JOHANNA PELLIKAINEN

Activator Protein -2 in Breast Cancer

Relation to Cell Growth, Differentiation and Cell-Matrix Interactions

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium L3, Canthia building, University of Kuopio, on Friday 1st October 2004, at 12 noon

Departments of Pathology and Forensic Medicine, Oncology, Surgery, and Otorhinolaryngology, Oral and Maxillofacial Unit, Faculty of Medicine University of Kuopio and Kuopio University Hospital

Tampere Medical School, Pathology University of Tampere
ABSTRACT

Breast cancer is the most common cancer among women in the western societies. Annually over 3500 new breast cancer cases are diagnosed in Finland and the incidence is on the increase. The breast cancer related mortality, fortunately, has shown a decline recently, mostly because of improved diagnostic and treatment modalities. However, tools for assessing prognosis more accurately are still required to select those patients who will benefit from aggressive adjuvant treatment.

In the present study the expression and prognostic value of factors related to regulation of cell growth, cell proliferation and differentiation (AP-2, p21^{WAF1}, p53, Ki-67, HER2) and cell-matrix interactions (MMP-2 and MMP-9) were evaluated in a large prospective series of 420 breast cancer patients diagnosed and treated at Kuopio University Hospital during the years 1990-1995. Expression of the biological markers was assessed by using immunohistochemistry. AP-2 expression was further evaluated by Western blotting and HER2 gene amplification was analysed by using chromogenic in situ hybridization (CISH). Interrelationships between the biological markers and clinicopathological parameters as well as relation to patient survival were investigated.

Nuclear AP-2 expression was reduced in malignant tissue compared to the strongly positive benign breast epithelium. Reduced nuclear AP-2 expression was associated with aggressive clinicopathological parameters and independently predicted shorter recurrence-free survival (RFS) in the whole patient group and in the axillary lymph node positive (ANP) patient subgroup, which is support for a tumour suppressive role for AP-2. p21^{WAF1} expression predicted the response to adjuvant hormone therapy but in survival analyses neither p21^{WAF1} nor p53 possessed any independent prognostic value for breast cancer outcome. Cell proliferation, disease stage and histological grade were the most important prognostic factors. HER2 overexpression was an independent predictor of shorter RFS in the whole patient group and in the ANP subgroup. Combining HER2 and AP-2 expression added information on breast cancer prognosis. MMP-2 expression was not related to survival. High MMP-9 expression in carcinoma cells predicted better RFS survival but did not have an independent prognostic value. Instead, positive stromal MMP-9 expression predicted reduced survival in a group of oestrogen receptor positive, small tumours.

The results indicate that nuclear AP-2 expression provides additional prognostic value in breast cancer both alone and in combination with HER2 overexpression. MMP-9 expression may add information on breast cancer prognosis, especially in early-stage cancer. In addition, this study confirms the value of HER2 overexpression, cell proliferation and traditional prognostic factors such as stage and histological grade.

National Library of Medicine Classification: QZ 200, WP 870, QZ 365

Medical Subject Headings: neoplasms, glandular and epithelial; carcinoma; breast neoplasms; prospective studies; prognosis; transcription factors; cell cycle; cell cycle proteins; genes, erbB-2; matrix metalloproteinases; gelatinase A; gelatinase B
ACKNOWLEDGEMENTS

The present study was carried out at the Department of Pathology and Forensic Medicine, University of Kuopio, and at the Departments of Pathology, Oncology, Surgery and Otorhinolaryngology, Oral and Maxillofacial Unit, Kuopio University Hospital, during the years 1999-2004.

I owe my deepest gratitude to my main supervisor, Professor Veli-Matti Kosma, M.D., Ph.D., Head of the Department of Pathology and Forensic Medicine, for introducing me the world of scientific research and for giving me the opportunity as well as facilities to perform this work. His expert guidance, support and enthusiasm have been invaluable for the completion of this thesis.

I am deeply grateful to my second supervisor, Docent Vesa Kataja, M.D., Ph.D., for sharing his experience in clinical oncology and for his ready comments when preparing the manuscripts. His devotion and encouragement during this study have been irreplaceable.

I wish to express my sincere thanks to my third supervisor, Professor Matti Eskelinen, M.D., Ph.D., for his never failing support and encouragement during these years.

