Emmi Peurala

REGULATORS OF HYPOXIA RESPONSE AND THE CELL CYCLE IN BREAST CANCER

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OULU UNIVERSITY HOSPITAL
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Abstract

Breast cancer is the most common cancer affecting the female population of the Western world. It is a heterogeneous disease entity that encompasses tumors with remarkably different forms of behaviour, and it is therefore vital to distinguish patients with good and poor prognoses. The classical prognostic and predictive factors for breast cancer serve as tools for clinical oncologists when planning treatment, but the growing awareness of breast cancer biology is bringing about a need for novel prognostic and predictive biomarkers.

This thesis examines the prognostic significance of hypoxia response and cell cycle regulators in ductal breast cancer and in triple-negative breast cancer (negative for hormone receptors and human epidermal growth factor receptor 2), concluding that PHD2 and PHD3 are associated with a good prognosis, while the role of PHD1 is controversial, as it is associated with proliferation in ductal breast cancer but with node-negative status in triple-negative breast cancer. In our experiments HIF-1α redeemed its role as a marker of an adverse prognosis, whereas the role of HIF-2α appeared to be the opposite. Our data suggest that PHDs can have other targets than the HIF-αs, and that triple-negative breast tumors express more HIF-1α and less HIF-2α and PHD3 than those with a good prognosis.

Furthermore, we identified cyclin D1 as a biomarker with independent prognostic significance in ductal breast cancer, being associated with good prognostic factors and a better outcome, whereas the opposite was seen in triple-negative breast cancer. CDK4 was associated with high proliferation in triple-negative breast cancer. In addition, high levels of p16 correlated with increased survival in breast cancer patients independently of receptor status.

Keywords: breast cancer, CDK4, cyclin D1, hypoxia-inducible factor (HIF), p16, PHDs 1-3
Peurala, Emmi, Hypoxiavasteen ja solusyklin säätelijät rintasyövässä.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Syöpäaudit ja särdehoito; Biolääketieteellinen laitos, Lääketieteellinen biokemia ja molekyylibiologia; Diagnostiikan laitos, Patologia; Oulun yliopistollinen sairaala
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä
Tässä väitöskirjatyössä tutkimme hypoksiavasteen ja solusyklin säätelijöiden ennusteellisuutta duktaalisessa rintasyövässä sekä kolmoisnegatiivisessa (ei ilmennä hormonirespooreita eikä epidermalikasvutegukareseptoria) rintasyövässä. PHD2 ja PHD3:n vahva ilmentyminen liittyi parempaan ennusteeeseen, mutta PHD1:n esiintymisen vaikutus oli ristiriitainen. PHD1:n ilmentyminen liittyi lisäänteeseen solujakautumiseen duktaalisessa rintasyövässä, mutta kolmoisnegatiivisessa rintasyövässä sen esiintyminen liittyi vähentymiseen imusulmukelmatasointiin. Tutkimassamme HIF-1α osoittautui huonoonennusteen merkiksi. Sitä vastoin HIF-2α:n ilmentymisen vaikutus näytti liittyvän parempaan ennusteeeseen. Tuloksemme osoittavat, että PHD-entsyymeillä on mahdollisesti muitakin kohteita kuin HIF-1α. Osoitimme myös, että HIF-1α:n ilmentyminen on yleisempää ja HIF-2α:n sekä PHD3:n ilmentyminen vähäisempää kolmoisnegatiivisessa kuin duktaalisessa rintasyövässä.

Asiasanat: CDK4, hypoxiassa induoituva tekijä (HIF), p16, PHDs 1-3, rintasyöpä, syklini D1
Ei peltoon kätketty, ei vuorten uumeniin, ei mereen, ei metsän syvyksiin. Se on sinussa itsessäsi.

Maija Paavilainen
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All my friends, I wish I could thank all of you here individually, but I’m afraid I would forget to name someone, so therefore – thank you all. I’ve learned that no matter how far I went or whatever I did, you will always be there for me. You have filled my life with joy, you have given me perspective when needed,
and you have stood by me even on the cloudiest of days. To have such friends is a privilege.

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This study was financially supported by the Finnish Breast Cancer Group.

Oulu, October 2013

Emmi Peurala
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AH</td>
<td>atypical hyperplasia</td>
</tr>
<tr>
<td>ADH</td>
<td>atypical ductal hyperplasia</td>
</tr>
<tr>
<td>ALH</td>
<td>atypical lobular hyperplasia</td>
</tr>
<tr>
<td>BLBC</td>
<td>basal-like breast cancer</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>breast cancer gene 1/2</td>
</tr>
<tr>
<td>CAK</td>
<td>cyclin-dependent kinase-activating kinase</td>
</tr>
<tr>
<td>CAV1/2</td>
<td>caveolin 1/2</td>
</tr>
<tr>
<td>CDK4/6</td>
<td>cyclin-dependent kinase 4/6</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DH</td>
<td>ductal hyperplasia</td>
</tr>
<tr>
<td>CAIX</td>
<td>carbonic anhydrase 9</td>
</tr>
<tr>
<td>CIS</td>
<td>carcinoma in situ</td>
</tr>
<tr>
<td>CISH</td>
<td>chromogenic in situ hybridization</td>
</tr>
<tr>
<td>CK5/6</td>
<td>cytokeratin 5/6</td>
</tr>
<tr>
<td>CMF</td>
<td>cyclophosphamide, methotrexate, 5-fluorouracil</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DFS</td>
<td>disease-free survival</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>for example</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EglN1/2/3</td>
<td>egg-laying defect nine homologue 1/2/3</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>FEC</td>
<td>5-fluorouracil, epirubicin, cyclophosphamide</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>glucose transporter 1</td>
</tr>
<tr>
<td>HCLK2</td>
<td>human homologue of the <em>C. elegans</em> biological clock protein 2</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HIF-1α/2α</td>
<td>hypoxia-inducible factor 1 alpha/2 alpha</td>
</tr>
<tr>
<td>HIF-1β</td>
<td>hypoxia-inducible factor 1beta</td>
</tr>
<tr>
<td>IC</td>
<td>invasive carcinoma</td>
</tr>
<tr>
<td>IDC</td>
<td>invasive ductal carcinoma</td>
</tr>
</tbody>
</table>
i.e. that is
IGF-2 insulin-like growth factor 2
IgG immunoglobulin G
IHC immunohistochemistry
ILC invasive lobular carcinoma
LCIS lobular carcinoma in situ
mRNA messenger ribonucleic acid
mTOR mammalian target of rapamycin
NF-κB nuclear factor-kappaB
OS overall survival
PAI-1 plasminogen activator inhibitor-1
PALB2 partner and localizer of breast cancer gene 2
PARP poly ADP-ribose polymerase
PBS phosphate-buffered saline
PDGF platelet-derived growth factor
PHD1/2/3 prolyl hydroxylase domain 1/2/3
PI3K phosphatidylinositol 3-kinase
PKM2 pyruvate kinase M2
PR progesterone receptor
pRB retinoblastoma tumor suppressor protein
pVHL von Hippel-Lindau tumor suppressor protein
Q-PCR real-time quantitative polymerase chain reaction
RB retinoblastoma tumor suppressor gene
RNA ribonucleic acid
TDLU terminal duct lobular unit
TGF-β transforming growth factor beta
TNBC triple-negative breast cancer
TNF-α tumor necrosis factor alpha
uPA urokinase-type plasminogen activator
VEGF vascular endothelial growth factor
WHO World Health Organization
List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


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1 Introduction

Once thought to originate from evil spirits, sin and bad deeds, a punishment from the gods, breast cancer was a ruthless killer, with women having to go through a painful death. Progress has been made since then, and the course of the disease has turned for many women today. However, the Western world still faces a breast cancer problem, in that its incidence is rising (Jemal et al. 2011). The high standard of living in the Western countries, including Finland, is reflected in a breast cancer risk, since its aetiological factors are becoming increasingly lifestyle-dependent (Gaudet et al. 2013, Hartz & He 2013, Jemal et al. 2011, Rose & Vona-Davis 2010). Every year approximately 4500–5000 women are diagnosed with breast cancer in Finland (Finnish Cancer Registry 2011), but fortunately, mostly due to advances in diagnosis and treatment, the prognosis for breast cancer patients is improving and mortality rates are declining (Jemal et al. 2011).

Breast cancer is a heterogeneous disease entity rather than one single disease (Almendro & Fuster 2011, Eroles et al. 2012). While some women have an excellent prognosis, others still have to face the unfortunate side of this malignancy (Eroles et al. 2012, Lin et al. 2012, Okugawa et al. 2005, Soerjomataram et al. 2008). Prognostic and predictive factors are needed in order to select patients efficiently for suitable treatment in an adjuvant setting (Morabito et al. 2003). In the search for ways to separate patients with good and poor prognoses, our attention was directed towards tumor hypoxia and regulation of the essential cell cycle.

The key mechanism of cellular adaptation to hypoxia is activation of the hypoxia-inducible factor (HIF) pathway (Kaelin Jr. & Ratcliffe 2008). The p16-cyclin D1-CDK4-RB pathway, on the other hand, regulates the G1/S transition point in the cell cycle (Weinberg 1995). Both overexpression of HIF and disruption of the RB pathway are commonly found in breast cancer (Semenza 2012a, Weinberg 1995).

This present work was designed to investigate potential new prognostic markers for breast cancer by focusing on the key regulators of the hypoxia response pathway, HIF-1α, HIF-2α and the HIF prolyl 4-hydroxylases PHDs 1–3, and the regulators of the cell cycle, cyclin D1, CDK4 and p16, in a series of ductal breast cancer patients and a series of triple-negative breast cancer (TNBC) patients.
2 Review of the literature

2.1 Breast cancer

2.1.1 Breast carcinogenesis

Carcinogenesis is the process by which normal cells transform into neoplastic cells as a result of cumulative genetic and epigenetic alterations. These mutational and non-mutational events result in autonomy over cell growth control, clonal proliferation and the formation of a malignant tumor (Rivenbark & Coleman 2012). The chain of carcinogenic events leading to neoplasm formation occurs over a long period of time, which explains why cancers normally emerge later in life.

Mammary gland development is a dynamic process which is under strict hormonal control (Anderson & Clarke 2004). A primitive ductal system is developed during embryogenesis, but the further differentiation takes place only during puberty and final maturation during pregnancy and lactation (Anderson & Clarke 2004, Pelekanou & Leclercq 2011). The mammary gland consists of epithelial cells and the underlying stroma, for illustration see Figure 1. Two sets of cells form the epithelium: luminal and myoepithelial (also basal) cells, under which lies the basement membrane (Anderson & Clarke 2004). The functional unit of the mammary gland, the terminal duct lobular unit (TDLU), and to be precise, one single epithelial cell in such a TDLU, is thought to give rise to invasive breast carcinoma (Allred et al. 2001, Anderson & Clarke 2004). It has also been proposed that specific breast cancer stem cells many serve as the origin of breast neoplasms (Bombonati & Sgroi 2011, Oliveira et al. 2010).
It seems that most invasive breast tumors arise from a series of precursor lesions. A linear histological model for breast cancer evolution has been put forward that encompasses progressive changes from the normal breast epithelium to hyperplasia, atypical hyperplasia (AH), carcinoma in situ (CIS) and finally invasive carcinoma (IC) with metastatic potential (Allred et al. 2001, Rivenbark & Coleman 2012, Simpson et al. 2005). This theory of an evolutionary relationship has been under extensive scrutiny and the outcomes have been favourable. It has been demonstrated that most alleged precursor lesions show similarities in DNA ploidy (Ottesen et al. 2000) and molecular phenotype (Allred et al. 2008, Tamimi et al. 2008) to the invasive lesions that they are thought to progress to. It should be remembered, however, that not all pre-malignant lesions progress to invasive ones, and that some invasive carcinomas may arise without a pre-existing lesion with malignant potential (Allred et al. 2001). The linear model can be criticized because of its simplicity, considering how extremely heterogeneous breast cancer is, both clinically and biologically. Hence the linear model has been expanded into a low grade-like and high grade-like molecular pathway model (Bombonati & Sgroi 2011, Simpson et al. 2005), see Figure 2.
Fig. 2. Biological heterogeneity in the multi-step process of breast carcinogenesis. TDLU, terminal duct lobular unit; DH, ductal hyperplasia; ADH, atypical ductal hyperplasia; ALH, atypical lobular hyperplasia; DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; basal cluster, expression of basal markers such as cytokeratins. See also Table 3.

In normal breast tissue there is an equilibrium between cell proliferation and cell death, but when this equilibrium faces disturbances it usually results in growth advantage (Rivenbark & Coleman 2012). The initiating step towards malignant progression is yet to be conclusively defined, but carcinogenesis is known to encompass a multitude of genetic and epigenetic errors. These alterations lead to the activation of oncogenes to remove the inhibitory control of cellular replication and the inactivation of tumor suppressor genes to enable self-stimulated proliferation and the avoidance of apoptosis, thus leading to the ultimate characteristic of neoplasms – immortalization (Allred et al. 2001, Dworkin et al. 2009, Rivenbark & Coleman 2012, Simpson et al. 2005). Probably the most famous proto-oncogene and tumor suppressor gene in human breast cancer are the human epidermal growth factor receptor 2 gene (HER2/neu) and tumor protein 53
gene (TP53), respectively (Allred et al. 2001, Taneja et al. 2010). Other known but less extensively studied proto-oncogenes contributing to breast cancer evolution include c-MYC and c-RAS (Donegan 1997). Most of the genes involved in mammary carcinogenesis are still unknown, however.

A major proportion of human breast cancers fall into the category of sporadic cancers, which have no recognizable strong genetic predisposing mutations but are the result of random de novo mutations (Rivenbark & Coleman 2012). Some of the genetic alterations associated with breast carcinogenesis are well-defined, such as specific gene amplifications, deletions, point mutations, chromosome rearrangements and aneuploidy (Dworkin et al. 2009), but while genetic changes attack the genome, epigenetic alterations do not represent changes in the genome itself but contribute to the phenotype of the genome, and as such still act strongly to promote breast carcinogenesis (Dworkin et al. 2009). These epigenetic mechanisms include DNA methylation, histone modifications and nucleosomal remodelling (Jovanovic et al. 2010).

In sporadic breast tumorigenesis oestrogen acts as a highly potent mitogen, and there is extensive evidence of oestrogen stimulating the growth of breast cancer (Platet et al. 2004). Oestrogen mediates its effects via the oestrogen receptor (ER), which regulates a multitude of genes that contribute to growth, differentiation and metabolism (Pelekanou & Leclercq 2011). There is a detectable increase in ER expression as the malignant progression advances from hyperplasia to atypical hyperplasia and on to carcinoma in situ and finally to invasive carcinoma (Anderson & Clarke 2004).

Approximately 5–10% of breast cancers are hereditary, out of which 90% are due to mutations in the breast cancer genes 1 and 2 (BRCA1 and BRCA2), the alleles of which are inherited in an autosomal dominant manner (Gage et al. 2012). The BRCA1s are tumor suppressor genes with a role in DNA damage response and proliferation (Teng et al. 2008). A smaller proportion of the hereditary breast cancers can be explained by other rarer inherited gene mutations, e.g. germline mutations of the partner and localizer of the BRCA2 gene (PALB2) (Njiaju & Olopade 2012, Southey et al. 2013). PALB2 functions in the same DNA damage response pathway as the BRCA1s, and is considered to entail a moderate risk of breast cancer (Njiaju & Olopade 2012, Southey et al. 2013). In addition, there are some genetic disorders that constitute a risk of breast cancer, e.g. Lynch syndrome, formerly known as hereditary non-polyposis colorectal cancer (Gage et al. 2012, Win et al. 2013).
2.1.2 Epidemiology and aetiology

Breast cancer is the most common cancer among women in the Western world, including Finland (Jemal et al. 2011). It is also the leading cause of cancer-related deaths in the female population. The incidence of breast cancer is continuously increasing, but at the same time mortality from the disease is decreasing (Jemal et al. 2011), on account of the growing general awareness of breast cancer among women, widespread mammographic screening programmes and the diagnostic and therapeutic improvements achieved in recent years (Jemal et al. 2011). Breast cancers are nowadays diagnosed more often at an early stage, so that they can be successfully treated. The methods of treatment have multiplied and offer a wide choice for uniquely tailored solutions.

