression both in stromal cells ($r = 0.25$, $p = 0.0050$) as well as in tumour epithelial cells ($r = 0.22$, $p = 0.0121$) (Figure 12B), while no significant correlation was seen with ER or PR. Neither Ki-67 nor bcl-2 expression showed any significant correlation with MMP-1 staining in tumour epithelial or stromal cells. p53 had a significant positive correlation with MMP-1 expression in tumour cells ($r=0.23$, $p=0.0113$), but not in stromal cells.

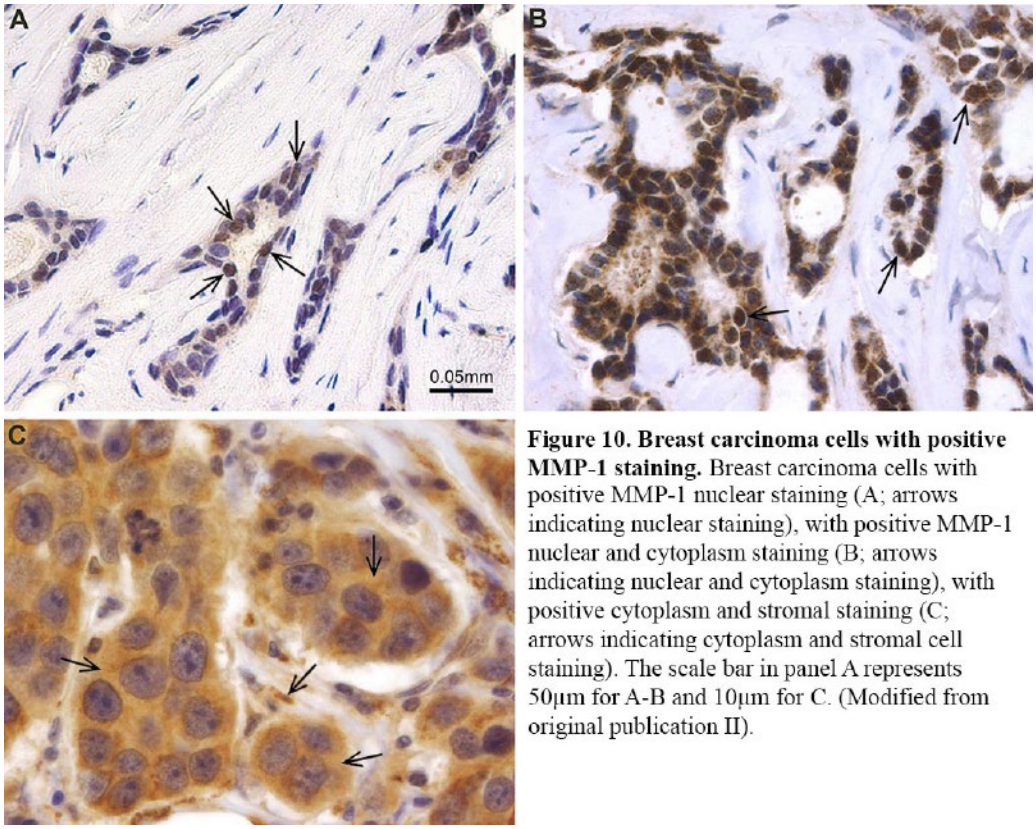

Figure 10. Breast carcinoma cells with positive MMP-1 staining. Breast carcinoma cells with positive MMP-1 nuclear staining (A; arrows indicating nuclear staining), with positive MMP-1 nuclear and cytoplasm staining (B; arrows indicating nuclear and cytoplasm staining), with positive cytoplasm and stromal staining (C; arrows indicating cytoplasm and stromal cell staining). The scale bar in panel A represents 50μm for A-B and 10μm for C. (Modified from original publication II).

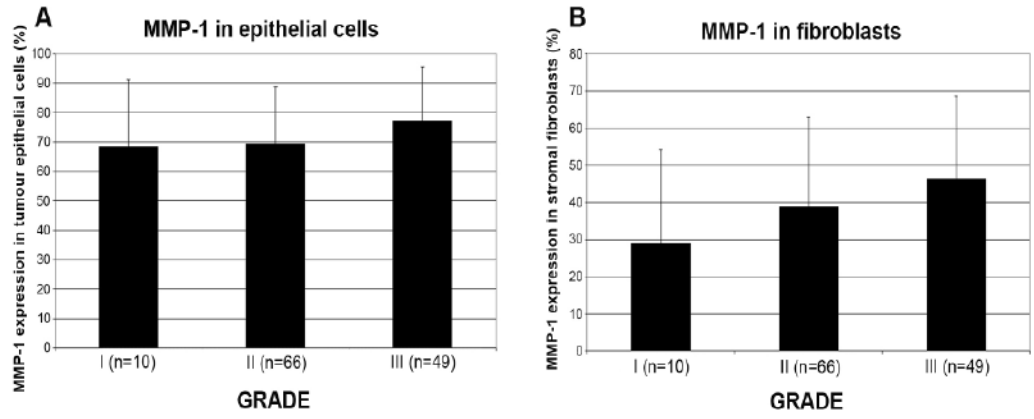

Figure 11. Correlation between tumour grade and MMP-1 reactivity. A significant correlation was found between the tumour grade and MMP-1 expression in tumour epithelial cell panel (A, r=0.23, p=0.0101) as well as in stromal fibroblasts (B, r=0.21, p=0.0170), the higher grade tumours showing the strongest MMP-1 positivity. (The nonparametric Spearman´s rank correlation r is a statistic for measuring the strength of the association between variables).

5.2.2 MMP-1 expression in different breast cancer subtypes (II)

MMP-1 expression in stromal cells showed significant differences (p = 0.0129) between breast cancer subtypes (luminal A, luminal B, HER2, triple-negative subtypes), while MMP-1 expression of cancer epithelial cells did not show any association with different breast cancer subtypes (Figure 12 A). In stromal cells MMP-1 expression was higher in luminal B type tumours than in luminal A subtype (p = 0.0258). In addition, MMP-1 expression in luminal B-type stromal cells was higher than in stromal cells in triple-negative subtype (p = 0.0336). When tumours were divided into triple-negative and non-triple-negative subtypes, no association with MMP-1 immunohistochemical staining in cancer cells or stromal cells was noted. Basal-like and non-basal-like groups had no association with MMP-1 expression in tumour epithelial cells or stromal cells.