I am also very grateful to Docent Jari Kellokoski, M.B., D.D.S., Ph.D., my fourth supervisor, for his expert advices in the complex field of AP-2 and molecular biology, as well as for his valuable comments during the writing process.

I express my special thanks to my coauthors: Kirsi Ropponen, M.D., Ph.D., for her friendliness and high expertise in pathology, her participation in the study has been invaluable during these years; Timo Pietiläinen, M.D., Ph.D., for introducing me the field of breast pathology, and for his valuable guidance at the beginning of this study; Jan Böhm, M.D., Ph.D., for his kind help at the first steps of this study as well as for his sense of humour and continuous support; Tiia Rissanen, M.D., for the great times in the laboratory as well as for her contribution in the study; Docent Anita Naukkarinen, Ph.D., and Jaana Rummukainen, M.D., Ph.D., for their expert knowledge of the HER2 procedures and friendly support.

I wish to sincerely thank Docent Paula Martikainen, M.D., Ph.D., Department of Pathology, Centre for Laboratory Medicine, Tampere University Hospital, and Professor Seppo Pyrhönen, M.D., Ph.D., Department of Oncology and Radiotherapy, Turku University Hospital, the official reviewers of my thesis, for their time and constructive comments during the final preparation of this thesis.

The staff of the Department of Pathology and Forensic Medicine in the University of Kuopio and Kuopio University Hospital is greatly acknowledged, especially Ms. Aija Parkkinen, Ms. Riikka Pennanen, Ms. Helena Kemiläinen, Ms. Mervi Malinen, Anna Taskinen, M.Sc., Ms. Irma Väänänen and Ms. Rauni Manninen for their skillful technical assistance. I also sincerely thank all of other personnel of the Departments for their friendly support during this study.
I want to warmly thank my fellow researchers Tero Leinonen, B.Sc., Kirsi Voutilainen, M.B., Sari Sillanpää, M.B., and Essi Hiltunen, M.D. for their humour and supporting conversations, as well as Risto Pirinen, M.D., Ph.D., Maarit Anttila, M.D., Ph.D., Jari Karjalainen, M.D., Ph.D., Arto Mannermäki, Ph.D., Hanna Tuhkanen, M.Sc., and Jaana Hartikainen, M.Sc. for their kind support.

I wish to thank Alpo Pelttari, M.Sc., for his skillful assistance with the microscope-camera. I also want to thank photographer Mr. Olli Horto for his valuable help in preparing the photographs for publication. The statistical advice of Pirjo Halonen, M.Sc. is highly acknowledged. The personnel of the scientific library of the University of Kuopio and Kuopio University Hospital also deserve my sincere thanks for their excellent service. The service of the Information Technology Center of the University of Kuopio is also acknowledged. I am grateful to Ewen MacDonald, D. Pharm., for revising the language of this thesis. The contribution of Anna-Kaisa Lytinen, R.N., to the project is also acknowledged. I also thank Ms. Eeva Oittinen for her technical assistance.

I wish to thank people at Kuopion Riento R.Y. boxing club, for keeping me in shape during this study as well as for their friendliness.

I wish to express my special thanks to my friends and colleagues: Mari Hyvärinen, M.D., Virpi Sidoroff, M.D., Tuulikki Uusitalo, M.D., and Minja Enväls, M.B., with whom I started my medical studies in 1997, for sharing my life during these years and for their still lasting friendship; as well as Merja Leppänen, M.B., Marjo Leppänen, M.B., Hanne Kuittinen, M.D., and Riina Korjamo, M.D., for fun parties, joyful discussions and support. I also wish to thank Henna Pirhonen, B. Sc. Pharm., and Aune Heikkinen, B. Sc. Pharm., for their relaxing companionship, as well as Ulla Nuutinen M.Sc., and Paula Paavola, M.D., for many laughs, interesting discussions about life and research, wild parties and other adventures. The support of others is also highly appreciated.

My most loving thanks belong to my family: I wish to warmly thank my dear parents, Eini and Raiimo Pellikainen, for giving me the steady basis of my life and for their continuous support and caring. I also wish to thank my sister, Hannele, and brother, Juha, for their great companionship and support.