Breast cancer accounts for approximately 23% of all cancers among women worldwide, but only 14% of cancer-related deaths (Jemal et al. 2011). According to the Finnish Cancer Registry, 4882 breast cancers were diagnosed in Finland in 2011, only 17 of which were in men (Finnish Cancer Registry 2011). This amounted to 32% of all cancers diagnosed in Finland in that year 2011. The lifetime risk of developing breast cancer for a woman in Finland is about 11% (Finnish Cancer Registry 2011), which means that every 9th woman is diagnosed with breast cancer at some point in her life. On the other hand, the lifetime risk of death from breast cancer is only 2% (Finnish Cancer Registry 2011). According to the Finnish Cancer Registry (2011), the relative survival figures at 1 and 5 years after diagnosis in 2007–2009 were 97% and 89%, respectively.

Some of the factors that contribute to the incidence of breast cancer, mainly hormonal and reproductive factors, are well-known, the most substantial risks being female gender and advanced age (Jemal et al. 2011, Kamangar et al. 2006). Other risk factors include a positive family history of breast cancer, benign breast disease, nulliparity, late age at first full-term pregnancy (Jemal et al. 2011, Kamangar et al. 2006), early menarche and late menopause (Collaborative Group on Hormonal Factors in Breast Cancer 2012). Postmenopausal hormone replacement therapy and obesity have also been associated with an elevated risk of breast cancer (Beral & Million Women Study Collaborators 2003, Rose & Vona-Davis 2010), and there is evidence that life-style factors such as smoking (Gaudet et al. 2013, Luo et al. 2011a) and alcohol consumption (Hartz & He 2013, Hayes et al. 2013, Lew et al. 2009) are notable additional risks.
2.1.3 Classical prognostic and predictive factors

A prognostic factor is defined as a marker that provides information on the natural history of a disease and the possible clinical outcome for each patient at the time of diagnosis, while a predictive factor is an indicator that gives valuable information for the selection of patients who might be likely to respond to a specific therapeutic intervention (Morabito et al. 2003, Okugawa et al. 2005). Numerous studies have been made of both prognostic and predictive factors for survival in breast cancer patients over past decades.

One of the most important factors affecting the prognosis is the clinical stage of the disease at the time of diagnosis (Donegan 1997, Taneja et al. 2010). Staging divides patients into four disease groups: early (stages I and II), locally advanced (stage III) and metastatic (stage IV) (Donegan 1997, Taneja et al. 2010). This staging is based on the TNM classification, which considers three factors: tumor size (T), primary nodal status (N) and the presence of distant metastases (M) (Tables 1 and 2).

Breast cancer is uncommon in women under 40 years of age (Cardoso et al. 2012), so that only 3% and 14% of breast cancer patients diagnosed in 2011 were under 40 and 50 years old, respectively (Finnish Cancer Registry 2011). Breast cancers in younger women more frequently have a hereditary background and a variety of gene mutations (Cardoso et al. 2012). The age of the patient at the time of the diagnosis is an independent marker of an adverse prognosis (Kroman et al. 2000), and it has been suggested that this may derive from the high prevalence of biologically aggressive subtypes of breast cancer in younger women (Cardoso et al. 2012).
Table 1. Classical TNM classification of breast cancer, modified from the AJCC Cancer Staging Handbook (AJCC 7th edition, 2010).

<table>
<thead>
<tr>
<th>TNM</th>
<th>Definition</th>
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<tr>
<td>Primary tumor (T)</td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1a</td>
<td>Tumor &gt; 1 mm but ≤ 5 mm in greatest dimension</td>
</tr>
<tr>
<td>T1b</td>
<td>Tumor &gt; 5 mm but ≤ 10 mm in greatest dimension</td>
</tr>
<tr>
<td>T1c</td>
<td>Tumor &gt; 10 mm but ≤ 20 mm in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor &gt; 20 mm but ≤ 50 mm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor &gt; 50 mm in greatest dimension</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor of any size with direct extension to the chest wall and/or to the skin</td>
</tr>
<tr>
<td>Primary lymph nodes (N)</td>
<td></td>
</tr>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastases</td>
</tr>
<tr>
<td>N1</td>
<td>Micrometastases or metastases in 1–3 axillary or internal mammary lymph nodes</td>
</tr>
<tr>
<td>N2</td>
<td>Metastases in 4–9 axillary or internal mammary lymph nodes</td>
</tr>
<tr>
<td>N3</td>
<td>Metastases in ≥ 10 axillary lymph nodes or in infraclavicular or ipsilateral supraclavicular lymph nodes</td>
</tr>
<tr>
<td>Distant metastases (M)</td>
<td></td>
</tr>
<tr>
<td>MX</td>
<td>Distant metastases cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No clinical or radiographic evidence of distant metastases</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastases determined by classic clinical and/or radiographic means</td>
</tr>
</tbody>
</table>
Table 2. Anatomical stage according to the TNM classification, modified from the AJCC Cancer Staging Handbook (AJCC 7th edition, 2010).

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IA</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IB</td>
<td>T0</td>
<td>N1 micrometastasis</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1 micrometastasis</td>
<td>M0</td>
</tr>
<tr>
<td>IIA</td>
<td>T0</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIB</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
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<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIIA</td>
<td>T0</td>
<td>N2</td>
<td>M0</td>
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<td></td>
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<tr>
<td>IIIB</td>
<td>T4</td>
<td>N0</td>
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<td></td>
<td>T4</td>
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<td></td>
<td>T4</td>
<td>N2</td>
<td>M0</td>
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<tr>
<td>IIIC</td>
<td>Any T</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>

The main factors driving prognostic predictions in primary breast cancer patients today are lymph node status, tumor size, histological grade of the tumor, proliferation index, steroid receptor status and HER2 status (Donegan 1997, Morabito et al. 2003, Soerjomataram et al. 2008, Taneja et al. 2010).

**Nodal status**

In the world of new, up-coming prognostic and predictive markers, the involvement of the axillary lymph nodes has continued to occupy a robust place as the single most important prognostic factor (Donegan 1997, Morabito et al. 2003). The axillary lymph nodes are the most common site for metastasis in breast cancer patients, and the existence of these metastatic lymph nodes can also serve as proof of the tumor’s capacity for distant metastasis (Donegan 1997). A direct correlation has been demonstrated between the number of metastatic nodes and an adverse clinical outcome (Arriagada et al. 2006, Fisher et al. 1983, Okugawa et al. 2005, Weiss et al. 2003). Involvement of the axillary nodes also
remains a major factor in deciding whether a patient receives systemic adjuvant therapy or not. The current standard is to treat node-positive patients with adjuvant chemotherapy (Morabito et al. 2003, Weiss et al. 2003).

Tumor size

The importance of tumor size is unequivocal, and it clearly comes second to nodal status as an independent prognostic factor (Donegan 1997). Tumor size is directly related to an increased risk of lymph node metastasis and to poor disease-free and overall survival (Soerjomataram et al. 2008). Tumors of 1 cm or less in diameter possess an especially low risk of recurrence, but as the diameter increases from less than 2 cm up to more than 5 cm, the risk of recurrence grows (Arriagada et al. 2006, Okugawa et al. 2005, Warwick et al. 2004).

Histological grade

Histological grade describes the differentiation of the tumor, and can serve as an independent indicator of tumor aggressiveness having prognostic value with regard to both overall and disease-free survival (Schumacher et al. 1993). Grading is based on three histological features: the degree of tubule formation, the number of mitoses and nuclear pleiomorphism (Donegan 1997). A running scale from 1 to 3 is used to indicate the progression from well to poorly differentiated tumors. Patients with grade 1 tumors have significantly better survival than patients with grade 2 or 3 (Elston & Ellis 1991, Okugawa et al. 2005, Warwick et al. 2004).

Ki-67

Ki-67 is a protein detected in cells during the active phases of the cell cycle, while it is absent in resting cells (Donegan 1997, Inwald et al. 2013). It is consequently used to describe cellular proliferation on a running scale from 0 (negative when < 5% of the invasive tumor cells show positivity) to 3 (high when > 30% of the invasive tumor cells show positivity). High proliferation activity is associated with poor histological differentiation, lymph node metastasis and a greater tumor size (Inwald et al. 2013, Morabito et al. 2003). It has been concluded that Ki-67 is an independent prognostic factor (Inwald et al. 2013, Luporsi et al. 2012), but its value as a predictive factor is still controversial (Luporsi et al. 2012).
Steroid receptors

Steroid receptors, also called hormone receptors, i.e. the oestrogen receptor (ER) and progesterone receptor (PR), mediate the effects of oestrogen and progesterone, respectively (Anderson & Clarke 2004). Oestrogen controls proliferation and differentiation in the normal mammary gland (Renoir et al. 2013), and it also plays a critical role in breast tumorigenesis, as approximately 50–80% of breast cancer tumors express ER, thus being highly dependent on the hormone for proliferation (Platet et al. 2004). ER-positive breast cancers are generally low in proliferation and more likely to be well differentiated (Morabito et al. 2003, Taneja et al. 2010). The expression of PR is controlled by functional oestrogen-ER-complexes that activate its transcription (Anderson & Clarke 2004, Donegan 1997).

ER/PR status is a weak independent prognostic factor, but it serves exceptionally well as a guide to endocrine therapy (Oldenhuis et al. 2008, Rastelli & Crispino 2008, Soerjomataram et al. 2008). The prevailing practise in clinical oncology is to use endocrine therapy whenever treating ER-positive breast cancer patients. The use of anti-oestrogens and aromatase inhibitors can improve the prognosis for ER-positive patients considerably relative to that of ER-negative patients (Soerjomataram et al. 2008). ER negativity in a tumor is related to a lack of benefit from adjuvant hormone therapy but ameliorated benefit from chemotherapy (Morabito et al. 2003).

HER2

Human epidermal growth factor receptor 2 (HER2) (also called HER2/neu, ErbB2) is encoded by the proto-oncogene ERBB2 (Donegan 1997). As a characteristic proto-oncogene, ERBB2 is involved in cell proliferation, and its mutation promotes neoplastic transformation (Taneja et al. 2010).

Approximately 15% of primary breast cancers show gene amplification and protein overexpression of HER2 (Patani et al. 2013), and it is a classical predictive prognosticator (Jahanzeb 2008, Patani et al. 2013). HER2 positivity is associated with an aggressive phenotype of breast cancer – node-positive, high-grade, large tumors – and poor prognosis (Ménard et al. 2002, Taneja et al. 2010). HER2 status is also a predictive factor for responsiveness to two sets of therapies. Firstly, the amplification and overexpression of HER2 is used for identifying patients suitable for treatment with the anti-HER2 monoclonal antibody
trastuzumab (Jahanzeb 2008, Murphy & Morris 2012). This has in fact improved the prognosis for HER2-positive breast cancer patients, bringing it close to that of patients without amplification or overexpression of ERBB2 (Jahanzeb 2008). In addition to trastuzumab, pertuzumab (a monoclonal antibody) and labatinib (a small molecule tyrosine kinase inhibitor) are HER2-targeted therapies used to treat patients with metastatic breast cancer (Murphy & Morris 2012, Saini et al. 2011). Secondly, HER2 positivity is associated with a beneficial outcome from adjuvant anthracycline and paclitaxel chemotherapy (Hayes et al. 2007, Pritchard et al. 2006).

### 2.1.4 Novel prognostic and predictive factors

Some new, promising biomarkers for breast cancer are now emerging. The urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) have already been taken into clinical use as invasion and metastasis markers for risk assessment and treatment planning in the case of breast cancer patients (Duffy & Duggan 2004, Harbeck et al. 2004, Harbeck et al. 2013). Similarly, the poly ADP-ribose polymerases (PARPs), which are involved in the response to DNA damage, have been linked to breast carcinogenesis, especially in BRCA-deficient breast tumors (Gibson & Kraus 2012), and PARP inhibitors are currently under investigation as new anti-cancer therapies. Hyperactivation of the phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) has been linked to resistance to both endocrine therapy and trastuzumab in breast cancer patients (Vinayak & Carlson 2013, Zagouri et al. 2012), while mutations of the tumor suppressor gene p53 are thought to be associated with a poor outcome of breast cancer (Taneja et al. 2010, Turner et al. 2013). Vascular endothelial growth factor (VEGF) is an important mediator of angiogenic effects in breast cancer and has been associated with poor survival (Taneja et al. 2010). The above factors are only a few examples, but it will suffice to say that the list of new markers being studied is extensive. Much research is yet to be done, however, before any of the new markers can establish a place for themselves in the group of classical prognostic and predictive factors.
2.1.5 Histopathological and biological types of invasive breast carcinoma

Histopathology has traditionally been the basis for the classification of breast cancer tumors. There is an abundance of histological diagnoses relevant to breast cancer, although just a small number of them cover the majority of the patients (Berg & Hutter 1995). Invasive ductal carcinoma is the most prevalent histological subtype, since about 60–80% of all breast tumors are of this type (Berg & Hutter 1995, Bombonati & Sgroi 2011). The second most common subtype is lobular carcinoma, so that the two together account for about 90% of all breast cancer tumors (Berg & Hutter 1995, Bombonati & Sgroi 2011). Other types include mucinous, medullary, tubular, papillary, cribriform and comedo carcinoma, Paget’s disease and inflammatory carcinoma (Berg & Hutter 1995, Li et al. 2005). Some of the histological subtypes have a specific prognostic meaning (Colleoni et al. 2012, Masuda 2012), in that inflammatory and medullary carcinomas are associated with an aggressive phenotype, for example, while mucinous, tubular and papillary carcinomas are associated with less aggressive phenotypes (Caldarella et al. 2013, Li et al. 2005). However, in view of the fact that the majority of breast tumors are of one single histological type but are at the same time biologically and genetically highly heterogeneous (Eroles et al. 2012), the importance of histological subtype as an independent prognosticator remains low.

The results of the vast amount of research into the molecular biology of breast cancer over the past decade have broadened our understanding of the complex diversity of the disease. The division of invasive breast cancer tumors into biological subtypes by reference to their molecular and genetic profiles has proved to be a superior means of classifying breast cancer patients, as these profiles provide extensive information on the possible clinical outcome (Simpson et al. 2005). Microarray technologies applied to study DNA, RNA, protein profiles and epigenetic changes can been used to portray a tumor’s phenotype (Sorlie 2004). Six intrinsic biological subtypes of breast cancer are currently recognised: luminal A, luminal B, HER2-enriched, basal-like, claudin-low and normal-like (Herschkowitz et al. 2007, Perou et al. 2000, Sorlie et al. 2001). In addition, there is a subtype of triple-negative breast cancer which will be discussed here separately.

Approximately 50–60% of breast cancers are of the luminal A type, and these tumors are usually ER/PR-positive, HER2-negative, well differentiated and low
in proliferation (Eroles et al. 2012). Luminal A-type patients have an excellent prognosis, although a tendency for a higher risk of late recurrence with bone metastases has been noted (Kennecke et al. 2010). Luminal B-type tumors may be distinguished from luminal A-type tumors mainly on the grounds of their distinct proliferation status (Prat & Perou 2011). Luminal B-type tumors show hormone receptor positivity, and in most cases HER2 negativity like the luminal A-type tumors, but are of high proliferation and normally poorly differentiated (Prat & Perou 2011) (Table 3). The luminal B type encompasses some 10–20% of breast cancer tumors, and these have a poorer prognosis than the luminal A tumors (Eroles et al. 2012).

The HER2-enriched subtype consists of tumors that are clinically HER2-positive, highly proliferative and usually ER-negative (Eroles et al. 2012) (Table 3). HER2 amplification and HER2 overexpression have been described in about 15% of all breast carcinomas (Patani et al. 2013), but it is significant that not all tumors showing HER2 positivity fall into the HER2-enriched category. Some 20% of luminal B-type tumors are HER2-positive, and in addition, around 30% of HER2-enriched tumors are in fact clinically HER2-negative (thus named HER2-enriched) (Prat & Perou 2011). HER2-enriched breast cancers are aggressive and entail a poor prognosis (Sorlie 2004).

The cluster of basal-like breast tumors accounts for 10–20% of all breast cancers (Eroles et al. 2012). Absence of the three prime receptors is a common feature of this subtype, although about 10% and 35% of basal-like tumors express ER and HER2, respectively (Carey et al. 2010). The name “basal-like” derives from the gene expression profile, which is similar to that of normal breast myoepithelial cells (Sorlie 2004). The genes expressed in basal-like tumors include cytokeratin 5 and 6 (CK5 and 6) and epidermal growth factor receptor (EGFR) (Eroles et al. 2012) (Table 3). Clinically, basal-like tumors are aggressive, with large tumor size, poor differentiation, high mitotic activity, tumor necrosis and a high frequency of axillary lymph node involvement (Eroles et al. 2012). Patients with basal-like tumors are often younger than the average of breast cancer patients at the time of diagnosis and have a poor prognosis, as this subtype has a pattern of aggressive early metastatic relapse in visceral organs (Kennecke et al. 2010, Prat & Perou 2011).