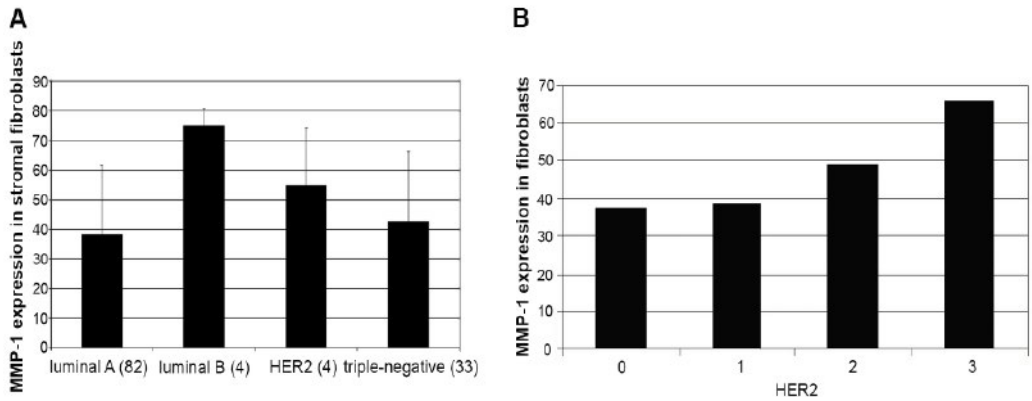

Figure 12. Correlation between MMP-1 expression in fibroblasts and different subgroups. MMP-1 expression in stromal fibroblasts showed significant differences (A, p = 0.0129) between different breast cancer subtypes. Luminal B type tumours demonstrated higher stromal cell MMP-1 expression than luminal A subtypes (p = 0.0258). In addition, MMP-1 expression in luminal B-type stromal cells was higher than in stromal fibroblasts in triple-negative subtype (p = 0.0336). HER2 immunohistochemical staining correlated with MMP-1 expression in stromal cells (B, r = 0.25, p = 0.0050).

5.2.3 Survival analysis for MMP-1 expression (II)

To examine whether the studied MMP-1 expression could be used as a prognostic indicator of breast cancer outcome, the influence of MMP-1 expression in tumour epithelial cells and stromal cells was studied using Cox univariate survival analyses. The median follow-up time was extensively long being over 20 years (range from 17 to 24 years). 44 patients (37%) included in the survival analyses were alive, 51 (43%) had died from breast cancer and 23 (19%) from some other reason at the end of follow up time.

In the breast cancer-specific survival there was a statistically significant difference between high and low MMP-1 expression in tumour cells (cut-off point 70%, p = 0.0171), grade I and III tumours (p = 0.0099), high and low ER expression (cut-off point 10%, p = 0.0013), low and high immunohistochemical bcl-2 expression (cut-off point 25%, p = 0.0003), basal-like and non-basal-like subtypes (p = 0.0103), triple-negative

and non-triple-negative breast cancers (p = 0.0137) (Figure 13). The differentiations in breast cancer-specific survival between Ki-67 subgroups proliferation index ≤15% and proliferation >15% were also statistically significant (p=0.0022). MMP-1 expression in cancer stromal cells showed no statistically significant association with breast cancer-specific survival, nor did patient age or p53 expression.