I wish to express my most heartfelt thanks to my fiance Pekka Eerikäinen, whom I met in the latter part of this work, for his loving and caring, as well as for the endless patience and encouragement that he has expressed towards my work.

This study has been financially supported by the Special Governmental Funding (EVO-funding) of Kuopio and Tampere University Hospitals, The North Savo Cancer Fund, The Paavo Koistinen Fund, The Kuopio University Fund, The Culture Fund of Finland (North Savo Fund), Research Foundation of Orion Corporation, and The Finnish Medical Society Duodecim, all of which are greatly acknowledged.

Kuopio, September 2004

[Signature]

Johanna Pellikainen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>avidin-biotin peroxidase complex</td>
</tr>
<tr>
<td>ANN</td>
<td>axillary lymph node negative</td>
</tr>
<tr>
<td>ANP</td>
<td>axillary lymph node positive</td>
</tr>
<tr>
<td>AP-2</td>
<td>activator protein –2</td>
</tr>
<tr>
<td>Bax</td>
<td>pro-apoptotic protein Bax</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>anti-apoptotic protein Bcl-2</td>
</tr>
<tr>
<td>BCRS</td>
<td>breast cancer-related survival</td>
</tr>
<tr>
<td>c-Myc</td>
<td>nuclear phosphoprotein c-Myc</td>
</tr>
<tr>
<td>c-Src</td>
<td>member of Src family kinases</td>
</tr>
<tr>
<td>CD44</td>
<td>hyaluronan receptor CD44</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CISH</td>
<td>chromogenic in situ hybridization</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>ECM</td>
<td>extra cellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenedinitril tetraacetic acid, disodium salt dihydrate</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>extracellular matrix metalloproteinase inducer</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>Ets</td>
<td>Ets family of transcription factors</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>GR</td>
<td>histological grade</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HAS</td>
<td>hyaluronan synthase</td>
</tr>
<tr>
<td>HER</td>
<td>human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head-and-neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor receptor 1</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IRS</td>
<td>immunoreactivity score</td>
</tr>
<tr>
<td>LCIS</td>
<td>lobular carcinoma in situ</td>
</tr>
<tr>
<td>LSAB</td>
<td>labeled streptavidin-biotin system</td>
</tr>
<tr>
<td>Ki-67</td>
<td>nuclear antigen associated with cell proliferation</td>
</tr>
<tr>
<td>MA1</td>
<td>mitotic activity index</td>
</tr>
<tr>
<td>MIB-1</td>
<td>monoclonal antibody towards Ki-67, clone MIB-1</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane type matrix metalloproteinase</td>
</tr>
<tr>
<td>M/V</td>
<td>volume-corrected mitotic index</td>
</tr>
<tr>
<td>ND</td>
<td>no data</td>
</tr>
<tr>
<td>NP</td>
<td>nuclear pleomorphism</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>OCT</td>
<td>OCT compound, tissue freezing medium</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>P21WAF1</td>
<td>cell cycle inhibitor / wild-type activated fragment 1</td>
</tr>
</tbody>
</table>
p27\(^{kip1}\) cell cycle inhibitor / kinase inhibitor protein 1
p57\(^{kip2}\) cell cycle inhibitor / kinase inhibitor protein 2
p53 nuclear phosphoprotein p53
PARP polyADP-ribose polymerase
PBS phosphate buffered saline
PCNA proliferating cell nuclear antigen
PKA protein kinase A
PMSF phenylmethylsulphonylfluoride
PR progesterone receptor
pRB retinoblastoma protein
RB-1 the gene encoding pRB
RFS recurrence-free survival
SDS sodium dodecyl sulphate
Sp1 specificity protein -1, transcription factor
Sp3 specificity protein -3, transcription factor
SPF S-phase fraction
Stage stage grouping according to TNM classification
TF tubule formation
TIMP tissue inhibitor of matrix metalloproteinase
TNF-\(\alpha\) tumour necrosis factor -\(\alpha\)
TNM tumour-node-metastasis classification
TRIS tris(hydroxymethyl)aminomethane
UICC International Union Against Cancer
WHO World Health Organization
LIST OF ORIGINAL PUBLICATIONS

This summary is based on the following original publications referred to in the text by their Roman numerals I-IV.