Clauclin-low tumors feature low expression of claudins, E-cadherin and other genes involved in tight junctions and intercellular adhesion (Prat & Perou 2011) (Table 3). A major proportion of these tumors are triple-negative, although about
20% are positive for hormone receptors (Eroles et al. 2012). Long-term survival is poor in the claudin-low subtype (Prat & Perou 2011).

The normal-like subtype encompasses about 5–10% of breast cancers. Their gene expression profile is close to that of adipose tissue and the status for all three receptors is negative (Eroles et al. 2012) (Table 3). It has been debated whether this subtype really exists, and a hypothesis has been put forward that it may be merely a technical artifact arising from the contamination of samples with normal tissue (Parker et al. 2009, Prat & Perou 2011).

**Table 3. The intrinsic subtypes of breast cancer relative to the prognostic and predictive markers for breast cancer.**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>ER/PR</th>
<th>HER2</th>
<th>Ki-67</th>
<th>Grade</th>
<th>Other markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>+</td>
<td>-</td>
<td>low</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>+</td>
<td>-</td>
<td>high</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>-</td>
<td>+</td>
<td>high</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>Basal-like</td>
<td>-</td>
<td>-</td>
<td>high</td>
<td>high</td>
<td>CK5/6 +, EGFR +</td>
</tr>
<tr>
<td>Triple-negative</td>
<td>-</td>
<td>-</td>
<td>high</td>
<td>high</td>
<td>CK5/14/17 +, EGFR +, CAV1/2 +, P-cadherin +</td>
</tr>
<tr>
<td>Claudin-low</td>
<td>-</td>
<td>-</td>
<td>low/high</td>
<td>high</td>
<td>Claudins -, E-cadherin -</td>
</tr>
<tr>
<td>Normal-like</td>
<td>-</td>
<td>-</td>
<td>profile close to adipose tissue</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Triple-negative breast cancer (TNBC)**

Although genetic profiling has expanded our view of the overwhelming diversity that characterizes breast cancer, the practical relevance of the intrinsic subtypes discussed above is still elusive, and the categorizing of breast tumors in such a way is hardly a routine clinical practise. Gene-expression profiling has been limited to clinical trials for the most part, but in the meantime the identification of TNBCs has been and still is an everyday diagnostic parameter in clinical oncology.

TNBC represent 10–20% of newly diagnosed breast cancer cases (Boyle 2012, Carey et al. 2010). The term “triple-negative” originates from the loss of ER/PR/HER2 expression in the tumor (de Ruijter et al. 2011). Triple-negative and basal-like breast cancers (BLBC) are frequently thought to be one single entity, because of their often similar negative receptor status, but in fact 10–35% of TNBCs are not BLBCs in terms of gene profiling, and up to 45% of BLBCs are not triple-negative in their receptor profile (Carey et al. 2010, Criscitiello et al. 2012, de Ruijter et al. 2011, Penault-Llorca & Viale 2012). It should be
remembered that the diagnosis of TNBC is based on immunohistochemistry and *in situ* hybridisation of only three gene products, while the diagnosis of BLBC would need gene-expression profiling of up to a few hundred genes (Penault-Llorca & Viale 2012). There is an overlap not only between the triple-negative and basal-like tumor subtypes but also between the triple-negative and claudin-low subtypes, and triple-negative and HER2-enriched subtypes (Prat & Perou 2011, Prat *et al.* 2013).

Triple-negative tumors are dominated by their ductal histology (Carey *et al.* 2010, Criscitiello *et al.* 2012). These tumors are fairly regularly aggressive, with a high grade, high proliferation activity and tumor necrosis (Bauer *et al.* 2007, Boyle 2012). There is a pattern of early relapses in the visceral organs, primarily the lungs (Carey *et al.* 2010), and in the central nervous system (Lin *et al.* 2012). Patients diagnosed with TNBC are often younger than average (<50 years old) (Bauer *et al.* 2007, Boyle 2012, Ovcaricek *et al.* 2011), and the expression of EGFR, basal makers such as cytokeratin 5, 14 and 17 (CK5/14/17) and myoepithelial markers such as caveolins (CAV1/2) and P-cadherin is common (Carey *et al.* 2010) (Table 3). An interesting finding has been the overexpression of another hormone receptor, the androgen receptor, which is found in about 30–35% of triple-negative tumors (Niemeier *et al.* 2010, Park *et al.* 2010). BRCA1-mediated breast cancers are associated with the triple-negative subtype, while some 50% of TNBCs show a BRCA1 deficiency (Criscitiello *et al.* 2012). It has been suggested that BRCA1 loss may play a significant role in TNBC carcinogenesis (de Ruijter *et al.* 2011).

Patients with TNBC have a poor prognosis, and the adverse effect on overall, breast cancer-specific and disease-free survival has been comprehensively reported (Bauer *et al.* 2007, de Ruijter *et al.* 2011, Ovcaricek *et al.* 2011). The 5-year overall survival rate among TNBC patients is approximately 75–80% (Bauer *et al.* 2007, de Ruijter *et al.* 2011, Ovcaricek *et al.* 2011). Because of the absence of ER, PR and HER2, no specific targeted therapies are available at the moment for treating patients with triple-negative disease, and thus, conventional chemotherapy is the standard for TNBC treatment. Fortunately, TNBC tumors are often sensitive to chemotherapy (Carey *et al.* 2010, de Ruijter *et al.* 2011), although a good initial response to cytotoxic treatment is often followed by early relapses in the form of distant metastases (Carey *et al.* 2010, de Ruijter *et al.* 2011).

Nevertheless, the future holds some prospects of developing an adjuvant treatment for TNBCs. The anti-VEGF monoclonal antibody bevacizumab and the
EGFR-targeted agent cetuximab are being studied in TNBC patients in clinical trials at the moment, in combination with chemotherapy (Duffy et al. 2012, Joensuu & Gligorov 2012). Likewise, the PARP inhibitor iniparib has entered clinical trials in cases of advanced TNBC with BRCA dysfunction (Duffy et al. 2012, Metzger-Filho et al. 2012).

2.1.6 Gene expression profiling in breast cancer

Gene expression profiling by means of microarray studies, whole genome sequencing and copy number analyses have allowed the expression of thousands of genes to be analysed in order to create a molecular profile of a tumor. The cancer genome encompasses a multitude of somatic genetic variations and a smaller proportion of inherited genetic aberrations (Campbell et al. 2008), and as a highly heterogeneous disease, breast cancer is no exception to this (Reis-Filho & Pusztai 2011). However, not all the genetic aberrations found in cancer tumors contribute to carcinogenesis or cancer progression (Bignell et al. 2010). Thus investigation of the genome and transcriptome of a tumor aims at searching for possible carcinogenetic drivers, perhaps potential targets for therapies in the future (Bignell et al. 2010, Campbell et al. 2008). For example, deletions in \textit{PPP2R2A}, \textit{MTAP} and \textit{MAP2K4} were identified in a recent large gene expression and copy number study as putative breast cancer genes, in addition to which novel genetic subgroups with distinct clinical outcomes were found (Curtis et al. 2012).

Gene expression profiling has become a significant tool, as it provides further information in addition to the traditional clinico-pathological factors, its specific aims being to predict the clinical outcome and responses to certain therapies (Arpino et al. 2013, Reis-Filho & Pusztai 2011). Early-stage breast cancer patients in particular are often given adjuvant treatment that they ultimately will not benefit from (Arango et al. 2013, Reis-Filho & Pusztai 2011). The best-known multigene analyses that are currently available for clinical use are MammaPrint and Oncotype DX (Nagaraj & Ma 2013). MammaPrint is a multigene assay designed for risk assessment in patients with node-negative breast cancer, regardless of ER status (Nagaraj & Ma 2013). Its 70-gene signature involves genes for proliferation, invasion, metastasis and angiogenesis, and it classifies patients according to their risk (high or low) of developing metastases (Arango et al. 2013, Arpino et al. 2013, Reis-Filho & Pusztai 2011). Oncotype DX is 21-gene signature that consists of 16 cancer-related genes involved in proliferation, invasion, ER and HER2 signalling and 5 reference genes (Arango et
It is fascinating to consider that maybe some day patients might be selected for treatment according to the genotype of their tumors. However, these analyses are not yet a routine diagnostic tool in clinical practise in Finland.

2.1.7 Treatment of primary invasive breast cancer

The management of patients with invasive breast cancer has gone through enormous advances over past decades, and it is the improvements in adjuvant treatment that are mainly to thank for the current decline in mortality rates.

Due to the biological diversity of breast cancer (Eroles et al. 2012, Prat & Perou 2011) there is no gold standard treatment that would suit all patients. However, surgical treatment should be considered as one for primary diseases with options that include radical mastectomy and, increasingly often nowadays, breast-conserving surgery (Aebi et al. 2011, White et al. 2011, White et al. 2011, Young 2001). Axillary lymph node evacuation has been the state-of-the-art for many years, but it is now considered that a sentinel node biopsy is an accurate enough predictor of axillary nodal status in many cases (Krag et al. 2010). If the sentinel node is found to be negative with respect to metastasis, axillary dissection can be omitted (Aebi et al. 2011, Goldhirsch et al. 2013).

Defining the risk of recurrence and death is the key to determining whether primary breast cancer patients need adjuvant treatment. Such decisions have traditionally been based on clinical and pathological parameters that discriminate between low, average and high-risk patients and predict the benefit achievable with certain treatments (Guarneri & Conte 2004). The classical prognostic and predictive factors employed in such cases have been discussed above.

Postoperative radiation therapy should always be recommended after breast-conserving surgery (Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) et al. 2011). However, post-mastectomy radiotherapy is currently recommended only for patients with positive nodal status or those with T3-T4 tumors regardless of nodal status, or else if there are additional risks factors present, e.g. young age of the patient or a positive posterior surgical margin (Aebi et al. 2011, Goldhirsch et al. 2013).
Endocrine therapy is indicated for patients with hormone receptor-positive tumors, with supporting clinical data pointing to a survival advantage over patients not treated with such therapy (Aebi et al. 2011, Burstein et al. 2010, Goldhirsch et al. 2013). The endocrine therapy options include the anti-oestrogen tamoxifen, the aromatase inhibitors letrozol, anastrozol and exemestane, and ovarian suppression treatment with the gonadotropin-releasing hormone agonists goserelin and leuprolein (Aebi et al. 2011, Goldhirsch et al. 2013). Aromatase inhibitors can only be used in post-menopausal women (Aebi et al. 2011). Patients with HER2 amplification detected in in situ hybridization should be treated with the monoclonal anti-HER2 antibody trastuzumab (Goldhirsch et al. 2013, Jahanzeb 2008, Joensuu et al. 2009).

The role of adjuvant chemotherapy in early breast cancer is much more controversial. Adjuvant chemotherapy improves disease-free and overall survival in the majority of breast cancer patients (Bergh et al. 2001), but the extent of its positive effect is greater in patients having a high risk of recurrence. Adjuvant chemotherapy is indicated if the likelihood of recurrence in a 10-year follow-up is more than 10%. High-risk biological features include positive axillary nodal status, tumor size over 2 cm, high proliferation activity, tumor of a high grade, age under 35 years, absence of endocrine responsiveness, HER2 positivity and triple-negative status (Aebi et al. 2011, Goldhirsch et al. 2013). Multigene tests, e.g. Mammaprint and Oncotype DX, can be used, if they are available for clinical use, to assess whether to recommend chemotherapy or not (Aebi et al. 2011, Goldhirsch et al. 2013, Nagaraj & Ma 2013).

The most frequently used chemotherapeutic agents in cases of breast cancer are anthracyclins (epirubicin, doxorubicin) and taxanes (docetaxel, paclitaxel) (Aebi et al. 2011). For the best possible effect, such agents are normally given in combination. CMF (cyclophosphamide, methotrexate, 5-fluorouracil) has been considered the standard adjuvant treatment for several years (Guarneri & Conte 2004), but anthracycline-based therapies such as FEC (5-fluorouracil, epirubicin, cyclophosphamide) tend to be preferred nowadays (Aebi et al. 2011). Taxanes, which are highly potent anti-cancer agents in breast cancer, are frequently added to this combination (Aebi et al. 2011, Joensuu et al. 2009).

If both hormonal and cytotoxic or radiation and cytotoxic therapies are indicated, hormonal and radiation therapies are administered after the completion of the chemotherapy (Aebi et al. 2011). Trastuzumab, however, can be administered concomitantly with chemotherapy (Aebi et al. 2011, Joensuu et al. 2009).
Certain clinical practise guidelines exist for treating early breast cancer patients, such as those of the European Society for Medical Oncology (ESMO), the American Society of Medical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) and the St. Gallen Consensus. Even so, the tailoring of treatment for each patient requires more detailed information about the patient than simply the biological nature of the tumor and the prognosis for the disease. Age is no reason to discriminate between breast cancer patients, however, but performance status and the presence of significant comorbidities will affect the choice of treatment (Guarneri & Conte 2004).

2.2 Tumor hypoxia

Hypoxia is defined as reduced oxygen (O₂) availability, and thus implies a reduced concentration of oxygen. Solid tumors often contain hypoxic regions because of their rapid, uncontrollable cell proliferation combined with a structurally and functionally abnormal vasculature (Semenza 2012b). Two conditions of hypoxia have been described: acute hypoxia is perfusion-limited, being caused by inadequate blood flow through an abnormal vessel, while chronic hypoxia is diffusion-limited, caused by distant location from the source of O₂ (Lundgren et al. 2007, Semenza 2012a). Hypoxic circumstances generate a hostile microenvironment in which tumor cells need the help of adaptive mechanisms in order to survive (Semenza 2012b). In this regard, hypoxia enables the clonal selection, in which only the cells that have adapted prosper. This adaptation of neoplastic cells is a pivotal driving force in the progression towards a more aggressive and resistant tumor phenotype (Milani & Harris 2008, Rohwer & Cramer 2011).

As an adaptive response, hypoxia exerts various effects on cellular and physiological functions, including angiogenesis, erythropoiesis, metabolism, cell proliferation, differentiation and apoptosis, to mention a few (Rohwer & Cramer 2011). The key mediator in this adaptation is the hypoxia-inducible factor (HIF), to be discussed later (Lundgren et al. 2007, Milani & Harris 2008, Rohwer & Cramer 2011, Semenza 2012a, Semenza 2012b). In addition, tumor hypoxia can contribute to cell cycle arrest, the mechanisms of which may be numerous (Pettersen & Lindmo 1983).

Tumor hypoxia in breast cancer and many other forms of solid cancers is associated with clinically aggressive tumor behaviour (Lundgren et al. 2007, Milani & Harris 2008, Semenza 2012a), and, as it is a crucial factor behind tumor
invasion, metastasis and resistance to radiotherapy and a variety of cytotoxic drugs, the overall result is a poor clinical prognosis (Lundgren et al. 2007, Rohwer & Cramer 2011). It has been debated, however, whether hypoxia contributes to more aggressive tumors or whether aggressive tumors entail more widespread hypoxia (Lundgren et al. 2007).

### 2.2.1 HIF

Hypoxia-inducible factor, or HIF, is a heterodimeric transcription factor composed of two subunits: an O₂-regulated α subunit and a stable β subunit. Three forms of α subunit exist in humans, HIF-1α, HIF-2α and HIF-3α, but only one β subunit, HIF-1β (Kaelin Jr. & Ratcliffe 2008). HIF-1α and HIF-2α have been the focus of extensive research due to their key roles in the heart of the adaptive response to hypoxia, while HIF-3α has not generally been regarded as a potent transcription factor but rather as a negative regulator of HIF-1α and HIF-2α (Semenza 2010). The two isoforms HIF-1α and HIF-2α are architecturally similar and undergo the same proteolytic regulation (Weidemann & Johnson 2008), but HIF-2α appears to be more restricted in its tissue expression (Semenza 2012b) and the transcriptional activity between the two isoforms may differ slightly, so that exclusive target genes for HIF-1α and for HIF-2α have been recognised in addition to genes that are responsive to both (Kaelin Jr. & Ratcliffe 2008). It has been proposed that the acute and chronic responses to hypoxia are mediated by HIF-1α and HIF-2α, respectively (Helczynska et al. 2008). The stability of the HIF-α subunits is regulated by the oxygen-sensing HIF prolyl 4-hydroxylases PHDs 1–3 (Myllyharju & Koivunen 2013).