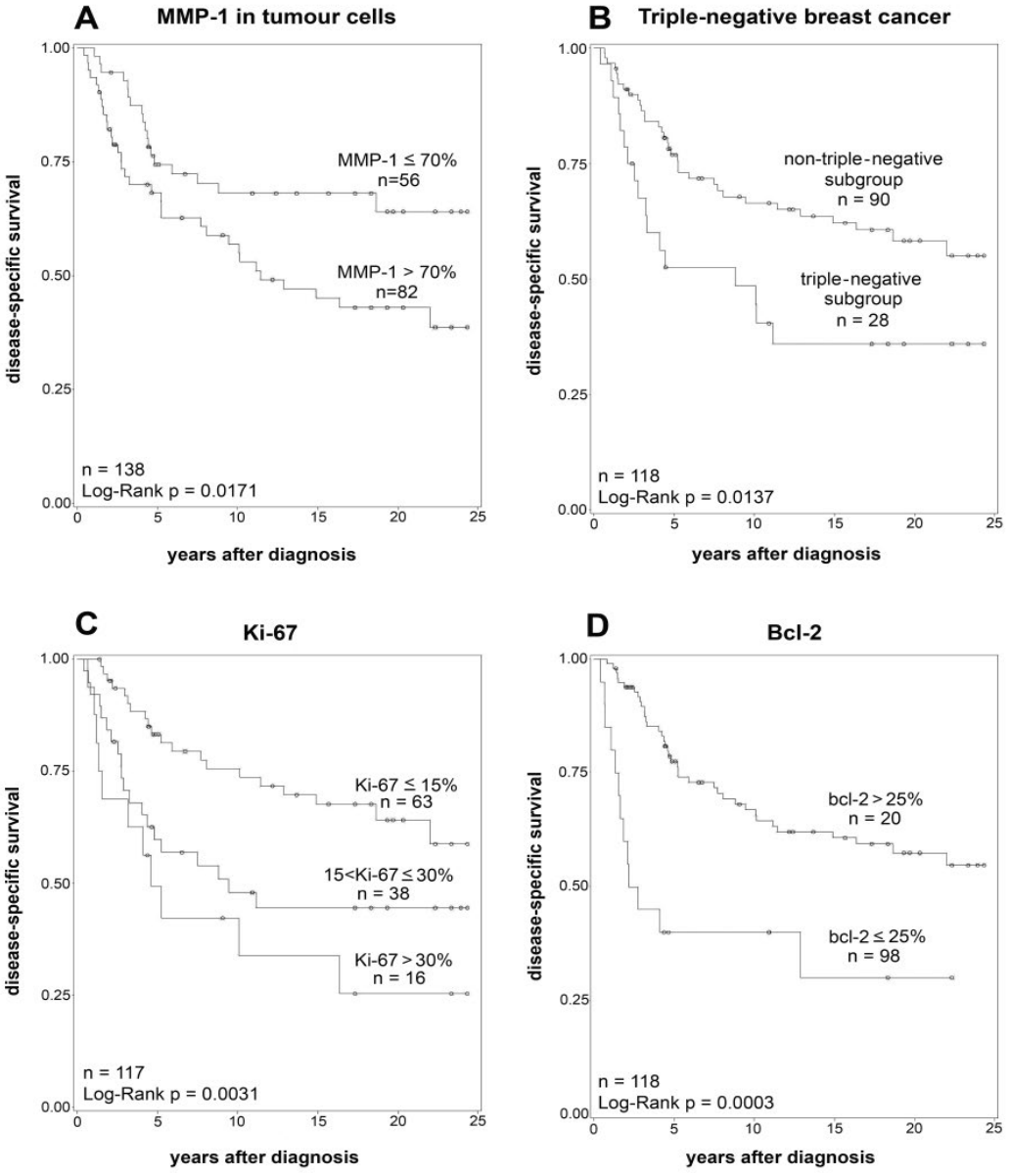

Figure 13. Kaplan-Meier analysis for disease-specifc survival of patients with breast cancer stratified according to MMP-1 positivity in tumour cells with a 70% cut-off level (A), triple-negative and non-triple-negative subgroups (B), Ki-67 subcategories (≤15%, 15%<Ki-67≤30% and > 30%) (C), bcl-2 with a 25% cut-off level (D). Comparison between curves was performed using the log-rank test.

The potential independent prognostic value of immunohistochemically detected MMP-1 expression in tumour cells was evaluated by stepwise Cox regression analysis. It was found that ER >10% (HR 0.41, 95% CI 0.24 to 0.72, p=0.0018), Ki-67 >15% (HR 2.35, 95% CI 1.34 to 4.13, p=0.0030), bcl-2 >25% (HR 0.33, 95% CI 0.18 to 0.63, p=0.0007), triple-negative subtype (HR 2.05, 95% CI 1.15 to 3.68, p=0.0158), basal-type subtype (HR 2.34, 95% CI 1.20 to 4.58, p=0.0127) and tumours cells with MMP-1 positivity with 70% (HR 1.99, 95% CI 1.12 to 3.53, p=0.0194) significantly associated with disease-specific survival in univariate models and were used as cut-off values. For p53 >10% (HR 1.73, 95% CI 0.98 to 3.06, p=0.0601) or for PR>10% (HR 0.71, 95% CI 0.40 to 1.25, p=0.2322) no significant association was found. Factors significantly associated with disease-specific survival in univariate models were included in multivariate analysis. Ki-67 >15% (p=0.0186), bcl-2 >25% (p=0.0158) and MMP-1 with tumour cells >70% positivity (p=0.0438) were independent prognostic parameters for breast cancer-specific survival (Table 9).

Table 9. Multivariate analysis of disease specific- survival analysis at 24 years (Study II).

Variable	HR (95%CI)	p (multivariate)
Ki-67>15%	2.01 (1.12-3.59)	0.0186*
Bcl-2>25%	0.45 (0.23-0.86)	0.0158*
MMP-1 tumour cells >70%	1.81 (1.01-3.22)	0.0438*

HR= Hazard Ratio

P-value less than 0.05 (*) were considered as significant.

Tumour grade was not included in disease specific -survival analysis, because none of grade I patients died of breast cancer.

5.3 Decorin expression in human breast tissue (III)

5.3.1 Localization of decorin mRNA in healthy human breast tissue, and in benign and malignant tumours of the human breast (III)

Analysis of publicly available GeneSapiens databank demonstrated that, although decorin expression is abundant both in healthy human breast tissue and in various human breast cancers, its expression is greater in healthy breast tissue than in ductal, lobular or other breast cancers (see the study III, Figure 1). Results of ISH analysis with DIG-labeled RNA probes for decorin clearly demonstrated that in healthy human breast tissue the expression of decorin takes place only in the cells within the stromal area surrounding the lobules and in the intralobular stroma, whereas the epithelial cells of normal ducts or lobules showed no decorin expression (Figure 14). Similar analysis of intraductal papillomas revealed that decorin expression is localized merely to the primary breast stroma around the dilated duct of papillomas, while the intraductal papilloma tissue was completely negative for decorin gene expression (Figure 15). Consistently with the

above, no decorin mRNA transcripts were detected within precancerous DCIS or LCIS. Furthermore, invasive epithelial cells from ductal, lobular and mucinous cancers lacked decorin mRNA, the expression of decorin taking place merely in the native stromal cells (Figure 16).

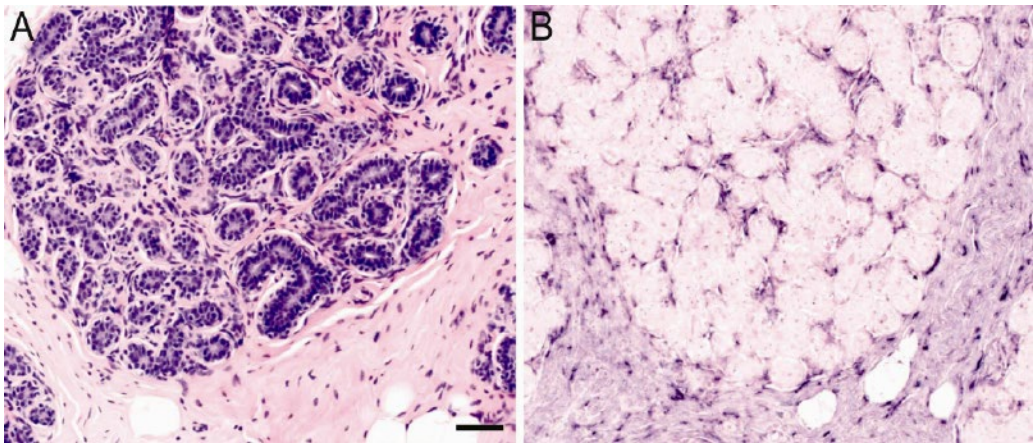

Figure 14. In normal human breast tissue, decorin mRNA is localized only in the stromal cells surrounding the lobulus and in the intralobular stromal cells. HE staining of normal lobulus and its stroma in panel A and in panel B ISH for decorin, serial section of the same normal lobulus as in panel A. The scale bar in panel A represents 50 μm for A-B. (Modified from original publication III).