This summary includes also unpublished data.
The original papers in this thesis have been reproduced with the permission of the publishers.
CONTENTS

1. INTRODUCTION ........................................................................................................... 15

2. REVIEW OF THE LITERATURE .................................................................................. 16

2.1 Epidemiology of breast cancer .............................................................................. 16

2.2 Risk factors for breast cancer .............................................................................. 16

2.3 Diagnosis and management of breast cancer ...................................................... 16

2.4 Traditional prognostic factors in breast cancer .................................................. 17

2.4.1 Tumour size ......................................................................................................... 17

2.4.2 Lymph node status ............................................................................................ 17

2.4.3 Stage .................................................................................................................. 19

2.4.4 Age and menopausal status .............................................................................. 19

2.4.5 Histopathological subtype .............................................................................. 20

2.4.5.1 In situ carcinoma .......................................................................................... 20

2.4.5.2 Invasive carcinoma ....................................................................................... 21

2.4.5.2.1 Invasive ductal carcinoma NOS ............................................................ 21

2.4.5.2.2 Invasive lobular carcinoma ................................................................. 21

2.4.5.2.3 Other invasive carcinoma types ............................................................ 22

2.4.6 Histopathological grade ................................................................................... 22

2.4.7 Hormone receptor (ER and PR) expression .................................................... 23

2.4.8 Blood and lymph vessel invasion .................................................................... 23

2.5 Prognostic value of factors related to cell proliferation, DNA-ploidy and
   apoptosis ..................................................................................................................... 23

2.5.1 Mitotic indices .................................................................................................... 23

2.5.2 PCNA, S-phase fraction, DNA-ploidy .............................................................. 24

2.5.3 Ki-67 ................................................................................................................... 24

2.5.4 Apoptosis .......................................................................................................... 24

2.6 Prognostic value of factors related to regulation of cell growth and
   differentiation ............................................................................................................ 25

2.6.1 pRB .................................................................................................................... 25

2.6.2 p53 ..................................................................................................................... 26

2.6.3 p21\textsuperscript{WAF1} ........................................................................................... 26

2.6.4 p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2} .................................................................. 27

2.6.5 AP-2 .................................................................................................................. 29
2.6.6 EGRF-family

2.7 Prognostic value of factors related to cell-matrix interactions, invasion and angiogenesis

2.7.1 Matrix metalloproteinases

2.7.2 Tissue inhibitors of metalloproteinases (TIMP)

2.7.3 Other factors related to cell-matrix interactions

2.7.3.1 Hyaluronan (HA)

2.7.3.2 CD44

2.7.3.3 Tenascin and versican

2.7.4 Angiogenesis

3. AIMS OF THE STUDY

4. MATERIAL AND METHODS

4.1 Patients

4.2 Treatment

4.3 Histology

4.4 Immunohistochemical staining of AP-2, p21\textsuperscript{WAF1}, and p53 (I-II)

4.5 Immunohistochemical staining of Ki-67, MMP-2, and MMP-9 (II, IV)

4.6 Immunohistochemical staining of HER2 (III)

4.7 Western blot (I)

4.8 Chromogenic in situ hybridization (CISH) (III)

4.9 Evaluation of the staining

4.9.1 AP-2, p21\textsuperscript{WAF1}, p53, Ki-67, MMP-2 and MMP-9 (I-IV)

4.9.2 HER2 (III)

4.10 Statistical analyses

4.11 Ethical aspects

5. RESULTS

5.1 Clinicopathological data (I-IV)

5.2 Expressions of biological factors

5.2.1 Expression of AP-2 (I-IV)

5.2.2 Expression of p21\textsuperscript{WAF1} and p53 (II)

5.2.3 Cell proliferation measured by Ki-67 expression (II-III)

5.2.4 Expression of HER2 (III-IV)

5.2.5 Expression of MMP-2 and MMP-9 (IV)
5.3 Relation of biological factors to clinicopathological data ................. 49
  5.3.1 Relation of AP-2 expression to clinicopathological data (I) ............ 49
  5.3.2 Relation of p21\textsuperscript{WAF1} and p53 expression to clinicopathological data (II) .... 51
  5.3.3 Relation of cell proliferation (Ki-67) to clinicopathological data ....... 51
  5.3.4 Relation of HER2 expression to clinicopathological data (III) .......... 51
  5.3.5 Relation of MMP-2 and MMP-9 expression to clinicopathological data (IV) 51