Under normoxic conditions HIF-α becomes 4-hydroxylated on one or two prolyl residues by PHDs, principally PHD2 (Kaelin Jr. & Ratcliffe 2008). This modification generates a binding site for the von Hippel-Lindau tumor suppressor protein (pVHL), a component of the E3-ubiquitin ligase complex, and as a result HIF-α is polyubiquitylated and subjected to proteosomal degradation (Bruick & McKnight 2001, Ivan et al. 2001, Jaakkola et al. 2001). Thus HIF-α subunits have a very short half-life under well-oxygenated conditions. In hypoxia, however, the oxygen-dependent PHDs cannot hydroxylate HIF-α, which is therefore able to escape degradation. The now stabilized HIF-α accumulates rapidly, relocates to the nucleus, dimerizes with the HIF-1β subunit and activates the transcription of several hundred genes (Kaelin Jr. & Ratcliffe 2008, Semenza 2010, Weidemann & Johnson 2008). These activated genes encode proteins that take part firstly in
adaptation to hypoxia but also in many other important aspects of cellular pathophysiology (Semenza 2010). Examples for HIF target gene products are the vascular endothelial growth factor (VEGF), which promotes angiogenesis, and EPO, which induces erythropoiesis (Kaelin Jr. & Ratcliffe 2008, Lundgren et al. 2007). Many activated genes also play a role in glucose, amino acid or nucleotide metabolism, in proliferation and in apoptosis (Lundgren et al. 2007), see Figure 3.

![Diagram of the HIF-PHD pathway](image)

**Fig. 3.** The HIF-PHD pathway. GLUT1, glucose transporter 1; VEGF, vascular endothelial growth factor; EPO, erythropoietin; IGF-2, insulin-like growth factor 2; PDGF, platelet-derived growth factor; CAIX, carbonic anhydrase 9.

In addition to hypoxia, the tumor microenvironment can provide other truculent compounds which inhibit the degradation of HIF-α, such as reactive oxygen and nitrogen species (Semenza 2010). Moreover, there are a range of genetic and epigenetic changes that can result in increased HIF-α function, e.g. the loss of pVHL function (Semenza 2010). HIF-1α can also be up-regulated by growth factors such as epidermal growth factor (EGF), insulin, insulin-like growth factor (IGF-2), tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) (Lundgren et al. 2007).
The vast majority of cancers overexpress HIF-1α and HIF-2α proteins, which is not surprising considering that intratumoral hypoxia is present in nearly all solid tumors to some degree (Kaelin Jr. & Ratcliffe 2008, Semenza 2012b, Weidemann & Johnson 2008). There are clinical data to demonstrate that HIF-1α loss of function results in decreased tumor growth, vascularization and metastasis, whereas HIF-1α gain of function has the opposite effects (Semenza 2010). Overexpression of HIF-1α and HIF-2α have been shown to increase the risk of mortality in cancers of the bladder, kidney, brain, stomach, colon and rectum, head and neck, prostate, lung, cervix and ovary (Semenza 2012b), and breast cancer is no exception in this respect. When HIF-1α is used as a marker of hypoxia in invasive breast cancer samples, around 25–40% of them show positivity, and it is significant that the frequency of HIF-1α-positive cells increases in parallel with the pathological stage (Milani & Harris 2008).

The association of HIF-1α with a poor prognosis has already become a generally admitted fact by virtue of the extensive studies carried out in this field. Here are a few examples of the findings. HIF-1α has been observed to correlate positively with HER2 status, VEGF expression and proliferation in lymph node-negative primary breast cancers, and its overexpression has been found to be an independent predictor of a poor prognosis, correlating with decreased overall survival (OS) and disease-free survival (DFS) (Bos et al. 2003). The same poor outcome has also been recorded in lymph node-positive diseases (Gruber et al. 2004, Schindl et al. 2002). Elevated expression of HIF-1α has been linked to an increased risk of distant metastases in ER-positive, HER-negative breast cancer patients (Dong et al. 2013), and HIF-1α positivity has been associated with the basal-like phenotype and BRCA1-derived breast tumors (Yan et al. 2009). Metastasis to the lungs has been found to be mediated at least to some extent by HIF-1α (Zhang et al. 2012). Furthermore, it has been shown that HIF-1α is an independent predictor of impaired response to primary chemoendocrine therapy, especially in ER-positive patients (Generali et al. 2006).

It is not HIF-1α alone, however, that has a profile of predicting a poor outcome in human breast cancer. HIF-2α has also been correlated with activation of VEGF expression, distant metastasis and impaired disease-free survival (Helczynska et al. 2008), and it has also been linked to poor differentiation and elevated proliferation, indicating more deleterious biological behaviour (Xiang et al. 2012).

Even though the evidence for HIF-1α and 2α as prognosticators of an adverse outcome is convincing, there are some studies that demonstrate that there might
be different sides to HIF-1α and 2α after all. HIF-1α has been shown to induce differentiation in myeloid leukaemic cells (Song et al. 2008), and its deficiency has been associated with aggressive progression of murine astrocytomas (Blouw et al. 2003). A protective role has even been suggested for HIF-1α, as hypoxia has been found to reduce proliferation and increase apoptosis in an HIF-1α-dependent manner in embryonic stem cells (Carmeliet et al. 1998). HIF-2α, on the other hand, has been described as performing a tumor suppressor role in rat gliomas, as its overexpression was reported to induce apoptosis and reduce tumor growth (Acker et al. 2005). In a series of human squamous cell carcinomas, HIF-1α overexpression was related to significantly improved overall and disease-free survival (Fillies et al. 2005).

In this regard, the inhibition of HIF-1α has presented itself as a lucrative target for novel anti-cancer drugs, principally because of its VEGF-promoting function, as the anti-angiogenic effects of most new therapeutic agents are primarily due to the inhibition of HIF-1 activity. Such new HIF-1 inhibitors include, for example, the anti-HER2 antibody trastuzumab as administered to HER2-positive breast cancer patients, the mTOR inhibitor temsirolimus and the anti-EGFR antibody cetuximab (Semenza 2010). There are experimental data to support the action of HIF-1 inhibitors as sensitizing agents to radiation therapy (Semenza 2010).

2.2.2 The HIF prolyl 4-hydroxylases

The family of HIF prolyl 4-hydroxylases consists of three members: prolyl hydroxylase domain 1 (PHD1), PHD2 and PHD3, also referred to as EglN2, EglN1 and EglN3, or HIF-P4H-1, HIF-P4H-2 and HIF-P4H-3, respectively (Myllyharju & Koivunen 2013). The HIF prolyl 4-hydroxylases, or PHDs, are involved in maintaining cellular oxygen homeostasis by hydroxylating conserved prolyl residues of the α subunit of the hypoxia-inducible factor (HIF) (Bruick & McKnight 2001, Ivan et al. 2001, Jaakkola et al. 2001). They act as oxygen censors, as their hydroxylase activity is highly oxygen-dependent (Hirsila et al. 2003, Kaelin Jr. & Ratcliffe 2008, Koivunen et al. 2006). In addition to O₂, PHDs require Fe²⁺, 2-oxoglutarate and ascorbate for their normal catalytic activities (Ivan et al. 2001, Myllyharju & Koivunen 2013).

All three isoforms contribute to the regulation of HIF-α, although tissue and cell-specific variations in the expression profile do exist (Appelhoff et al. 2004). Furthermore, the contribution that each isoform makes to HIF-regulation differs
under varying conditions, PHD2 being the dominant regulator of HIF in normoxia, whereas in hypoxia it is PHD3 that is considered to take the lead in this respect (Appelhoff et al. 2004). It should be noted that the isoforms may act differently on HIF-1α and HIF-2α, as PHD3 has been found to contribute more substantially to the regulation of HIF-2α than HIF-1α, while PHD2 has the reverse preference (Appelhoff et al. 2004). The PHDs also vary in their subcellular location, PHD1 being exclusively a nuclear protein, while the major part of the PHD2 is expressed in the cytoplasm (Metzen et al. 2003). By contrast, PHD3 is distributed more evenly between the cytoplasm and nucleus (Metzen et al. 2003).

There is increasing evidence that the PHDs are involved in the oxygen-dependent regulation of several additional proteins, including the large subunit of RNA polymerase II (Mikhaylova et al. 2008), the β2 adrenergic receptor (Xie et al. 2009), the glycolytic pyruvate kinase M2 (PKM2) that acts as a co-activator of HIF-1 (Luo et al. 2011b), nuclear factor-kappaB (NF-κB) (Cummins et al. 2006) and the DNA damage response protein, the human homologue of the C. elegans biological clock protein 2 (HCLK2) (Xie et al. 2012b). The HIF-independent functions of the prolyl hydroxylases are currently being investigated, and there are implications that PHDs may have other targets as well as HIF-α.

The PHDs are inhibited by acute hypoxia, but chronic hypoxia is thought to overactivate all the PHD isoforms (Ginouves et al. 2008) by inhibiting mitochondrial activity thereby increasing the availability of intracellular O₂ for the PHDs (Ginouves et al. 2008). PHD2 and PHD3 have actually been reported to be hypoxia-inducible (Aprilikova et al. 2004, Henze et al. 2010). There could be several mechanisms for this induction, but they have been shown to be themselves subject to feedback up-regulation by HIF-1 (Fong & Takeda 2008).

Although several reports and reviews have been published on the HIF 4-prolyl hydroxylases, their prognostic relevance has still not been fully elucidated.

PHD1

Of the prolyl 4-hydroxylases, PHD1 is the one which has to date been the most firmly related to breast cancer tumorigenesis, having been shown to be induced by oestrogen and to be a direct transcriptional target of ER in breast cancer cells (Seth et al. 2002). It has been suggested that oestrogen may regulate the levels of PHD1 in several alternative ways: via direct transcriptional regulation, by influencing mRNA stability or indirectly through other signalling pathways (Seth
et al. 2002). Additionally, the same report underlines the observation that PHD1 stimulates the proliferation of breast cancer cells in vitro (Seth et al. 2002). PHD1 inactivation has been associated with decreased levels of cyclin D1, a cell cycle regulator to be discussed later, breast cancer tumorigenesis and suppression of mammary gland proliferation in an in vivo mouse model (Zhang et al. 2009). Also, a positive correlation has been observed between PHD1 and VEGF expression in human breast cancer (Fox et al. 2011), but PHD1 has not been found to correlate with survival functions, although it has been identified as an independent negative prognostic factor for disease-specific survival in cases of non-small cell lung carcinoma (Andersen et al. 2011). PHD1 is thought to be primarily a nuclear protein (Metzen et al. 2003), but in the non-small cell lung carcinoma study it was detected in the cytoplasm (Andersen et al. 2011).

PHD2

PHD2 is considered to be the HIF-1α low steady-state setting prolyl 4-hydroxylase under normoxic conditions, since its silencing results in HIF-1α stabilization while the silencing of PHD1 or PHD3 has little effect on HIF levels (Berra et al. 2003). PHD2 is expressed physiologically in the cytoplasm (Metzen et al. 2003), but translocation to the nucleus can occur, as has been seen in aggressive (less differentiated and strongly proliferating) tumors of the head and neck (Jokilehto et al. 2006). The same effect has also been detected in cases of colon carcinoma, in association with anchorage-independent cell growth (Jokilehto et al. 2010). Similarly, nuclear PHD2 expression has been correlated with a larger tumor size and higher stage in pancreatic tumors (Couvelard et al. 2008). It has been proposed that PHD2 may have additional important functions that are HIF-independent. Its loss has been observed to contribute to tumor growth through an effect on angiogenesis (Chan et al. 2009), and similarly it has been suggested that it normally functions to inhibit angiogenesis and that its silencing angiogenesis, the regulation of which is totally independent of HIF and the prolyl 4-hydroxylase activity of PHD2 (Chan & Giaccia 2010). PHD2 has also been discovered to induce cell cycle arrest in the G1/S phase through direct modulation of cyclin D1, again independently of HIF (Su et al. 2012). There are few studies that have recognized PHD2 as an independent prognostic factor, although its low cytoplasmic expression has been associated with shortened survival in gastric (Kamphues et al. 2012) and colorectal cancers (Xie et al.)
2012a), and this effect appears to be HIF-independent at least in colorectal cancer (Xie et al. 2012a).

PHD2 has been called a tumor suppressor, and in the light of the above there is evidence to validate this argument. Interestingly, however, one study carried out in lung cancer patients concluded that even cytoplasmic expression of PHD2 was associated with an unfavourable prognosis, as it correlated with poorer disease-specific survival (Andersen et al. 2011).

**PHD3**

The role of PHD3 as a prognostic factor is highly variable. It has been reported to possess the unique function of inducing apoptosis under normoxia, as has been seen in neural cells, where it exercises its pro-apoptotic effects in a prolyl 4-hydroxylase-dependent but HIF-independent manner (Lee et al. 2005, Schlisio et al. 2008). In cancer cells, PHD3 has been reported to activate protein aggregation, suggesting that this aggregation might be one of the mechanisms behind its apoptosis-enhancing function (Rantanen et al. 2008). Since the pro-apoptotic function is dependent on oxygen, it has been suggested that PHD3 may have a cell survival supporting role under hypoxic conditions, e.g. in glioblastoma cells (Henze et al. 2010). It has been demonstrated with head and neck squamous carcinoma cells that given a low oxygen supply, PHD3 depletion leads to cell cycle arrest at the G1/S boundary by reducing the amount of phosphorylated pRB and the amount of cyclin D1 (Hogel et al. 2011).

In some cases PHD3 has been seen to possess a tumor suppressor role. PHD3 knockout was found to enhance tumorigenesis in colorectal cancer cells, and its decreased expression was associated with higher tumor grade and nodal metastasis in human colorectal cancer (Xue et al. 2010). In non-small cell lung cancer, however, PHD3 proved to be a prognosticator of unfavourable disease-specific survival (Andersen et al. 2011).

A positive correlation has been demonstrated between PHD3 and VEGF in breast cancer patients, which might suggest a pro-angiogenic role (Fox et al. 2011). One subtype of hereditary breast cancer, BRCA1-mutant breast cancer, has been correlated with down-regulation of PHD3 (Yan et al. 2009), and PHD3 negativity has also been associated with the basal-like phenotype, a more aggressive subtype of breast cancer (Yan et al. 2009).
2.3 Cell cycle regulators

2.3.1 The cell cycle

The cell cycle is a carefully controlled process, the essential task of which is to replicate DNA and make two identical copies of a dividing cell (Sherr 2000). The doubling of the genome takes place in the S (synthesis) phase, while halving of the genome occurs during the M (mitosis) phase. The period between the M and S phases is called the G1 (Gap 1) phase, and that between the S and M phases the G2 (Gap 2) phase (Sherr 2000), see Figure 4. These two phases, G1 and G2, give the cell time to repair any DNA damage or replication errors that might have taken place earlier in the cycle (Massague 2004). Influenced by extracellular signals during G1 phase, the cell makes a decision regarding whether to replicate its DNA and divide, to exit the cell cycle by passing into a resting state, G0, to differentiate or to die (Massague 2004). Once the decision in favour of cell division is made at a point late in the G1 phase called the “restriction point”, the cell is committed to completing the cell cycle (Lundberg & Weinberg 1999). A quiescent cell in the G0 phase can re-enter the cell cycle under the stimulus of growth factors (Sherr 1996). The cell cycle is illustrated in Figure 4.

The above scheme implies that the cell cycle possesses a high oncogenic potential, so that a complex machinery is needed to control it. Malignant cells tend to have escaped this control by having modifications in their cell cycle regulating gene profile, and it is this that gives cancer its typical characteristic of uncontrolled cell proliferation (Sherr 1996).

Fig. 4. The cell cycle. M, mitosis; G0, quiescent phase; G1, Gap 1 phase; S, synthesis; G2, Gap 2 phase.
2.3.2 The RB pathway

The retinoblastoma tumor suppressor gene (RB) encodes a nuclear phosphoprotein (pRB) that plays a central role in regulating the G1-to-S phase of the cell cycle (Weinberg 1995). The loss of RB and disruption of the RB pathway have been well documented in many human tumor types (Sherr 1996).