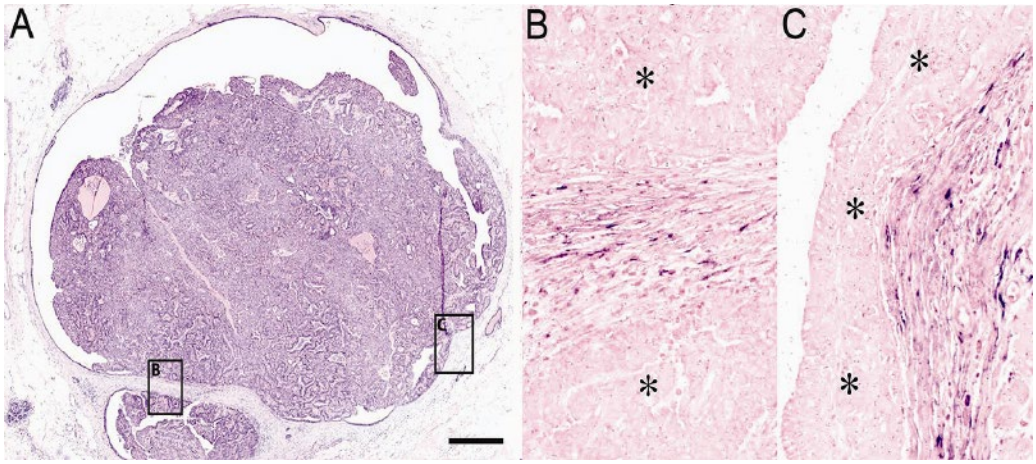

Figure 15. HE staining of benign intraductal papilloma (A). The cells of intraductal papilloma (indicated by asterisks) do not express decorin (B, C). Decorin mRNA is localized to the primary stroma around the dilated duct of the papilloma (B, C). Positive DIG reaction in ISH indicating the localization decorin mRNA can be seen in purple. The scale bar in panel A represents 500 μm for A and 50 μm for B-C. (Modified from original publication III).

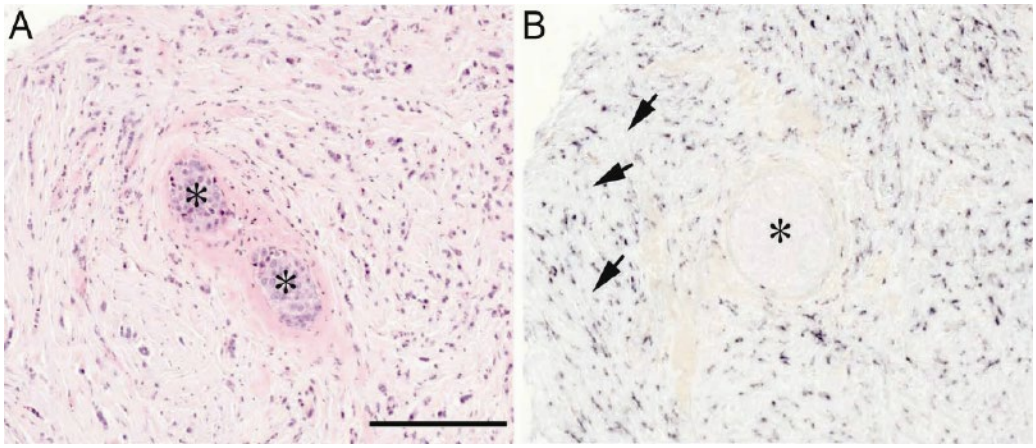

Figure 16. Epithelial cells of invasive lobular breast cancer are negative for decorin expression. HE staining of invasive lobular carcinoma with lobular carcinoma in situ (A) and ISH for decorin (B). Positive DIG reaction of stromal cells in ISH indicating decorin expression can be seen in purple. Arrows in panel B indicate areas of infiltrating cancer cells negative for decorin expression. No decorin mRNA was detected in lobular carcinoma in situ (asterisk). The scale bar in panel A represents 200 μm for A-B.

5.3.2 Effect of adenoviral decorin transduction on MCF7 cells (III)

Next, human breast adenocarcinoma cell line MCF7 and decorin producing adenoviral vector were applied to examine the effects of targeted decorin transduction on the behaviour of breast cancer cells. Similarly to the above results *in vivo*, cultured MCF7 cells did not express decorin as shown by RT-qPCR. When these cells were transduced with decorin adenoviral vector, their growth pattern changed markedly, i.e., their proliferation was inhibited and cell cohesion was decreased. Decorin-transduced MCF7 cells also exhibited a number of morphologic alterations. Particularly, several of the decorin-transduced MCF7 cells contained a large vacuole within another larger cell with a crescent-shaped nucleus at its periphery (Figure 17). These so-called cannibal cells were also observed among the MCF7 cells transduced with the control vector and among those without any transduction, but to a much lesser number.