5.4 Interrelationships between biological factors .................................... 51
  5.4.1 Relation of AP-2 to p21\textsuperscript{WAF1} and p53 expression (II) ........... 51
  5.4.2 Relation of AP-2 to HER2 expression (III) .................................. 53
  5.4.3 Relation of AP-2 to MMP-2 and MMP-9 expression (IV) .................. 53
  5.4.4 Relation of HER2 to p21\textsuperscript{WAF1} and p53 expression ................ 53
  5.4.5 Relation of HER2 to MMP-2 and MMP-9 expression (IV) ................. 53

5.5 Relation of biological factors to cell proliferation (II-III) ..................... 54

5.6 Prognostic factors for breast cancer ................................................ 54
  5.6.1 Clinicopathological factors (I-IV) ............................................ 54
  5.6.2 Biological factors ................................................................... 55
  5.6.2.1 Relation of AP-2 expression to survival (I) ................................. 55
  5.6.2.2 Relation of p21\textsuperscript{WAF1} and p53 expressions to survival (II) ........ 55
  5.6.2.3 Relation of cell proliferation (Ki-67) to survival (II-III) ................. 56
  5.6.2.4 Relation of HER2 expression to survival (III-IV) ............................ 56
  5.6.2.5 Relation of MMP-2 and MMP-9 expressions to survival (IV) ............. 57
  5.6.3 Multivariate survival analysis of all cases ..................................... 57

6. DISCUSSION ...................................................................................... 60
  6.1 Evaluation of the study material ....................................................... 60
  6.2 Evaluation of the study methods ......................................................... 60

6.3 Clinicopathological prognostic factors in breast cancer ...................... 61

6.4 AP-2 – expression and breast cancer ............................................... 62
  6.4.1 Expression of AP-2 and its relation to clinicopathological data .......... 62
  6.4.2 Role of AP-2 in the regulation of cell proliferation, p21\textsuperscript{WAF1} and p53 .... 62
  6.4.3 Relation of AP-2 to HER2 and MMPs ........................................... 63
  6.4.4 Prognostic value of AP-2 ............................................................ 65

6.5 Expression of 21\textsuperscript{WAF1} and its relation to p53, clinicopathological factors
and prognosis in breast cancer ........................................................... 66
6.6 Cell proliferation and breast cancer......................................................... 68
6.7 HER2 expression, relation to p21WAF1, p53, MMPs and survival in breast cancer................................................................................. 69
6.8 Expression of MMP-2 and MMP-9, relation to clinicopathological factors and prognosis in breast cancer .................................................. 71
7. SUMMARY AND CONCLUSION ................................................................. 73
8. REFERENCES ............................................................................................ 75
9. ORIGINAL PUBLICATIONS
1. INTRODUCTION

Breast cancer is the most common cancer among women in the western societies. It accounts for 30% of total cancers in women, and it is the leading cause of cancer deaths in Finland, responsible for 848 deaths in 2001\(^1\). Annually over 3500 new breast cancers are diagnosed in Finland and the incidence is increasing\(^1\). However, at the same time, breast cancer related mortality has started to decline\(^1\). Diagnostics of breast cancer has generally improved and tumours are found as small, local tumours\(^2\). Currently, the 5-year overall survival rate of breast cancer patients is close to 80\% in Scandinavia\(^3\), reaching 85\% in Finland\(^1\). However, despite early diagnosis and improved adjuvant therapies, metastatic breast cancer is still an incurable disease and the mechanisms leading to malignant transformation as well as to the formation of distant metastases are unclear.

Several clinicopathological factors such as disease stage and hormone receptor expression, as well as many biological factors such as HER2 are used to predict the behaviour of breast cancer\(^4\)\(^7\). Despite intensive research, there are still inadequate tools available for assessing prognosis, for example is not known why some tumours with seemingly good prognosis show more aggressive behaviour compared to others with otherwise similar properties. An improved understanding of breast cancer biology and the steps involved in malignant transformation would make it easier to predict the disease outcome as well as the response to treatment, and thus more accurately select patients for intensive adjuvant therapies. In addition, deeper knowledge could reveal new potential targets for novel treatment modalities.