Passage through the restriction point late in the G1 phase and entry into the S phase are governed by cyclins and cyclin-dependent kinases (CDKs) (Lundberg & Weinberg 1999). To be functional, the CDKs need an activating association with cyclins (Lundberg & Weinberg 1999, Musgrove et al. 2011). On the other hand, cyclins are highly labile proteins and are degraded rapidly without their CDK counterparts unless influenced by a continuous mitogenic stimulus (Sherr 2000, Weinberg 1995). The D-type cyclins (cyclins D1, D2 and D3) serve as growth factor sensors (Sherr 2000), so that when cells enter the cell cycle, extracellular signals induce the expression of cyclin D1, which then forms holoenzymes with its catalytic partners CDK4 and CDK6 (Lundberg & Weinberg 1999, Massague 2004, Sherr 1996, Weinberg 1995). The resulting complexes in turn phosphorylate the pRB (Lundberg & Weinberg 1999, Massague 2004, Sherr 1996, Weinberg 1995). By binding to transcription factors such as the members of the E2F family, the pRB then suppresses the transcription of genes that are required for the progression of the cell cycle to the S phase (Lundberg & Weinberg 1999, Massague 2004, Sherr 1996, Weinberg 1995). When phosphorylated, the pRB cannot bind to E2Fs, which leads to dissociation of the pRB-E2F complexes. Thereby pRB loses much of its growth inhibitory power, while the E2Fs can freely carry out their cell growth promoting actions (Lundberg & Weinberg 1999, Massague 2004, Sherr 1996, Weinberg 1995) (Figure 5).

The CDKs are negatively regulated by the cyclin-dependent kinase inhibitors (CDKIs) (Sherr 2000). One CDKI, p16, a member of the INK4 family, acts as a tumor suppressor by directly inhibiting CDK4 and CDK6 and maintaining pRB in its hypophosphorylated E2F-associated form, thereby resulting in G1 phase arrest (Lundberg & Weinberg 1999, Massague 2004, Sherr 1996, Weinberg 1995). Inactivation of p16 results in a loss of the inhibition of pRB phosphorylation, facilitating a loss of control over cell cycle arrest (Witkiewicz et al. 2011b) (Figure 5). Other CDKIs include e.g. the so called Cip/Kip family members, p21 and p27, the latter of which also inhibits the G1 progression in the cell cycle (Lim & Kaldis 2013).
Interestingly, hypoxia has been shown to induce cell cycle arrest at the G1/S border (Pettersen & Lindmo 1983), an effect that has been described as being RB-dependent, as hypoxia has been found to induce the accumulation of pRB (Krtolica et al. 1998).

As stated above, the inactivation of growth inhibitory functions and uncontrolled cell proliferation is favoured by pRB phosphorylation (Weinberg 1995). In the case of breast cancer tumors there may be genetic events upstream of RB which can negatively affect pRB function by promoting its phosphorylation. These may include up-regulation of CDK4 (Thoms et al. 2007), loss of p16 function (Rocco & Sidransky 2001), cyclin D1 gene amplification or cyclin D1 protein overexpression (Musgrove et al. 2011).

![Fig. 5. The p16-cyclin D1-CDK4-RB pathway.](image)

### 2.3.3 Cyclin D1

The D-type cyclins (cyclins D1, D2 and D3) are induced by various oncogenes and mitogenic growth factors (Roy & Thompson 2006), and they perform a growth-promoting role in the cell cycle (Musgrove et al. 2011). Of these three
cyclins, it is D1 that is primarily linked to human tumorigenesis (Roy & Thompson 2006), and in fact is nowadays well established as a human oncogene (Musgrove et al. 2011, Tashiro et al. 2007).

The CCND1 gene, of which cyclin D1 is the product, is located on chromosome 11q13 (Roy & Thompson 2006). There is a vast amount of evidence that CCND1 amplification and cyclin D1 overexpression are involved in most human cancer types, including breast, colon, lung, bladder and liver cancers, squamous carcinomas of the head, neck and oesophagus, B cell lymphomas, and human parathyroid adenomas (Tashiro et al. 2007). CCND1 is amplified in many common cancers (up to 15–40%), but the mRNA and protein overexpression rates are even higher (Musgrove et al. 2011). In the case of breast cancer, the reported rates are 5–20% for gene amplification and 40–90% for protein overexpression (Roy & Thompson 2006). These figures provide an insight to the fact that high cyclin D1 expression is not always due to gene amplification, insinuating at the same time that there are other forms of maintaining the overexpression (Musgrove et al. 2011, Tashiro et al. 2007).

The information we have about the role of cyclin D1 in breast cancer is convincing. In addition to the above, it has been shown that cyclin D1 possesses a significant role in mammary gland development and carcinogenesis (Sicinski & Weinberg 1997). Normal mammary gland epithelium proliferation was attenuated during pregnancy in cyclin D1 knockout mice (Sicinski et al. 1995), while tissue-specific expression of the protein resulted in mammary hyperplasia and adenocarcinoma in transgenic mice (Wang et al. 1994).

Oestrogens, which are still the most important proliferative factors in mammary epithelial cells, convey their mitogenic effects via oestrogen receptors (ER), which are ligand-regulated transcription factors situated in the cell nucleus (Platet et al. 2004). CCND1 is one of oestrogens target genes, and thus cyclin D1 mediates some of the effects of oestrogen (Altucci et al. 1996). It has been proposed that cyclin D1 in turn derives part of its oncogenic activity in breast cancer from the ER, this effect being totally independent of hormones and CDK4/6 (Bernards 1999, Neuman et al. 1997, Zwijsen et al. 1997).

The general consensus achieved in several studies is that CCND1 amplification in human breast cancer is related to a poor prognosis and increased disease recurrence (Bieche et al. 2002, Lundgren et al. 2012, Quintayo et al. 2012, Roy et al. 2010, Seshadri et al. 1996), but the role of CCND1 gene product cyclin D1 overexpression in breast cancer is much more controversial. It has been widely demonstrated that cyclin D1 is associated with features of a good
prognosis, for it is predominantly expressed in well-differentiated, low-grade, slow-growing, ER-positive subtypes of human breast cancer (Bostrom et al. 2009, Musgrove et al. 2011, Roy & Thompson 2006). The expression of cyclin D1 has been shown to correlate with reduced disease recurrence (Bieche et al. 2002, Lundgren et al. 2012), although there are reports that show just the opposite. In ER-positive patients, cyclin D1 expression has been associated with an increased risk of relapse, local recurrence and distant metastasis (Kenny et al. 1999). The risk of disease recurrence has been found to be altered in postmenopausal women overexpressing cyclin D1 who have been treated with either tamoxifen (an anti-oestrogen) or anastrozole (an aromatase inhibitor) (Lundgren et al. 2012), which is consistent with the notion that cyclin D1 may possibly predict resistance to endocrine therapy (Musgrove et al. 2011).

In addition, there is evidence of that cyclin D1 may be associated with resistance to some cytotoxic drugs (Biliran et al. 2005, Noel et al. 2010), but these studies have been carried out on other forms of cancer rather than breast cancer. In another hormone-dependent cancer type, adenocarcinoma of the prostate, overexpression of cyclin D1 has been correlated with a high proliferative index and a metastatic disease, and it has been suggested that high cyclin D1 may be related to the evolution of an androgen-independent disease form (Drobnjak et al. 2000). One experiment with a human oesophageal cell line has provided the insight that cyclin D1 might be able to enhance tumor angiogenesis through VEGF activation (Tashiro et al. 2007).

Proliferating cells are programmed to arrest their cell cycle progression when their DNA is damaged, and it has been suggested that degradation of cyclin D1 might be one of the mechanisms behind this (Jirawatnotai et al. 2012). There is evidence of a link between cyclin D1 and proteins engaging in DNA repair, this being completely independent of both the evident cell cycle promoting role of cyclin D1 and its role in activating CDK4/CDK6 (Jirawatnotai et al. 2012).

Cyclin D1 offers an attractive target for a therapeutic strategy because of its strong overexpression profile in human breast cancer and many other forms of cancer. Previous studies in this field have concentrated on targeting cyclin D-associated kinase activity (Cicenas & Valius 2011), but now that we have more knowledge of the CDK-independent functions of cyclin D1, e.g. DNA damage repair in cell proliferation, it may represent a more promising anti-cancer target for clinical oncology (Jirawatnotai et al. 2012).
2.3.4 CDK4

CDK4 belongs to a large family of cyclin-dependent kinases (CDKs), the activity of which derives from their ability to phosphorylate serine and threonine residues on their substrates (Lim & Kaldis 2013). There are currently over 20 members in the CDK family (Lim & Kaldis 2013). Each of the CDKs guards a specific checkpoint in the cell cycle with the co-operation of the cyclins in order to ensure genomic integrity and normal proliferation (Malumbres & Barbacid 2001). Cyclin D1, as discussed above, binds specifically to otherwise incompetent CDK4 and CDK6 (Malumbres & Barbacid 2001). In addition to cyclin D1, CDK4 and CDK6 can form complexes with the other D-type cyclins, cyclin D2 and D3 (Paternot et al. 2010). CDK4 and CDK6 are close homologues in terms of their structure and function, but it has been suggested that CDK4 may be the primary D-type cyclin-dependent kinase in most cells (Paternot et al. 2010). To achieve its complete catalytic activity, CDK4 itself needs to be phosphorylated by the CDK-activating kinase (CAK) (Bockstaele et al. 2006). The principal substrate of CDK4 in the mammalian cell cycle is the pRB at the gateway to the G1-to-S transition (Malumbres & Barbacid 2001, Paternot et al. 2010). A cyclin-dependent kinase inhibitor, p16, targets CDK4 as a negative regulator to maintain a balance in the RB pathway (Paternot et al. 2010).

CDK activity is normally strictly controlled by post-transcriptional modifications (Roberts et al. 2012), but deregulation of this cell cycle control due to aberrant CDK activity is a common feature of most cancer types (Cicenas & Valius 2011). Viral infections, Alzheimer’s and Parkinson’s diseases, ischaemia and some proliferative disorders also show abnormal CDK activity (Cicenas & Valius 2011).

Of the family of CDKs, CDK1 has been shown to be sufficient to drive the mammalian cell cycle alone (Santamaria et al. 2007), so that CDK4 is dispensable to some extent. CDK4 is still essential, however, for cellular differentiation during development, the proliferation of specialized tissues and the installation of non-proliferative states (Malumbres & Barbacid 2009, Paternot et al. 2010). Tumor cells also represent a type that seems to require CDK4 activity for achieving higher levels of proliferation (Malumbres & Barbacid 2009, Santamaria et al. 2007).

CDK4 activity is deregulated in many human tumors (Bockstaele et al. 2006, Paternot et al. 2010), and as it has been shown to be absolutely crucial for various oncogenic transformation processes such as immortalization, cell proliferation
and malignant transformation, it appears that many cancer cells might be addicted to high CDK4 activity (Semczuk et al. 2004, Sgambato et al. 1995, Tetsu & McCormick 2003, Yu et al. 2006, Zou et al. 2002).

CDK4 overexpression can be the result of gene amplification, a mutation that alters its sensitivity to its direct inhibitor p16 (Paternot et al. 2010), loss of p16 function or cyclin D1 overexpression (Bockstaele et al. 2006, Malumbres & Barbacid 2001). Gene amplification and consequent overexpression of CDK4 protein have been found in gliomas, sarcomas and breast cancer, for example (An et al. 1999, Khatib et al. 1993, Reifenberger et al. 1994). In one human breast cancer study the amplification of the CDK4 gene was seen in 16% of the carcinoma cells (An et al. 1999). Overexpression of the protein has been much more widely examined, however, and high levels of the gene product have been detected additionally in endometrial, ovarian, lung and hepatocellular carcinomas and oral squamous cell carcinomas (Dobashi et al. 2004, Kusume et al. 1999, Lu et al. 2013, Poomsawat et al. 2010, Semczuk et al. 2004).

Increased CDK4 expression has been found to be common in carcinogen-induced rat mammary tumors (Sgambato et al. 1995), and where carcinogens are concerned, there may be a special connection between CDK4 and the HER2 oncogene. CDK4-deficient mice have been shown to be resistant to breast cancers initiated by HER2 (Landis et al. 2006), and there is evidence that a continued presence of CDK4 is required to maintain breast tumorigenesis, especially in HER2-driven tumors (Yu et al. 2006). The inhibition of CDK4 has been found to suppress proliferation, and thus tumor growth, to induce G1 arrest and to promote apoptosis ex vivo (Baughn et al. 2006, Dean et al. 2012a, Fry et al. 2004, Thoms et al. 2007). Overexpression of CDK4 in human breast cancer has previously been associated with a larger tumor size and higher tumor stage (Ito et al. 1997), and both gene amplification and the protein have been correlated with a high proliferation index in the same setting (An et al. 1999). Some reports have identified CDK4 as an independent prognostic factor, predicting impaired overall survival for lung (Wu et al. 2011) and hepatocellular carcinoma (Lu et al. 2013) patients, for example.

In view of the wide spectrum of diseases in which the CDKs play a role, they have been the most attractive factors in the RB pathway to try to harness for therapeutic use (Cicenas & Valius 2011, Thoms et al. 2007). An intensive search for CDK inhibitors has taken place in recent years, and some of the findings hold some promise for clinical oncology (Canavese et al. 2012, Cicenas & Valius 2011). Considering the role of CDK4 and the effect of CDK4 inhibition
mentioned above, the specific targeting of CDK4 in cancer research is not surprising. In one study focusing on selective CDK4/6 inhibitors these were found to inhibit tumor growth in HER2-driven tumors in mice and to reduce dose-limiting myelosuppression of cytotoxic treatment in RB-deficient tumors, the proliferation of which is not mediated by CDK4/6 (Roberts et al. 2012). In addition, a selective CDK4/6 inhibitor was found to be effective in a triple-negative RB-proficient cell line in concomitant use with anthracyclines and taxanes (Dean et al. 2012b), which are the two main groups of cytotoxic drugs used in breast cancer treatment. Clinical trials are already in progress with some of the CDK-inhibitor molecules that have been discovered (Johnson & Shapiro 2012).

2.3.5 p16

There are two families of CDKIs, the INK4 family and the Cip/Kip family (Lim & Kaldis 2013). The product of the CDKN2 gene, p16, belongs to the INK4 family and is, hence, also known as p16INK4a. p16 acts as a tumour suppressor (Lukas et al. 1995), and inactivation of its gene is a common event in nearly half of all human cancers studied (Rocco & Sidransky 2001).

The role of p16 as a tumor suppressor is well-established. It has an important function in limiting cell proliferation through the cyclin D1-CDK4/6-RB pathway (Witkiewicz et al. 2011a), and its best-known feature is its ability to bind to and inhibit the catalytic activities of the CDK4/6-cyclin D1 holoenzyme (Lukas et al. 1995, Rocco & Sidransky 2001, Witkiewicz et al. 2011a). However, it also contributes to cell cycle progression through alternative, cyclinD1-CDK4/6-independent pathways (Li et al. 2011), e.g. through phosphorylation of the RNA polymerase II needed in DNA transcription (Serizawa 1998). Besides all this, there is evidence of p16 promoting cellular senescence, cell spreading, angiogenesis and anoikis (apoptosis after loss of epithelial cell anchorage) (Rocco & Sidransky 2001), although the mechanisms behind these functions are yet to be properly elucidated.

Silencing of CDKN2 has been one of the most prominent genetic changes identified in human cancers to date, being found in nearly 50% of all such cancers (Li et al. 2011). Its genetic inactivation can be established in three alternative ways: by deletion, methylation or point mutation (Rocco & Sidransky 2001). However, the genetic status does not always reflect the status of p16 function, which may vary a great deal in human cancers. The physical up-regulation and
down-regulation that takes place is a summation of the genetic alterations and the oncogene-mediated deregulation of function (Li et al. 2011).

Normal proliferating cells do not express significant levels of p16 prior to extensive rounds of cell division, which may suggest a late-stage antiproliferative role for p16, as in the senescence of replicative cells (Witkiewicz et al. 2011a). The activation of p16 expression can be triggered by DNA damage, oncogenic stress or physiological ageing (Witkiewicz et al. 2011a).

Despite its role as a tumor suppressor, aberrant levels of p16 are commonly observed in cancer (Li et al. 2011). It is very likely that overexpression of p16 is induced by stress or oncogenic environmental risk factors through an undefined feedback loop, but its inhibition of cell proliferation is bypassed or counteracted (Li et al. 2011). An example will enlighten this. It has been demonstrated that p16-mediated cell cycle arrest is RB-dependent (Rocco & Sidransky 2001). This suggests that the control mechanism could be bypassed by the loss of RB (Witkiewicz et al. 2011a). It has been proposed that the loss of RB can generate a stress that could induce p16 expression, and since RB is already compromised, the induced levels of p16 are not able arrest the cell cycle, so that these tumors develop high p16 levels (Witkiewicz et al. 2011a). Furthermore, tumor hypoxia is presented as one potential stress mechanism, since p16 has been shown to be hypoxia-inducible (Zygunt et al. 2002). However, cell cycle arrest due to hypoxia cannot be explained completely by the act of p16.