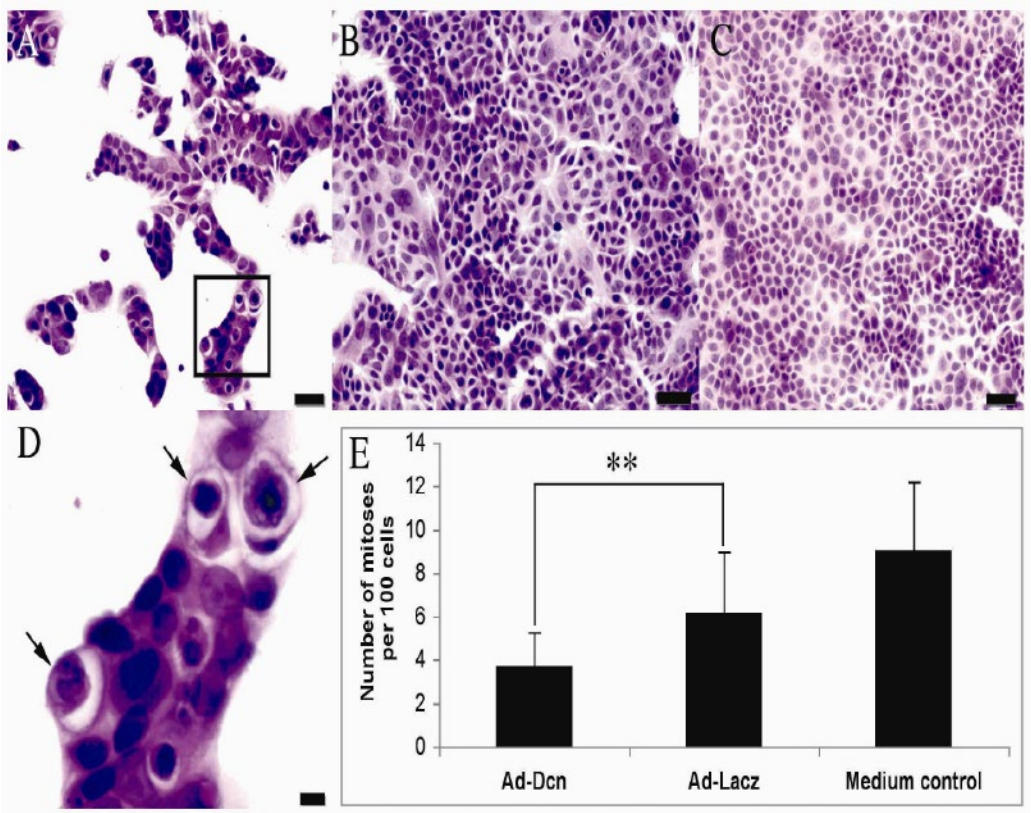

Figure 17. Decorin gene transduction mediated by a recombinant adenovirus has a significant effect on the growth pattern of human breast adenocarcinoma cells (MCF-7) and inhibits their proliferation. HE staining of MCF-7 cell cultures transduced with human decorin cDNA containing adenovirus (Ad-Dcn) (A) and lacZ gene containg adenovirus (Ad-LacZ) (B). Control vector Ad-LacZ encoding lacZ gene (C). Magnified illustration of the boxed region shown in A (C). Arrows indicate the presence of cannibal cells after Ad-Dcn transduction of MCF-7 cells (D). Number of observed mitoses per 100 cells, 2 days after in vitro incubation with Ad vectors. A – B, scale bar 50 μm; C, scale bar 10 μm; **, $p < 0{,}001$, Student´s t-test. (Modified from original publication III).

6. DISCUSSION

6.1 Prognostic value of cyclin A, B1, D1 and E in breast cancer (I)

As a result of regular mammography screening, a shift toward the detection of earlier-stage breast cancers with better prognosis has occurred (Mook *et al*., 2011). Additional specific biomarkers are however required to provide safer, more individualized treatment and to spare patients from unwanted side effects and complications of over-treatment (Weigel and Dowsett, 2010).

The molecular interaction between cell function and cyclins in cancer is far from being fully understood (Casimiro *et al*., 2012). In cancer cells, cyclin expression is found to be highly upregulated leading to uncontrolled cell division and proliferation (Rastogi and Mishra, 2012). Although many previous studies have analysed the protein expression and prognostic role of different cyclins in breast cancer, little is known about their combined expression, especially in combination with classic prognostic factors.

Overexpression of cyclins A, B1, and E have been shown in several studies to associate with poor prognosis in breast cancer (Baldini *et al*., 2006; Aaltonen *et al*., 2009a; Scaltriti *et al*., 2011). Also, in the present study, the immunohistochemical expression of cyclins A, B1 and E showed significant association with tumour grade, mitotic index as well as with HER2 and Ki-67 expression. Furthermore, the expression of cyclins A, B1 and E correlated with each other, but not with the expression of cyclin D1. In the study I none of the cyclins A, B1, E or D1 showed correlation with metastasis. Triple-negative breast cancers are typically high grade tumours and frequently associated with high expression of Ki-67 and high level of cyclin E, but low expression of cyclin D1 (Branham *et al*., 2012), which was also confirmed in the present study.

The prognostic value of histological grade has been accepted in most breast cancer types and it is a valuable prognostic factor, particularly in early breast cancer without lymph node involvement (Rakha *et al*., 2010b; Reyal *et al*., 2013). A recent immunocytology study has shown overexpression of cyclins A and E to appear after 11 months from the tamoxifen exposure in the endometrial cells in women with breast cancer (Metwally *et al*., 2013). This cyclin overexpression in these patients may be a marker of malignant transformation (Metwally *et al*., 2013). Alterations of cyclin expression levels in control breast tissue after cancer treatment could also be easily detected and this could provide a new and valuable tool for follow-up for positive cyclins breast cancer patients.

Cyclin E has been shown to be a sensitive and specific prognostic indicator in patients with breast cancer (Keyomarsi *et al*., 2002). The results of the study I showed that cyclin E has a negative correlation with ER and PR status and a positive correlation with triple-negative breast carcinomas thus associating high cyclin E expression with a

high risk of cancer progression. In addition, the positive correlation between cyclin E and histological grade, Ki-67, HER2 and standardized mitotic index are in agreement with poor clinical outcome in breast cancer. Cyclin E overexpression is shown to lead to acquired trastuzumab resistance, which results a lower progression free survival (Scaltriti *et al*., 2011). The mechanism of trastuzumab is mediated by inhibiting the kinase activity and exposion of CDK2 (Le *et al*., 2006). Trastuzumab has also shown to suppress the level and activity of CDK6, cyclin A and cyclin D1 (Le *et al*., 2006). An overload of cyclin E may therefore make cells independent from trastuzumab-mediated cell cycle arrest (Scaltriti *et al*., 2011). Trastuzumab-resistant cells have higher CDK2 activity and are more sensitive to CDK2 inhibitors suggesting that treatment with CDK2 inhibitors may be a suitable strategy in breast cancer patients with high cyclin E and HER2 expression (Fiszman and Jasnis, 2011; Scaltriti *et al*., 2011).

The mRNA level of cyclin E was upregulated in all 12 breast cancer samples studied and the highest cyclin E mRNA expression was detected in grade III samples when compared to normal breast tissue by quantitative PCR. A discrepancy between the cyclin E immunohistochemical and mRNA expression has previously been demonstrated (Potemski *et al*., 2006). This may be explained by the fact that frozen tumour tissues examined by RT-PCR might contain some fat tissue, fibrotic tissue or lymphocytic infiltrates from the adjacent stroma, while immunostaining results are specific for tumour cells (Oda *et al*., 2010). Furthermore, the amount of mRNA does not always correlate to the protein level (Potemski *et al*., 2006).