Numerous studies have documented the loss of p16 and its aberrant cell proliferation-promoting effect as a widespread occurrence in human cancer, including breast, colon, pancreatic, gastric, oesophageal, bladder and non-small cell lung carcinomas, mesothelioma, squamous carcinomas of the head and neck, myeloma and leukaemia (Li et al. 2011, Rocco & Sidransky 2001). In the case of breast cancer the frequency of p16 inactivation is about 20% (Li et al. 2011). The significance of p16 overexpression, however, is not fully understood. High levels of p16 have been associated with both a better and a poorer prognosis for cancer. In human breast cancer, an association of ER negativity, higher grading and high proliferation activity with the overexpression of p16 has been detected (Milde-Langosch et al. 2001), and also an association of p16 with the basal-like phenotype (Abou-Bakr & Eldweny 2013, Bohn et al. 2010, Herschkowitz et al. 2008) and infiltrative tumor border pattern (Chae et al. 2011). p16 overexpression has been connected with an increased risk of ductal carcinoma in situ (DCIS) progressing to an invasive disease (Witkiewicz et al. 2011b), although patients overexpressing p16 with atypical hyperplasia, a putative precursor for DCIS, did
not run a risk of breast cancer (Radisky et al. 2011). p16 has recently been reported to down-regulate VEGF expression, thus inhibiting angiogenesis, and to inhibit metastasis in breast cancer cells (Zhang et al. 2010). In another study, p16 was shown to inhibit the proliferation and migration of breast cancer cells (Li & Lu 2010).

Targeting p16 per se as an option for developing new cancer therapies has so far been only marginally explored, but the predictive value of p16 expression as a response to different forms of cancer therapy has been explored to some extent. In luminal B-type breast cancer (ER-positive and often high grade), overexpression of p16 is related to failure to respond to endocrine therapy and benefit from cytotoxic chemotherapy (Hershkowitz et al. 2008), whereas in head and neck cancer and prostate cancer, tumours that express high levels of p16 show an improved response to radiation therapy (Witkiewicz et al. 2011a).
3 Aims of the present thesis

Early diagnosis and adequate treatment for each patient remains the best way to improve the prognosis for breast cancer patients. Bearing in mind that breast cancer is a heterogeneous disease with patient outcomes varying from extremely good to disastrously poor, it is becoming more and more important to distinguish patients who need aggressive oncological therapy from those who can be spared from being overtreated.

The work for this thesis was aimed at examining the prognostic significance of HIF-1α, HIF-2α, PHD1–3, cyclin D1, CDK4 and p16 in invasive ductal breast cancer patients and in a subgroup of TNBC patients, with the main focus on the following issues:

1. the prognostic value of the immunoreactivity of the hypoxia response markers HIF-1α, HIF-2α and the HIF-prolyl 4-hydroxylases PHDs 1–3 in patients with invasive ductal breast cancer,

2. the prognostic significance of the immunoreactivity of the cell cycle regulatory markers cyclin D1, CDK4 and p16 in patients with invasive ductal breast cancer, and

3. the prognostic value of the immunoreactivity of HIF-1α and HIF-2α, the individual PHDs, cyclin D1, CDK4 and p16 in patients with TNBC.
4 Materials and methods

4.1 Patients and tumor specimens (I-III)

The work for papers I and II was based on a series of 102 invasive ductal breast cancer patients treated at Oulu University Hospital during the years 2000–2007, and that for paper III on a series of 111 TNBC patients treated at Oulu University Hospital and Kuopio University Hospital in 2000–2009. An additional 59 hormone receptor-positive, HER2-negative cases selected from the series of 102 invasive ductal breast cancer patients were included in paper III as controls.

The formalin-fixed, paraffin-embedded tumor specimens used in the histological and RNA analyses were collected from the archives of the Department of Pathology, Oulu University Hospital, and the Department of Pathology, Kuopio University Hospital. Approval for the use of these specimens was obtained from the local Ethical Committees and the Finnish National Supervisory Authority for Welfare and Health. Clinical information on patient characteristics and follow-up data were obtained from the clinical and pathological records of the two hospitals. The diagnoses were re-evaluated according to the WHO classification by the pathologist who graded the immunohistochemical stainings.

The median age of the unselected ductal breast cancer patients reported on in papers I and II was 59 years (range 28–87 years), while that of the TNBC patients in paper III was 57 years (range 32–86 years). 13 of the TNBC patients were under 40 years of age and 31 were under 50. All the 102 patients in papers I and II had invasive ductal mammary carcinoma, as did the majority of the TNBC patients while 8% had medullary and 2% lobular carcinomas. Only 12% of the patients in papers I and II did not receive any kind of adjuvant treatment. 80% of the TNBC patients in paper III received adjuvant chemotherapy compared to the 18% of the 59 selected ER+/PR+/HER2- control patients who received adjuvant chemo therapy. 53% of the control patients received endocrine therapy. For further patient characteristics relevant to papers I-III, see Table 3.
Table 4. Patient characteristics (I-III).

<table>
<thead>
<tr>
<th>Clinicopathological factors</th>
<th>Invasive ductal carcinoma (I-II) n = 102 (%)</th>
<th>Triple-negative breast cancer (III) n = 111 (%)</th>
<th>ER+/PR+/HER2- control patients (III) n = 59 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>66 (64.7)</td>
<td>45 (40.5)</td>
<td>43 (72.9)</td>
</tr>
<tr>
<td>T2</td>
<td>27 (26.5)</td>
<td>61 (55.0)</td>
<td>13 (22.0)</td>
</tr>
<tr>
<td>T3</td>
<td>6 (5.9)</td>
<td>1 (0.9)</td>
<td>3 (5.1)</td>
</tr>
<tr>
<td>T4</td>
<td>3 (2.9)</td>
<td>4 (3.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><strong>Node</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>58 (56.9)</td>
<td>64 (57.7)</td>
<td>35 (59.3)</td>
</tr>
<tr>
<td>Positive</td>
<td>42 (41.2)</td>
<td>47 (42.3)</td>
<td>24 (40.7)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (1.9)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30 (29.4)</td>
<td>2 (1.8)</td>
<td>25 (42.4)</td>
</tr>
<tr>
<td>2</td>
<td>35 (34.3)</td>
<td>15 (13.5)</td>
<td>24 (40.7)</td>
</tr>
<tr>
<td>3</td>
<td>37 (36.3)</td>
<td>94 (84.7)</td>
<td>10 (16.9)</td>
</tr>
<tr>
<td><strong>Oestrogen receptor (ER)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24 (23.5)</td>
<td>111 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>78 (76.5)</td>
<td>0 (0.0)</td>
<td>59 (100.0)</td>
</tr>
<tr>
<td><strong>Progesterone receptor (PR)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>35 (34.3)</td>
<td>111 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>67 (65.7)</td>
<td>0 (0.0)</td>
<td>59 (100.0)</td>
</tr>
<tr>
<td><strong>HER2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>84 (82.4)</td>
<td>111 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>18 (17.6)</td>
<td>0 (0.0)</td>
<td>59 (100.0)</td>
</tr>
<tr>
<td><strong>Ki-67</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>21 (20.6)</td>
<td>7 (6.3)</td>
<td>15 (25.4)</td>
</tr>
<tr>
<td>1+</td>
<td>37 (36.3)</td>
<td>11 (9.9)</td>
<td>26 (44.1)</td>
</tr>
<tr>
<td>2+</td>
<td>20 (19.6)</td>
<td>19 (17.1)</td>
<td>14 (23.7)</td>
</tr>
<tr>
<td>3+</td>
<td>24 (23.5)</td>
<td>74 (66.9)</td>
<td>4 (6.8)</td>
</tr>
</tbody>
</table>

4.2 Immunohistochemistry (IHC) (I-III)

The surgical specimens for routine light microscopy and immunohistochemical analysis were fixed in formalin and embedded in paraffin. Immunostaining for HIF-1α, HIF-2α, PHD1–3, cyclin D1, CDK4 and p16 was carried out as follows. Sections of 3µm were deparaffinized and treated with TRIS/EDTA with pronase for epitope retrieval. To block non-specific binding of IgGs, the specimens were incubated in a blocking solution (EnVision® Detection System, Dako, Denmark)
before the application of primary antibodies. The sections were then incubated with polyclonal antibodies for HIF-1α, HIF-2α, PHD1–3, cyclin D1, CDK4 and p16 (details in Table 4). The colour was developed with diaminobenzidine tetrahydrochloride (DAB) (EnVision Detection System, Dako, Denmark). Each step was followed by careful rinses with tween/phosphate-buffered saline (PBS). The sections were also lightly counterstained with haematoxylin. For negative controls, sections were incubated with PBS instead of the primary antibodies.

Table 5. Details of the antibodies used (I-III).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Paper</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>I</td>
<td>NB100–105</td>
<td>1:100</td>
<td>Novus Biologicals, Littleton, USA</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>III</td>
<td>NB100–134</td>
<td>1:2000</td>
<td>Novus Biological, Littleton, USA</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>I,III</td>
<td>ep190b</td>
<td>1:500</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>PHD1</td>
<td>I,III</td>
<td>NB100–310</td>
<td>1:400</td>
<td>Novus Biologicals, Littleton, USA</td>
</tr>
<tr>
<td>PHD2</td>
<td>I,III</td>
<td>NB100–138</td>
<td>1:200</td>
<td>Novus Biologicals, Littleton, USA</td>
</tr>
<tr>
<td>PHD3</td>
<td>I</td>
<td>ab4562</td>
<td>1:200</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>PHD3</td>
<td>III</td>
<td>NBP1–30440</td>
<td>1:400</td>
<td>Novus Biologicals, Littleton, USA</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>II-III</td>
<td>M3635</td>
<td>1:25</td>
<td>Nako North America Inc., CA, USA</td>
</tr>
<tr>
<td>CDK4</td>
<td>II-III</td>
<td>DCS-35</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology Inc., CA, USA</td>
</tr>
<tr>
<td>p16</td>
<td>II</td>
<td>CINtec</td>
<td>non-diluted</td>
<td>mtm Laboratories AG, Heidelberg, Germany</td>
</tr>
<tr>
<td>p16</td>
<td>III</td>
<td>IMD-16</td>
<td>1:125</td>
<td>Boston Biological Technology Ltd., CA, USA</td>
</tr>
</tbody>
</table>

To assess the specificity of the commercial antibodies available for PHD1–3 in paper I, we stained breast carcinoma specimens with these in the presence and absence of the recombinant purified antigens. This gave us values for the specific recognition of PHD1–3 in the specimens.

The stainings of the tumor specimens were analysed by multi-headed light microscopy and scored in the following way. The cytoplasmic and nuclear stainings were evaluated individually for all eight factors and the intensity of expression was scored on a scale from 0 to 3, where 0 = negative, 1 = weak, 2 = moderate and 3 = strong. The percentage of nuclei stained was assessed on a running scale from 0–100% in each case.

Information regarding the ER, PR, HER2 and Ki-67 statuses was obtained from the clinical and pathological records. The cut-off point for hormone receptor (ER and PR) positivity was 10%, i.e. 0–9% = negative and ≥ 10% = positive in papers I and II. However, the TNBC tumors did not show any ER or PR positivity in paper III. The cut-off points for Ki-67 were the following: ≤ 5 = negative, 5–15% = 1+, 16–30% = 2+, > 30% = 3+. HER2 was considered positive in IHC.
when the result was either 2+ or 3+ and positive in chromogenic in situ hybridization (CISH) when \( \leq 6 \) gene copies were detected.

4.3 Real-time quantitative PCR (II)

The level of cyclin D1 (II) was measured by real-time quantitative PCR as follows. RNA was extracted from the paraffin sections of breast carcinoma tumors with NucleoSpin FFPE RNA/DNA (Macherey-Nagel). 100ng of RNA was used for cDNA synthesis performed with iScript (Bio-Rad). The amount of cyclin D1 mRNA relative to 18S RNA in the samples was analysed by Q-PCR performed in a Stratagene MX3005 thermocycler with iTaq SYBR Green Supermix and ROX (Bio-Rad). The sequences of the forward and reverse primers are shown in Table 5.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (forward)</th>
<th>Primer (reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCND1</td>
<td>5'-GCTCCTGGTGAACAAGCTCAA-3'</td>
<td>5'-TTGGAGAGGAAGTGTTCAATGAAA-3'</td>
</tr>
<tr>
<td>18S</td>
<td>5'-GACTCAACACGGGAACCTC-3'</td>
<td>5'-AGCATGCCAGAGTCTCGTTC-3'</td>
</tr>
</tbody>
</table>

4.4 Statistical analyses (I-III)

The statistical analyses were carried out with SPSS 17.0 (I-II) and 20.0 (III) (SPSS Inc., Chicago Illinois, USA). The clinical characteristics were expressed as percentages, and the significances of associations between the factors studied in the clinical samples were evaluated using Pearson’s Chi-square test or the 2-sided Fisher's exact test as appropriate. A 2-tailed P-value was used in all the analyses, and a P-value < 0.05 was considered statistically significant. The Kaplan-Meier method and Cox regression model were used for the analyses of disease-free survival, breast cancer-specific survival and overall survival. Hazard ratios (HR) and 95.0% confidence intervals (95.0% CI) are indicated.

The staining results for the proteins were combined for the statistical analyses to form a positive and a negative group. HIF-1\( \alpha \) was considered negative when \( \leq 2\% \) of the nuclei were stained positive, while HIF-2\( \alpha \) was considered negative when no nuclear staining was observed, all other cases being scored positive. PHD1 was considered negative when the percentage of stained nuclei was \( \leq 2\% \) and when the intensity of nuclear staining was negative or weak, whereas other cases were scored positive. PHD2 and PHD3 were scored negative when their
cytoplasmic intensities were negative or weak and positive when the intensities were moderate or strong. Cyclin D1 was considered positive when the proportion of stained nuclei was > 40% and when the intensity of the nuclear staining was strong, and negative in all other cases. CDK4 and p16 were scored as negative when ≤ 2% of the nuclei were stained and the nuclear intensity was negative or weak, and as positive in all other cases.
5 Results

5.1 Prognostic significance of hypoxia-related factors in invasive ductal carcinoma (I)

HIF-1α and HIF-2α showed only nuclear staining in our cohort of invasive ductal carcinoma, about 30% of the specimens staining for HIF-1α and 80% for HIF-2α. HIF-1α did not correlate statistically significantly with any of the classical prognostic or predictive factors for breast cancer, but a trend for a correlation between HIF-1α expression and PR negativity was detected ($P = 0.082$). In addition, patients with high levels of HIF-1α had a tendency for decreased breast cancer-specific survival ($P = 0.126; \text{HR} = 2.4; \text{95.0% CI} = 0.75, 7.5$). The only statistically significant correlation for HIF-2α was that with tumor stage (T1 and T2 tumors) ($P = 0.020$). HIF-2α did not show any independent prognostic significance in breast cancer patients.

Nuclear PHD1 expression was detected in 33% of the invasive ductal carcinoma specimens and was found to correlate statistically significantly with ER negativity ($P = 0.036$) and a higher proliferation rate ($P = 0.043$), factors implying a poor prognosis for breast cancer.

In the case of PHD2 both cytoplasmic and nuclear staining was detected. PHD2 is primarily a cytoplasmic protein but translocation to the nucleus has been reported to be possible, and so we analysed both. About 50% of the tumor samples showed cytosolic PHD2 expression, while only 7.8% showed nuclear expression. Neither correlated with any of the main clinico-pathological factors studied. The patients with high levels of PHD2 expression showed a tendency for increased breast cancer-specific survival ($P = 0.150; \text{HR} = 2.6; \text{95.0% CI} = 0.68, 9.6$) and longer disease-free survival ($P = 0.109; \text{HR} = 2.2; \text{95.0% CI} = 0.82, 5.8$).

Approximately 60% of the breast tumor samples stained positive for PHD3, the expression of which was associated with good prognostic factors: low tumor stage ($P = 0.011$), low tumor grade ($P = 0.000$), ER positivity ($P = 0.031$) and low proliferation rate ($P = 0.026$). PHD3 positivity as such nevertheless failed to show any independent prognostic significance.