The prognostic value of ***cyclin D1*** as a breast cancer biomarker is a somewhat controversial issue, while both positive and negative findings have been observed (Mylona *et al*., 2013; Peurala *et al*., 2013; Xu *et al*., 2013). High *CCND1* amplification is associated with poor prognosis in breast cancer, whereas high protein expression of cyclin D1 has been linked to both poor and good clinical outcome (Roy *et al*., 2010; Lundgren *et al*., 2012). Apart from the role as a prognostic marker, cyclin D1 overexpression alone or cyclin D1 overexpression together with *CCND1* amplification have been associated in ER+ breast cancer to tamoxifen resistance in some studies (Roy *et al*., 2010). While estrogen rapidly induces cyclin D1 expression, antiestrogens cause an inhibition of CDK activity and a reduction in cyclin D1 expression. Some published studies suggest that high cyclin D1 expression is not solely associated with *CCND1* gene amplification but is also affected by ERα status (Quintayo *et al*., 2012).

In a recent meta-analysis *CCND1* overexpression has been detected to affect the prognosis of ER+ breast cancer patients, but not patients with unselected primary breast cancer or patients treated with neoadjuvant chemotherapy (Xu *et al.,* 2013). Cyclin D1 overexpression has been demonstrated to correlate with poor prognosis in invasive breast cancer when no adjuvant treatment is given (Lin *et al*., 2013). A positive relationship between cyclin D1 overexpression and ER is a repeated result in almost all studies (Mylona *et al*., 2013). From a clinical point of view, overexpression and amplification

of cyclin D1 associates with poor endocrine response (Patani *et al*., 2013). The study I confirmed a significant positive correlation between the immunohistochemical staining of cyclin D1 and the expression of hormone receptors ER and PR. Furthermore, cyclin D1 overexpression correlated with non-triple negative and non-basal-like breast carcinomas. The connection between ER and cyclin D1 expression implies that cyclin D1 might contribute to the prognosis of ER+ patients. In addition, the lack of correlation between cyclin D1 and tumour grade, Ki-67, HER2 suggests that increased expression of cyclin D1 might indicate a good prognosis for breast cancer patients.

In RT-PCR study, cyclin D1 gene expression had variable results and cyclin D1 was up-regulated in 7/12 (58%) tumours studied, which is higher than in previous reports (5% to 30%) (Elsheikh *et al*., 2008; Roy *et al*., 2010). When cyclin D1 mRNA level was compared with the immunohistochemically detected expression, cyclin D1 protein expression was constantly higher suggesting that translation of cyclin D1 is not always secondary to gene transcription, and pathogenic activation of cyclin D1 can occur via potential additional pathways, including transcriptional and post-transcriptional dysregulation (Arnold and Papanikolaou, 2005).

Increased expression of ***cyclin A*** has been associated with poor prognosis in a variety of invasive human cancers (Mrena *et al*., 2006) including advanced breast cancer, where high cyclin A expression has been demonstrated to correlate with tumour grade, mitotic index and Ki-67 status (Poikonen, 2005). The results of the study I showed correlation with cyclin A expression and tumour grade, Ki-67, HER2, standardized mitotic index as well as expression of cyclins B1 and E suggesting that cyclin A overexpression is linked to poor prognosis. Triple-negative or basal-like tumours did not correlate with cyclin A expression. There are also opposite results concerning the role of cyclin A as a prognostic marker, so the impact of cyclin A expression on clinical outcome is not clear (Ahlin *et al*., 2009). The differences between published results may be due to the lack of definition of optimal cut-off value for cyclin A expression in tumour cells (Ahlin *et al*., 2007).

A previous study has shown unexpectedly that the overcome from distant metastases to death was significantly longer for patients with tumours expressing high cyclin A expression compared with low cyclin A tumours (Ahlin *et al*., 2009). Patients with distant metastases receive palliative chemotherapy, which is more effective in highly proliferating tumours than in slowly proliferating ones. As cyclin A synthesis is active during cell cycle progression from the start of S phase to the beginning of mitosis, high cyclin A expression detects the cells that are actively proliferating and thus most sensitive to chemotherapy (Huuhtanen *et al*., 1999). It is also possible that other mechanisms than gene mutation or amplification might have a role in the overexpression of cyclin A in different tumour subtypes. Depending on which mechanism causes the protein overexpression, the influence on patient survival in breast cancer may vary (Sørby *et al*., 2012).

Cyclin B1 is a key initiator of mitosis in cell cycle and cyclin B1 overexpression has been found to associate with various clinico-pathologic parameters including high tumour grade and distant metastasis. Therefore cyclin B1 has been identified as a prognostic marker for poor patient outcome in several tumours, including breast cancer (Aaltonen *et al*., 2009a; Chae *et al*., 2011; Khan *et al*., 2013). Cyclin B1 is first situated in the cytoplasm, and at the beginning of mitosis it is translocated to the nucleus (Suzuki *et al*., 2007). However, a significant part of cyclin B1-Cdk1 complex remains in the cytoplasm (Gavet and Pines, 2010). The different intracellular localization of cyclin B1 can have different effects on the results of the immunohistochemical expression as well as on the malignant potential of cyclin B1 (Suzuki *et al*., 2007).

The immunohistochemical expression of nuclear cyclin B1 in the study I was observed in 0% to 30% of the tumour samples. The mRNA expression of cyclin B1 was up-regulated in 9/12 cases (75%) and the mRNA levels tended to be higher in poorly differentiated breast cancers. In addition to mRNA, immunohistochemically detected protein expression of cyclin B1 showed a significant association with tumour grade, standardized mitotic index, Ki-67 and Her-2/neu. These observations are in agreement with earlier studies, which show that nuclear cyclin B1 immunoreactivity is significantly associated with adverse clinical outcome in breast cancer patients (Suzuki *et al*., 2007; Aaltonen *et al*., 2009a). Cyclin B1 had a significant positive correlation with cyclins A and E, but unlike in some previous studies the study I showed that cyclin B1 was not correlated to triple-negative or basal-like breast cancer subtypes (Agarwal *et al*., 2009).

Most published TMA validation studies have correlated one to three cyclins with clinico-pathological characteristics of breast cancers (Aaltonen *et al*., 2006), but in the study I cyclins A, B1, D1 and E were evaluated together. The results of the TMA slides were quite well comparable with the staining results of the traditional large section slides in breast cancer. Thus TMA can be considered to replace the whole section slides when studying the cyclin expression in breast cancer.

In conclusion, the findings of the study I suggest that overexpression of cyclins A, B1 and E associate with aggressive breast cancer. Cyclin D1 expression is independent of other cyclins and is correlated with ER and PR status, non-triple negative and non-basal-like breast carcinomas suggesting that cyclin D1 expression might be a marker of good breast cancer prognosis. Since cellular proliferation is an essential factor in the biologic behaviour of breast cancer (Jonat and Arnold, 2011), the expression of Ki-67 could be worth of studying together with the expression of cyclins A, B1, D1 and E when determining breast cancer prognosis.

6.2 MMP-1 as a prognostic marker in breast cancer (II)

Recent studies have revealed that breast cancer progression is a multistep process and it is thought to be driven by complex and reciprocal interactions between epithelial cancer

cells and cells in the surrounding microenvironment including leukocytes, fibroblasts, macrophages and endothelial cells (Khamis *et al*., 2012; van Rooyen *et al*., 2013).

Several studies have shown that breast cancer progression, invasion and metastatic potential may be associated with the overexpression of various matrix metalloproteinases (MMPs), which were originally recognized as extracellularly acting proteolytic enzymes (Mannello and Medda, 2012). The study II shows MMP-1 positivity both in the nuclei and in the cytoplasm of breast cancer epithelial cells as well as in cancer associated stromal cells, which is in line with recent works (Mannello and Medda, 2012). The earlier conflicting results may occur due to the used antibody (Köhrmann et al 2009), while there are antibodies that are specific for the latent form of MMP-1 and show no reaction with the active form. In addition, different pretreatment protocols and other MMP family members can cause non-specific staining results.

The most important finding of the present study was that MMP-1 expression in tumour epithelial cells carries unexpectedly an independent prognostic value in breast cancer. Identification of the nuclear localization of immunohistochemically detected MMP-1 expression represents a new and interesting viewpoint of MMP-1 function showing that the acitivity of MMP-1 is not limited to the ECM. While MMP-1 is involved extracellularly in the breakdown of many secreted factors and matrix proteins critical for tumour progression and invasion, the role of intracellularly located MMP-1 is still poorly understood. There are studies showing that MMP-1 accumulates within the cells during the mitotic phase of the cell cycle, and thus nuclear localization of MMPs could be associated with apoptosis (Limb *et al*., 2005; Hadler-Olsen *et al*., 2011). Future studies should aim at the role of the nuclear MMP-1 in breast cancer development and additional studies are required to understand the interaction between MMP-1 activity at various subcellular and extracellular localization.

In the study II the MMP-1 expression was also analysed in different breast cancer subtypes. One important finding was the significantly different MMP-1 expression in stromal cells in different breast cancer subtypes. The MMP-1 positivity in stromal cells in triple-negative subtype and HER2 overexpression breast cancer can help to recognize the patients with risk of metastatic behaviour. Luminal B subtype showed higher MMP-1 expression in stromal cells than the triple-negative subtype. In addition, recent studies have shown breast cancer subtypes to have distinct interactions with the surrounding microenvironment (Breuer *et al*., 2013). Stromal gene expression has been shown to correlate with histological tumour grade (Ma *et al*., 2009) and the latest studies have presented that the molecular profiling of the cancer-associated microenvironment may be a better predictor of patient outcome in breast cancer than the tumour epithelium (Farmer *et al*., 2009). In the study II the patient follow up time was more than 20 years and the survival analyses revealed a significant link between high MMP-1 expression both in cancer-associated fibroblasts and in tumour cells with metastatic tumour progression and shortened survival. The interaction with breast cancer epithelial cells and surrounding

stroma emphasize the importance of the wider MMP molecular profiling of a "lethal stromal phenotype" when identifying patients at the greatest risk (Mannello, 2011).

Over the past decades, the pharmaceutical industry has made an impressive attempt to develop synthetic metalloproteinase inhibitors (MMPIs) for the treatment of cancer and other diseases. MMPIs have been tested in clinical research studies to treat various cancers, but tests have failed to improve patient outcome. In addition, patients have had harmful side effects from joint toxicity that is thought to be due to broad-spectrum nature of MMP1 inhibition (Bingham, 2005). The administration of MMPIs should be made after thorough consideration, because the expression profile of MMPs, as well as the activity of MMPs, is not the same in different cancer types and varies in the early stage compared to advanced cancer (Galley *et al*., 2011). In the future, studies should be focused on new selective MMP-1 inhibitors that are capable of minimizing undesired interaction with other metalloenzymes (Fisher and Mobashery, 2006).

In conclusion, the results of the study II reveal that high MMP-1 expression both in tumour epithelial cells and in cancer associated stromal cells are significantly associated with breast cancer progression, poor prognosis and shortened survival. In addition, correlation between p53 and MMP-1 expression in tumour epithelial cells is in line with tumour aggressiveness. The results point out that cancer epithelial cells and their microenvironment interact together creating the process of carcinogenesis (Albini and Sporn, 2007). Further clarification of the microenvironmental mechanisms involved in tumour progression and targeting these components gives a great promise for future cancer therapy (Muppalla *et al*., 2013).

6.3 Localization of decorin expression in benign and malignant breast tissue and the effect of decorin transduction on breast cancer cells (III)

Previous reports of decorin expression levels in breast cancer have been variable. Low level of decorin has been associated with invasive breast cancer and poor clinical outcome (Buraschi *et al.,* 2012). On the other hand, it has been reported that decorin overexpression is associated with high number of lymph node metastasis and poor survival in breast cancer (Cawthorn *et al.,* 2012). Moreover, decorin has been shown to be overexpressed in the stroma of human laryngeal cancer and to be associated with the histological gradus (Stylianou *et al.,* 2008). In addition to these varied results, there has also been uncertainty whether breast cancer epithelial cells express decorin (Gu *et al.,* 2010; Oda *et al.,* 2012).

In the study III the publicly available GeneSapiens databank offered the possibility to study decorin gene expression level between different tumour types and the corresponding normal human tissue (Kilpinen *et al*., 2008). The analysis revealed that the relative decorin gene expression level is significant in both healthy breast tissue and

in different breast tumours. Decorin expression was found to be lower in most common types of breast cancer, but higher in breast carcinomas without known special type, when compared to decorin expression in healthy breast tissue. The level of epithelial cells in malignant tumours can be predominant over the stromal component and thus malignant tumours are expected to accumulate less decorin than normal breast tissue (Goldoni and Iozzo, 2008). Thus, reduced levels of decorin can possible explain the association with more aggressive tumours (Oda *et al.,* 2012).