Comparison of the expression levels of PHD1–3 showed a trend for a positive correlation between PHD2 and PHD3 ($P = 0.068$), but no interrelations between PHD1 and PHD3, or between PHD1 and PHD2. The expression patterns of HIF-1α and HIF-2α did not correlate with each other. One statistically significant
correlation emerged between the PHDs and the HIFs in our cohort, a positive correlation between HIF-2α and PHD3 expression \( (P = 0.002) \), while a trend for a positive correlation was detected between HIF-2α and PHD1 expression \( (P = 0.071) \).

### 5.2 Prognostic significance of the cell cycle regulatory factors in invasive ductal carcinoma (II)

Cyclin D1 showed both nuclear and cytoplasmic staining in invasive breast carcinoma cells. In addition, the lymphocyte nuclei present in the breast tumor specimens stained positive for cyclin D1. Since the main function of cyclin D1 takes place in the nucleus, only nuclear staining was taken into account in the present analyses. Approximately 60% of the tumor specimens showed nuclear cyclin D1 expression. When the amplification rate of the cyclin D1 gene \( CCND1 \) was measured by Q-PCR and the relation between cyclin D1 protein expression and gene amplification was evaluated, tumors with high protein expression had 1.8 times higher expression of cyclin D1 mRNA than those with low or negative protein levels. This suggests that cyclin D1 protein expression is at least partially due to increased expression of \( CCND1 \).

Statistically significant correlations were found between cyclin D1 expression and a low tumor grade \( (P = 0.013) \), hormone receptor positivity \( (P = 0.000 \) for ER positivity and \( P = 0.024 \) for PR positivity) and a negative or low proliferation rate \( (P = 0.031) \). About 73% of the cyclin D1-positive tumor samples were T1, 62% node-negative, 97% ER-positive, 77% PR-positive, 64% negative or low in proliferation activity, and 86% HER2-negative, whereas 53% of the cyclin D1-negative tumor samples were high-grade, 55% moderate or high in proliferation activity and 55% ER and PR-negative. Our data clearly demonstrate the close relation between cyclin D1 expression and markers of a good prognosis in invasive ductal breast cancer. Cyclin D1 expression also showed independent prognostic value, as it correlated with increased breast cancer-specific and overall survival \( (P = 0.020; \text{HR} = 4.26; \ 95.0\% \ CI = 1.12, 16.1; \text{and} \ P = 0.013; \text{HR} = 3.93; \ 95.0\% \ CI = 1.23, 12.6, \text{respectively}) \).

CDK4 protein was detected only in the tumor cell nuclei, and approximately 70% of the tumor samples stained positive for the antibody. However, no differences in correlation with the clinical prognostic factors were found between the CDK4-positive and negative tumors, and hence no statistically significant correlations were found either.
p16 is a nuclear protein with an expression rate of 55% in the ductal invasive breast cancer specimens. Additionally, the adjacent stromal fibroblasts often showed positive staining for p16. The only statistically significant association between p16 and the clinical prognostic factors was that of p16 positivity with HER2 negativity ($P = 0.002$). A further association of p16 expression with a better clinical outcome for invasive ductal breast carcinoma patients was a correlation between p16 expression and increased breast cancer-specific survival ($P = 0.028; \text{HR} = 4.0; 95.0\% \text{ CI} = 1.0, 15.0$) and disease-free survival ($P = 0.004; \text{HR} = 4.1; 95.0\% \text{ CI} = 1.5, 12.0$).

The correlations between the RB pathway factor, cyclin D1, CDK4 and p16 were analysed, but no significant associations were found.

5.3 Prognostic significance of the hypoxia response and cell cycle regulators in TNBC (III)

As TNBC tumors are by definition negative for ER, PR and HER2, we correlated nuclear HIF-1$\alpha$, HIF-2$\alpha$, PHD1, CDK4, p16 and cyclin D1 and cytoplasmic PHD2 and PHD3 with the four other main clinico-pathological factors for breast cancer: tumor size, nodal status, grade and proliferation index Ki-67. The survival functions of these patients were also studied in the case of each marker.

The HIFs, PHD1, CDK4 and p16 showed exclusively nuclear staining in this cohort of 111 TNBC tumors, while PHD2 and PHD3 and cyclin D1 showed both nuclear and cytoplasmic staining. HIF-1$\alpha$ and PHD1 were expressed in most lymphocytic nuclei seen in the tumor samples as well.

Approximately 80% of the tumor samples stained positive for HIF-1$\alpha$, and HIF-1$\alpha$ positivity correlated statistically significantly with high grade ($P = 0.034$) and high proliferation ($P = 0.009$). 88% of the HIF-1$\alpha$-positive tumors were of grade 3 and 88% were moderate or high in proliferation activity. About 60% of the tumor samples showed HIF-2$\alpha$ positivity. Of the remaining HIF-2$\alpha$-negative tumors, 93% were of high grade, but this was not statistically significant, as 79% of the HIF-2$\alpha$-positive tumors were of high grade ($P = 0.080$) as well. HIF-1$\alpha$ or HIF-2$\alpha$ did not act as independent prognostic factors in this cohort.

About 60% of the tumor samples were positive for PHD1 and 65% for PHD2. The majority of the tumor samples were PHD3-negative, as 60% did not show any cytoplasmic PHD3 staining. PHD1 negativity was associated with a positive nodal status ($P = 0.049$), as 55% of the PHD1-negative tumors were node-positive and 66% of PHD1-positive tumors were node-negative. Interestingly, there was a
trend for an association between PHD1 negativity and high proliferation \((P = 0.096)\), as a high proliferation index was detected in 70% of the PHD1-negative tumors, but also in 64% of the PHD1-positive ones. PHD2 and PHD3 did not correlate statistically significantly with the prognostic factors studied here, but trends towards correlations between greater tumor size and PHD2 negativity \((P = 0.061)\) and between PHD3 positivity and high proliferation activity \((P = 0.091)\) were detected. The PHD2-negative tumors were T2 in 71% of cases, while the distribution between T1 (46%) and T2 (47%) was more even in the PHD2-positive tumors. 81% of the PHD3-positive tumors had high proliferation activity, while only 58% of the PHD3-negative tumors did so. No correlations between the PHDs and overall, disease-free or breast cancer-specific survival were detected.

Cyclin D1 expression was detected in 71% of the tumor specimens, whereas the proportions of CDK4-positive and negative tumors were almost equal (49% vs. 51%). About 70% of the tumors expressed p16. Cyclin D1 was not associated with any of the clinical prognostic markers in a statistically significant way, but a trend for a correlation \((P = 0.064)\) was observed between cyclin D1 negativity and greater tumor size (63% were T2). CDK4 expression was associated significantly with a high proliferation index, as 76% of the CDK4-positive tumors were highly proliferative \((P = 0.023)\). High p16 expression was associated with increased breast cancer-specific survival \((P = 0.0048; \text{HR} = 2.3; 95.0\% \text{CI} = 1.5, 6.5)\) and overall survival \((P = 0.003; \text{HR} = 3.1; 95.0\% \text{CI} = 1.5, 6.5)\), and showed a trend for increased disease-free survival as well \((P = 0.065; \text{HR} = 2.1; 95.0\% \text{CI} = 0.96, 4.77)\). Cyclin D1 and CDK4 did not exhibit any independent prognostic significance in this cohort.

In addition to the 111 TNBC samples, we had previously stained 59 ER+/PR+/HER2- invasive ductal breast carcinoma specimens for all eight markers as reported in papers I-II, and we used these as controls, comparing the expression patterns of the hypoxia response-related and cell cycle-related markers between these two groups. Comparison in terms of the clinico-pathological factors demonstrated as associations between the TNBC tumors and aggressive features of breast cancer, in that they were of higher grade \((P = 0.000)\), of higher proliferation activity \((P = 0.000)\), larger in size \((T2 \ (P = 0.000))\) and more often node-positive \((P = 0.000)\) than the control tumors, which were well-differentiated, of low proliferation, smaller in size \((T1)\) and node-negative.

The TNBC tumors were more often positive for HIF-1\(\alpha\) \((P = 0.000)\) and negative for PHD3 \((P = 0.000)\) and HIF-2\(\alpha\) \((P = 0.005)\) than the ER+/PR+/HER2-controls. 80% of the TNBC tumors stained positive for HIF-1\(\alpha\), while 54% of the
control tumors did not show any staining for HIF-1α. 71% of the control tumors showed PHD3 expression, but 60% of the TNBC tumors failed to express PHD3. Also, 81% of the control tumors were HIF-2α-positive, while the distribution of HIF-2α expression was more even in the TNBC tumors. The other hypoxia or cell cycle-related factors did not show any differences in expression pattern between the two subgroups.

Lastly, we examined the prognostic significance of each marker by comparing the TNBC and ER+/PR+/HER2- patients. Here Cyclin D1 was the only marker to stand out as an independent prognosticator, in that the Cyclin D1-positive ER+/PR+/HER2- patients had a significant increase in overall survival (P = 0.042; HR = 4.69; 95.0% CI = 1.1, 20.8) and disease-free survival (P = 0.025; HR = 4.73; 95.0% CI = 1.2, 18.4) relative to the cyclin D1-positive TNBC patients, and a tendency for increased breast cancer-specific survival was also detected in this setting (P = 0.059; HR = 7.22; 95.0% CI = 0.92, 56.4).

5.4 Interrelations between the hypoxia related and cell cycle regulatory factors (I-III)

The interrelations between the factors of constituting the HIF pathway were examined in invasive ductal carcinoma tumors in paper I, where comparison of the expression levels of PHD1–3 showed a trend for a positive correlation between PHD2 and PHD3 (P = 0.068) but no interrelations between PHD1 and PHD3, or between PHD1 and PHD2. The expression patterns of HIF-1α and HIF-2α did not correlate with each other. One statistically significant correlation between the HIF prolyl 4-hydroxylases and the hypoxia-inducible factors was found to exist, a positive correlation between HIF-2α and PHD3 expression (P = 0.002), in addition to which a trend was detected for a positive correlation between HIF-2α and PHD1 expression (P = 0.071).

Likewise, the correlations between the factors of the RB pathway, cyclin D1, CDK4 and p16, were examined in invasive ductal carcinoma samples in paper II, but no significant associations were found.

In addition, the associations between hypoxia-related and cell cycle-related factors were examined in invasive ductal carcinoma tumors in paper II, yielding a positive correlation between p16 and the expression of both PHD1 (P = 0.032) and PHD2 (P = 0.027) and a correlation between CDK4 positivity and PHD3 negativity (P = 0.031).
Paper III examines these interrelations in TNBC tumor specimens, and points to a statistically significant positive correlation between PHD2 and PHD3 \((P = 0.032)\), and a trend for an association between PHD1 positivity and PHD3 negativity \((P = 0.070)\). Similarly HIF-1\(\alpha\) and HIF-2\(\alpha\) expression correlated in a positive manner \((P = 0.004)\), and positive correlations were similarly detected between PHD1 and both HIF-1\(\alpha\) \((P = 0.008)\) and HIF-2\(\alpha\) \((P = 0.003)\) expression. PHD2 expression, in turn, was associated with negative HIF-1\(\alpha\) status \((P = 0.018)\) and negative HIF-2\(\alpha\) status \((P = 0.011)\). Only one correlation was found between cyclin D1, CDK4 and p16 in this cohort, a negative correlation between CDK4 and p16 expression \((P = 0.002)\). In addition, some correlations were found between the cell cycle regulators and the hypoxia response regulators. CDK4 positivity was associated with PHD1 and HIF-1\(\alpha\) positivity \((P = 0.000\) and \(P = 0.005\), respectively) and PHD3 negativity \((P = 0.031)\), while p16 positivity was associated with PHD1 positivity \((P = 0.040)\). The expression of cyclin D1 showed a trend towards a positive correlation with PHD1 \((P = 0.087)\).
6 Discussion

The purpose of this work was to investigate the prognostic relevance of the hypoxia response and the cell cycle regulators in human breast cancer. The cohort of ductal mammary carcinoma patients comprised mainly patients with good prognostic features, as the majority had a node-negative disease and the tumors were small (T1), hormone receptor-positive, HER2-negative and of low proliferation. Most of the patients in the TNBC cohort similarly had a node-negative disease, but the tumors were larger (T2) and poorly differentiated. This highlights the general understanding that the majority of breast cancer patients have a good prognosis, but there are some, such as those with triple-negative disease who have a poor prognosis (Lin et al. 2012, Soerjomataram et al. 2008).

6.1 HIF pathway factors in human breast cancer

Overexpression of HIF is a hallmark of many human cancers and their metastases (Semenza 2010). The two HIFs, HIF-1α and 2α, have been firmly associated with a poor outcome and treatment resistance in the vast majority of cancers, including breast cancer (Semenza 2010). Our findings are consistent with this notion in the case of HIF-1α, but differ with respect to the role of HIF-2α.

Approximately 30% and 80% of the present ductal breast carcinoma tumors showed HIF-1α and HIF-2α expression, respectively, while 80% of the TNBC tumor cohort stained positive for HIF-1α and 60% for HIF-2α. Since degradation of HIF-αs is oxygen-dependent, these are stabilized under hypoxic conditions (Kaelin Jr. & Ratcliffe 2008). TNBC tumors are highly proliferating tumors that outgrow their blood supply fast and frequently contain instances of tumor necrosis (Carey et al. 2010), which implies that hypoxia is a significant factor in the tumor microenvironment. This might be one of the reasons to explain why the TNBC tumors were so much more frequently HIF-1α-positive than the unselected ductal breast tumors. Yan et al. (2009) detected an association between HIF-1α expression and the basal-like phenotype which mirrors our TNBC tumor cohort finding, in that HIF-1α expression correlated significantly with high proliferation activity and high grade. Interestingly, however, we found no correlation between high HIF-1α expression and greater tumor size suggesting that other factors than outgrowth of oxygen supply due to larger size are actually responsible for HIF-1α stabilization in TNBC. Like many others before us (Gruber et al. 2004, Kronblad et al. 2006, Yan et al. 2009), however, we did find a trend for decreased breast
cancer-specific survival in ductal breast cancer patients with HIF-1α-positive tumors, but surprisingly, this effect was not seen in our TNBC patient cohort. HIF-2α, on the other hand, correlated significantly with a smaller tumor size in the unselected ductal breast cancer patients. Furthermore, the TNBC tumors were less often positive for HIF-2α than the unselected ductal breast cancer tumors. These results support the idea that HIF-2α positivity in breast cancer correlates with less aggressive tumors. There is much evidence, however, to contradict this finding. Xiang et al. (2012) associated HIF-2α expression with a high grade and high proliferation activity, and Helczynska et al. (2008) found its expression to correlate with a poor outcome in cases of invasive breast cancer. Thus it seems that the prognostic significance of HIF-2α needs further investigation.

The HIF prolyl 4-hydroxylases, PHDs 1–3, have been less extensively studied in clinical breast cancer samples. In our material 60% of the triple-negative tumors stained positive for PHD1 but only 30% of the unselected ductal breast tumors. Additionally, PHD1 expression correlated with high proliferation activity and ER negativity in the ductal breast tumor cohort, both being factors pointing to a poor prognosis. PHD1 has previously been related to breast cancer tumorigenesis and the stimulation of breast cancer cell proliferation (Seth et al. 2002, Zhang et al. 2009). Seth et al. (2002) have also shown that the expression of PHD1 can be induced by oestrogen and that PHD1 is a direct transcriptional target of ER. In view of the correlation between PHD1 expression and ER negativity and the high proportion of PHD1-positive TNBC tumors in our material, one must speculate that not all PHD1-mediated proliferation is oestrogen-dependent. Nevertheless, we did not find any positive correlation between PHD1 expression and proliferation activity in the TNBC tumors, but rather a trend for an association between PHD1 negativity and high proliferation. Significantly high PHD1 levels were also detected in the TNBC cases with node-negative disease, suggesting a role for PHD1 in preventing breast cancer progression. This would have to be regarded as specific to TNBC, however, since no such association was found in our ductal breast cancer cohort.

Our results associated PHD3 with good prognostic factors in the ductal breast tumor cohort as it correlated significantly with a low tumor grade, smaller tumor size, ER positivity and low proliferation activity. PHD3 has been considered to have tumor suppressor qualities (Chan & Giaccia 2010, Rantanen et al. 2008, Schlisio et al. 2008, Xue et al. 2010), and the expectations of a tumor suppressor were confirmed here. PHD3 has previously been reported to induce apoptosis in cellulo (Lee et al. 2005, Schlisio et al. 2008), and to our knowledge this is the
first clinical study to show that it may also be an important regulator of apoptosis in breast tissue in vivo, as tumors of larger size, poor differentiation and increased proliferation appeared when its expression was low. On the other hand, PHD3 failed to show any independent prognostic significance and it is relevant to ask whether this was due to the general good outcome of the cases in this cohort. PHD3 was expressed in 60% of the unselected ductal tumors and in 70% of the selected ER+/PR+/HER2- tumors with an excellent prognosis, but in only 40% of the tumors in the TNBC cohort. Our results are in line with the previous finding of an association of PHD3 negativity with basal-like breast cancer (Yan et al. 2009). This argues for a pattern of PHD3 expression that associates it with less aggressive breast cancers.

Although PHD2 did not correlate with any of the clinico-pathological factors studied in either of our cohorts, we did find a trend for increased breast cancer-specific and disease-free survival among ductal breast cancer patients with high levels of PHD2, giving a hint that PHD2 may possess tumor suppressor qualities as well. Very little clinical information exists on PHD2, and it has been regarded as a physiological rather than a pathological regulator of HIF-α (Berra et al. 2003). Nevertheless, its loss has been shown to contribute to tumor growth (Chan et al. 2009), and our finding is consistent with this. Since PHD2 is normally a cytosolic protein that is able to relocate to the nucleus, we examined its nuclear expression as well, but no correlations with the clinical prognostic factors were found in that case, either.

The PHDs 1–3 are dedicated HIF-α down-regulators which need oxygen for their enzymatic activity (Myllyharju & Koivunen 2013). It is known that insufficient HIF degradation occurs even in the presence of oxygen, e.g. in cancer cells (Kaelin Jr. & Ratcliffe 2008), and therefore it was relevant to ask whether insufficient expression of the PHDs may contribute to the HIF overexpression in cancer. Surprisingly, PHD1–3 expression did not correlate with down-regulation of either HIF-1α or HIF-2α in our cohort of ductal breast cancer patients, suggesting that the PHDs may have other targets in addition to the HIF-αs. It might also be that the PHDs function differently in hypoxic cancerous tissue than in normal tissue. In the TNBC cohort, however, PHD2 positivity was associated with both HIF-1α and HIF-2α negativity, perhaps indicating that the down-regulation may work to some extent but can be somehow counteracted or bypassed, because HIF-1α was still so widely expressed in these tumors. On the other hand, high levels of PHD1 were associated with both HIF-1α and HIF-2α positivity in the TNBC cohort. As stated before, our data associate both PHD1
and HIF-1α expression with poor prognosis, and thus it is no surprise that the factors correlated in such a manner.

HIF-1α and 2α did not correlate with each other in the unselected ductal breast cancer tumor cohort, but they did correlate in a positive manner in the TNBC cohort. This finding is rather controversial, as we found HIF-1α to be associated with a poor prognosis in breast cancer and HIF-2α with a better prognosis. However, in view of the fact that they are hypoxia-inducible factors, the finding that the HIF-αs were correlated in such a manner in the often highly hypoxic TNBC tumors may not be so surprising. PHD2 and PHD3 were also correlated in a positive manner in our TNBC patient cohort and showed a trend towards the same correlation in our unselected ductal breast cancer patient cohort, linking these two markers of a good prognosis together. In addition, PHD2 and PHD3 are themselves both hypoxia-inducible genes, and therefore they are likely to be regulated in the same direction (Aprelikova et al. 2004, Henze et al. 2010). The fact that a positive correlation was also found between PHD3 and HIF-2α in the unselected ductal breast cancer cohort confirms our suggestion that HIF-2α is a marker of a better prognosis.

6.2 RB pathway factors in human breast cancer

Disruption of the p16-cyclin D1-CDK4-RB pathway frequently occurs in human cancers, including breast cancer (Sherr 1996). Cyclin D1 is a key regulator of the G1/S phase progression, and its overexpression is likely to promote cell proliferation and differentiation (Musgrove et al. 2011, Roy & Thompson 2006). Cyclin D1 overexpression is a common event in breast cancer and has been associated with subtypes that are more indolent, oestrogen receptor-positive and have a good prognosis (Bostrom et al. 2009, Roy & Thompson 2006). We detected a similar pattern in our material, where cyclin D1 was expressed in 60% of the unselected ductal breast tumors, while over 70% of the TNBC tumors were cyclin D1-positive, since cyclin D1 overexpression correlated statistically significantly with lower tumor grade, oestrogen and progesterone receptor positivity and lower proliferation activity the ductal breast cancer cohort, that with good prognostic features.

As mentioned before, it has been proposed that cyclin D1 promotes differentiation (Musgrove et al. 2011, Roy & Thompson 2006), and tumors of a lower grade are by definition well differentiated (Schumacher et al. 1993). It is also thought that the expression of hormone receptors is a sign of differentiation
in breast tumors (Platet et al. 2004). In our material cyclin D1 expression correlated significantly with increased breast cancer-specific and overall survival in the ductal tumor patients, but its expression was nevertheless high in the TNBC tumor cohort as well. It is actually quite understandable that cyclin D1 expression should be higher in the more proliferating TNBC tumors than in the well differentiated ductal breast carcinoma tumors. When we compared the independent prognostic significance of cyclin D1 expression between a group of TNBC patients and a group of ER+/PR+/HER2- control patients, we found that the high levels in the TNBC patients were associated with a poor clinical outcome, whereas this was not the case in the control patients. It is a known fact that TNBC patients have a poorer prognosis than those with other breast cancer phenotypes (Bauer et al. 2007, Lin et al. 2012). Bearing this in mind, it may not be that cyclin D1 is associated with a poor outcome for TNBC per se, but rather that it does not have the same protective effect than as it does in ER+/PR+/HER2-tumors.

The relationship of CCND1 gene amplification to cyclin D1 protein expression is a point of controversy. CCND1 is reported to be associated with an increased risk of breast cancer recurrence, while nuclear expression of its protein has been associated with reduced risk of recurrence (Lundgren et al. 2012). The amplification rate of the CCND1 gene is lower than the incidence of cyclin D1 protein overexpression, considering that amplification of the CCND1 gene has been identified in approximately 15–20% of human cancers while protein expression has been demonstrated in 50–70% (Musgrove et al. 2011). Given that high cyclin D1 protein expression was correlated with a high cyclin D1 mRNA level in our cohort of ductal breast cancer tumors, at least some part of the increased protein expression is likely to be due to gene amplification, but there must be other forms maintaining this overexpression.

CDK4 was expressed in about 70% of the present unselected ductal breast cancer tumors and in about 65% of the TNBC tumors. Aberrant CDK4 activity is common in most cancer types (Cicenas & Valius 2011), and it has also been proposed that tumor cells may need CDK4 activity in order to achieve higher levels of proliferation (Malumbres & Barbacid 2009, Santamaria et al. 2007). Our finding of a correlation between CDK4 positivity and high proliferation activity in TNBC tumors fits in well with this notion. TNBC tumors are known to be highly proliferative, and abnormal expression of CDK4 may be one reason for this. Even though we found a correlation between CDK4 positivity and high proliferation activity, no correlation was noted between CDK4 positivity and
larger tumors. It is interesting that CDK4 is so widely expressed in TNBC tumors, because its expression has been connected earlier with HER2-driven breast cancer in a mouse model (Landis et al. 2006) and in human breast tumors (Yu et al. 2006). The reason behind such a high proportion of CDK4-positive tumors in the ductal breast cancer cohort is unknown, although it has been stated that many cancer cells may be addicted to high CDK4 activity (Semczuk et al. 2004, Sgambato et al. 1995, Tetsu & McCormick 2003, Yu et al. 2006, Zou et al. 2002).

As a tumor suppressor, p16 has a cell cycle limiting function (Lukas et al. 1995), and silencing of this gene is common in human cancers (Li et al. 2011, Rocco & Sidransky 2001). Nevertheless, overexpression of p16 is commonly found in cancer (Li et al. 2011). According to our data, 55% of the unselected ductal carcinoma tumors and about 70% of the TNBCs showed p16 expression, with a statistically significant correlation between p16 expression and HER2 negativity existing in the former group. HER2 is an oncogene, the expression of which is thought to be a sign of a poorer prognosis in breast cancer patients (Ménard et al. 2002, Taneja et al. 2010). A few previous studies have associated p16 expression with the basal-like phenotype of breast cancer (Abou-Bakr & Eldweny 2013, Bohn et al. 2010, Herschkowitz et al. 2008), and BLBC and TNBC certainly overlap in many ways, one mutual characteristics being the negative hormone receptor and HER2 status (Carey et al. 2010). Although the expression rate of p16 is high in TNBC patients, it seems too hasty to conclude that this is the reason for the poor prognosis that tends to be attached to TNBCs.

In our material p16 functioned as a tumor suppressor, so that its overexpression was associated with increased breast cancer-specific and disease-free survival in the ductal breast cancer patients and with increased breast cancer-specific and overall survival in the TNBC patients, with a tendency towards increased disease-free survival. It can thus be concluded that p16 acts as a positive prognosticator in human breast cancer independently of hormone receptor and HER2 status.

Surprisingly, we did not find any significant correlations between the three cell cycle regulators, cyclin D1, CDK4 and p16, in the unselected ductal breast carcinoma cohort, although there was a correlation between p16 negativity and CDK4 negativity in the TNBC cohort. The TNBC tumors that were positive for CDK4 were significantly more proliferative than the CDK4-negative ones, and the p16-positive TNBC tumors were more proliferative than the p16-negative ones. This finding is not in line with the physiological roles of p16 and CDK4, as p16 should act as a down-regulator of CDK4 activity (Massague 2004, Sherr
1996, Weinberg 1995), and thus it suggests that other factors must be involved in the control of the cell cycle in TNBC tumors.

### 6.3 Interrelations between the hypoxia response regulators and cell cycle regulators

To our knowledge this is the first study to investigate the correlations between the regulators of the hypoxia response, HIF-1α, HIF-2α and PHD1–3, and the regulators of the cell cycle, cyclin D1, CDK4 and p16, in human breast cancer.

Hypoxia has been shown to induce cell cycle arrest at the G1/S border (Pettersen & Lindmo 1983), an effect that has been described as being RB-dependent, as hypoxia has been shown to induce the accumulation of pRB (Krtolica et al. 1998). p16, the brake at the heart of the cell cycle that governs G1/S transition, has itself been shown to be hypoxia-inducible (Zygmunet al. 2002) but has not been proven to explain the G1 arrest. Both PHD1 and PHD2 have been proposed previously as furnishing the link between hypoxia response and cell cycle arrest, as they have been shown to capable of directly modulating cyclin D1, and thus of inducing cell cycle arrest (Su et al. 2012, Zhang et al. 2009). This effect has been demonstrated with a mammary cell line in the case of PHD1 (Zhang et al. 2009) and with a pancreatic cell line in the case of PHD2 (Su et al. 2012).

We did not find any correlation between PHD2 and cyclin D1 in terms of their expression in our cohorts, however, although there was an interesting trend towards a positive correlation between PHD1 and cyclin D1 expression in the TNBC tumors. Zhang et al. (2009) have suggested that PHD1 inactivation reduces cyclin D1 levels and suppresses mammary gland proliferation, and our findings support this. The trend is an interesting one, however, because we found that cyclin D1 correlated with a good prognosis in ductal breast cancer tumors and not with proliferation, while PHD1 correlated with high proliferation. In TNBC tumors, on the other hand, cyclin D1 was associated with a poorer prognosis, while PHD1 expression was associated with negative nodal status. Thus the significances of these two factors seem to be the opposite in TNBC to those observed in otherwise unselected ductal breast cancer cases.

This finding of a statistically significant positive correlation between p16 and the expression of both PHD1 and PHD2 in the ductal breast cancer tumors is surprising, because both p16 and PHD2 are associated with a good prognosis in
breast cancer, while our findings underline a role for PHD1 as a negative prognostic factor.

Furthermore, we found positive correlations between CDK4 and PHD1 expression and between CDK4 and HIF-1α expression in the TNBC cohort. CDK4 and HIF-1α were both associated with high proliferation activity in the TNBC tumors, as was PHD1 in ER-negative ductal breast cancer. In addition, we found a correlation between CDK4 positivity and PHD3 negativity in the TNBC tumor cohort. In conclusion, the expression of CDK4, HIF-1α and PHD1 may be regarded on the evidence presented here as being associated with a poor prognosis and that of PHD3 with a good prognosis, and thus these correlations appear to be rational.

6.4 The prognostic significance of the hypoxia response regulators and the cell cycle regulators

We showed here that cyclin D1 and p16 are independent prognostic factors having impact on breast cancer patient survival. We also associated the expression of PHD3 and HIF-2α with good prognostic factors and that of HIF-1α and CDK4 with poor prognostic factors. According to our data the prognostic significance of PHD1 might differ between breast cancer subtype.

We recognize that the assessment of new prognostic biomarkers require studies with much power. Our data consisted of 102 ductal breast cancer patients for papers I and II and 111 TNBC patients and 59 ER+/PR+/HER2- control patients for paper III. There is a recently published data on the expression of 22,277 genes in a cohort of 2,977 breast cancer patients (Kaplan Meier Plotter). The gene expression and patient survival data are used as a database for a survival analysis tool available online. This tool can be accessed online at www.kmplotter.com.

When using this data analysis tool to test the effect of our markers, HIF-1α, PHDs 1–3, cyclin D1, CDK4 and p16, on the outcome of breast cancer patients, the results were mainly similar to ours yet no statistically significant results emerged. HIF-2α was not possible to test in this analysis tool. It is noteworthy, however, that we studied protein expression in contrast to gene expression and mRNA levels. In addition, the end points used in this analysis tool were the relapse-free, distant metastasis-free and overall survival and the palliative performance scale, which slightly differ from the ones we used in our study.
Summary and conclusions

Along with our expanding knowledge of the heterogeneity of breast cancer, prognostic biomarkers are increasingly needed to predict the behaviour of individual tumors. Furthermore, treatment methods are evolving in the direction of targeted therapies. This thesis examines the prognostic significance of the hypoxia response regulators HIF-1α, HIF-2α and PHDs 1–3 and the cell cycle regulators cyclin D1, CDK4 and p16 a series of ductal breast cancer patients and a series of TNBC patients.

The main conclusions to be drawn from this work are:

1. The results suggest a tumor suppressor role for PHD3 in breast cancer, as its expression was associated with good prognostic features, while down-regulation of its expression was seen in TNBC which is known to be an aggressive phenotype.

2. Cyclin D1 expression was associated with good prognostic features and a better outcome in the cases of ductal breast cancer, but with a poorer clinical outcome in TNBC.

3. p16 positivity was closely correlated with increased survival in both the ductal breast cancer patients and TNBC patients.

4. HIF-1α expression showed a trend towards reduced survival in the ductal breast cancer patients and was associated with poor prognostic factors in TNBC.

5. The results highlighted the controversial role of PHD1 in breast cancer, as it seems to be associated with high proliferation in ductal ER-negative breast cancers, but may have a role in preventing the progression of TNBC, since it correlated with node negativity.

6. It may be suggested that CDK4 can serve as a marker of proliferation in TNBC.

7. The expectations regarding the physiological regulatory mechanisms associated with the HIF and RB pathways were not fulfilled here, suggesting that there may be other factors contributing to the regulation of the hypoxia response and the cell cycle in both ductal breast cancer and TNBC.
References


Original publications


Reprinted with permission from Springer (I) and BioMed Central (II).

The original publications are not included in the electronic version of this thesis.
1203. Yannopoulos, Fredrik (2013) Remote ischemic preconditioning as a means to protect the brain against hypothermic circulatory arrest: an experimental study on piglets

1204. Arvonen, Miika (2013) Intestinal immune activation in juvenile idiopathic arthritis


1206. Penttilä, Matti (2013) Duration of untreated psychosis: association with clinical and social outcomes and brain morphology in schizophrenia


1212. Tilkkanen, Jani (2013) Early repolarization in the inferolateral leads of the electrocardiogram: prevalence, prognosis and characteristics


1214. Kaakinen, Pirjo (2013) Pitkääikaisairaiden ohjaustyön toimialusten järjestely

1215. Pasanen, Anna Kaisa (2013) A translational study on the roles of redox molecules, cell cycle regulators and chemokine receptors as prognostic factors in diffuse large B-cell lymphoma

1216. Malo, Elina (2013) The role of low birth weight and resistin in metabolic syndrome

1217. Karjalainen, Jaana (2013) Cardiovascular autonomic function in coronary artery disease patients with and without type 2 diabetes: Significance of physical activity and exercise capacity
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