Significant variations exist in the results of publicly available datasets depending, e.g., on the number of patients in each database, the quality of specimens collected, sample processing and instrumentation used for gene expression read-out (Freeman *et al.,* 2013). Furthermore, the samples contain various amounts of stroma (Sainio *et al.,* 2013) and the amount of epithelium in breast tissue decreases with menopausal status (Reid *et al*., 1996). One reason for the variation in decorin expression could be the composition of the stromal component among different tumours, which can vary markedly from abundant elastic tissue to mucinous stroma (Connolly *et al.,* 2003). The heterogeneity of samples can cause discrepancies among results (Chou *et al*., 2013), and thus previously reported analyses of decorin gene expression profiling have not been able to separate invasive breast cancer from in situ breast cancer or identify different subtypes of breast cancer.

Localization of decorin in normal human breast tissue and in breast tumour *in vivo.* The immunohistochemically detected expression of decorin was restricted to the ECM adjacent to the normal epithelium and to the peritumoural stroma, while cancer epithelial cells as well as normal breast epithelium showed no decorin expression. Although much utilized method, immunohistochemistry can reveal only the existence of a protein after production not the exact site of protein expression on a cellular level. While GeneSapiens database does not reveal the cellular origin of a specific molecule, we used ISH with DIG-labeled decorin probes in the study III, and demonstrated that epithelial cells from normal human breast tissue, benign intraductal papillomas or different human breast cancers did not express decorin mRNA. Instead, the expression of decorin was localized to the cells of the stroma both in normal human breast tissue and in breast tissues containing benign or malignant breast epithelial tumours. This same finding was also showed to be true for human breast adenocarcinoma MCF7 cells and in a recent study showing that human bladder cells lack the expression of decorin in vitro and in vivo (Sainio *et al.,* 2013).

ISH is a useful method to localize and detect specific mRNA, e.g. decorin, in morphologically preserved tissues sections or cell preparations to a particular cell or a particular region of the tissue of interest (Uehara *et al.,* 2007; Kirsch *et al.,* 2012; Yoshihara *et al.,* 2013). On the contrary, Northern blot and RT-PCR analyses use pooled tumour samples that in addition to tumour cells contain tumour-associated normal epithelial cells and original stromal cells. Thus, the pooled tumour samples give only information on the presence or absence of mRNAs without the information of the cellular source of mRNA (Wilcox, 2000).

Radiologically dense breast tissue is known to be one of the strongest risk factor for all types of breast cancer (Guo *et al.,* 2001; Bertrand *et al.,* 2013). Alteration in stromal composition has been correlated with increased mammographic density, because breast stroma is the major tissue compartment by volume (Alowani *et al.,* 2003). Decorin accumulation has been shown to be involved in the formation of a collagenous rich stroma associated with mammographic density (Skandalis *et al.,* 2011). Up-regulation of decorin may lead to an organized ECM, potentially providing a physical barrier against tumour cell migration (Bi, 2013). However, the abundant expression of decorin may lead to changes in cytoskeletal organization in the ECM and thus impede the diffusion of chemotherapeutic agents (Provenzano and Hingorani, 2013).

Influence of targeted decorin transduction on the behaviour of human breast cancer cell line *in vitro*.

ISH results clearly demonstrated that human breast cancer epithelial cells are not able to express decorin. In the study III, decorin transduction caused significant changes in the behaviour of decorin-negative MCF7 human breast carcinoma cells. Adenoviral-mediated expression of decorin has earlier been shown to cause morphological changes in the human tumour xenografts including sharper tumour border, reduced invasiveness and angiogenesis as well as evidence of cyto-differentiation (Reed *et al.,* 2002). Earlier preclinical studies have also demonstrated that exogenous decorin added to the tumour microenvironment evokes endothelial cell autophagy (Neill *et al.,* 2013). In the study III, the discovered cannibalism in decorin-transduced MCF7 cells can be an evidence of process where cancer cells are under starvation (Wang *et al.,* 2013). Autophagy can also indicate the potential oncosupressive function by acting to remove critical cell components that would otherwise be involved in tumour growth (Buraschi *et al.,* 2013). Because decorin is a well-known non-toxic natural biological product and an anti-oncogenic molecule, it could be a new therapeutic target in the treatment of breast cancer (Santra *et al.,* 2000; Neill *et al.,* 2012b).

Despite of decorin´s tumour suppressor role in carcinogenesis, it has also been shown to have opposite roles in tumour progression depending on the type and background of the cancer analysed (El Behi *et al.,* 2013). A recent experiment with aggressive human bladder tumour cells has shown that decorin overexpression is required unexpectedly for cancer progression by promoting angiogenesis and tumour cell invasiveness (El Behi *et al.,* 2013). This opposite role is explained by different decorin forms and/or protein localization, for example with nuclear localized decorin (Dil and Banerjee, 2012). The mechanism of decorin-induced growth suppression on MCF7 breast cancer cells has not been the research subject in this study. However, the observed decreased proliferation of MCF7 is most likely mediated by the interaction of decorin core protein with EGFR and other ErbB family proteins (Feugaing *et al*., 2013). Alternative possible mechanisms include other tyrosine kinase pathways, whose activity is regulated by decorin (Hu *et al*., 2009).

7. CONCLUSION

Based on the results of the present study, the following conclusions can be drawn:

1. The overexpression of cyclin A, B1 and E correlated with aggressive breast cancers. Cyclin D1 expression was independent of the other cyclin and could be considered as a marker of good prognosis. The expression of cyclins A, B1, D1 and E may be beneficial to study together for determining prognosis for invasive breast cancer.

2. The immunohistochemical staining of tissue sections and tissue microarrays were comparable with each other. Intraclass correlation coefficients between the immunohistochemical stainings of tissue sections and tissue arrays were good.

3. MMP-1 expression associated strongly with tumour evolution, poor prognosis and shortened survival. High MMP-1 expression in both cytoplasm and nuclei of breast cancer cells with respect to stromal cells revealed an unexpected role of nuclear MMP-1. Both tumoural and stromal cells were involved in breast cancer progression suggesting that breast cancer outcome is driven by interactions between epithelial cancer cells and stromal microenvironment. .

4. Cancer cells independently of the type of breast cancer do not express decorin mRNA. Decorin expression was localized in the cells of the original stroma both in normal breast tissue and in breast tissue containing benign or malignant breast epithelial tumours. Transduction of decorin in decorin-negative human breast cancer cells markedly modulated the growth pattern of these cells. The result of this study suggests that decorin might have therapeutic value in the treatment of breast cancer.

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