In the present study the expression and prognostic value of factors related to regulation of cell growth, cell proliferation and differentiation (AP-2, p21\(^WAF1\), p53, Ki-67, HER2), as well as cell-matrix interactions (MMP-2 and MMP-9) were examined in a large prospective series of breast cancer patients.
2. REVIEW OF THE LITERATURE

2.1 Epidemiology of breast cancer
Breast cancer is the most common cancer among women in the western societies and the leading cause of cancer related deaths in Finland. The proportion of breast cancer is 30% of all cancers in women. In Finland over 3500 new breast cancers are diagnosed each year. The age-adjusted incidence of breast cancer was 82.6 per 100 000 person years in the year 2001 in Finland. In the same year the annual number of breast cancer deaths was 848 i.e. the age-adjusted mortality rate is 15.9 per 100 000 person years. The incidence of breast cancer is still increasing but mortality has started to decline in Finland. Breast cancer in males is rare, accounting for <1% of all diagnosed breast cancers annually in Finland.

2.2 Risk factors for breast cancer
The risk of developing breast cancer is related to life-time oestrogen exposure. Accordingly, breast cancer is uncommon at age younger than 35 years but increases with advanced age. Risk factors for breast cancer are early onset of menstruation, nulliparity or delayed first childbirth, short duration of breast-feeding, low number of children, late menopause, postmenopausal obesity, extended use of oral contraceptives, long-term oestrogen replacement therapy, which all are surrogates for oestrogen exposure. Other risk factors are higher education and socio-economical status, often reflecting late parity, exposure to ionizing radiation, high alcohol consumption, physical inactivity, and dietary habits including high consumption of fat and processed red meat. In addition, family history of breast or ovarian cancer, and history of certain benign breast disease, such as sclerosing adenosis, hyperplasia, atypical hyperplasia, and ductal papillomatosis, increase the breast cancer risk. The proportion of familial breast cancer is 5-10% of all breast cancers. A germline mutation in BRCA1 or BRCA2 tumour suppressor genes accounts for approximately 20% of familial breast cancers in Finland.

2.3 Diagnosis and management of breast cancer
Diagnosis of breast cancer is based on the clinical examination conducted by the physician, mammography, ultrasonography when appropriate, and stereotactic core needle
biopsy. The aim of modern surgical treatment is breast conservation with wide local tumour resection if possible. Modified radical mastectomy is the surgical mode in more advanced cases. Both modalities are combined with sentinel node biopsy and axillary lymph node dissection in sentinel node positive cases. All patients treated with local resection, unless there is ductal carcinoma in situ with diameter less than 15 mm, as well as those with axillary lymph node positive cases and large tumours having been treated with mastectomy, are postoperatively offered radiotherapy. Patients with factors related to increased recurrence risk equal or higher than 10% in 10 years, such as lymph node positivity, receptor negativity and/or histological grade 2 or 3, or age < 35 years, are given adjuvant anthracycline based chemotherapy. In addition, patients with hormone receptor positive tumours receive antioestrogen therapy for five years. Treatment of metastatic breast cancer is palliative with endocrine therapy, chemotherapy, radiotherapy, and surgery depending on the biological properties and spread of the disease. In recent years also targeted therapy with monoclonal antibodies has been possible in selected cases.

2.4 Traditional prognostic factors in breast cancer

2.4.1 Tumour size

A large tumour size has been shown to be an adverse prognostic factor in breast carcinoma. Tumour size is an independent prognostic factor and an additive factor to lymph node status. The probability of lymph node metastases and poor differentiation increases with increasing tumour size. In tumours ≤ 1 cm in diameter, lymph node metastasis have been diagnosed in 18% of the cases, in tumours 1-1.5 cm in 26%, in 1.6-2.0 cm tumours in 34%, in tumours 2-3 cm in 44%, and in large 3-5 cm tumours in 58% of the cases. The 20-year adjusted survival rate for tumours < 1 cm was 88%, for tumours 1.0 - 1.9 cm 78%, for tumours 2.0-4.9 cm 68% and for tumours > 5 cm 58%.

2.4.2 Lymph node status

The histologically assessed axillary lymph node status is the most important single independent prognostic factor in breast cancer. The appearance of lymph node metastases is associated with significantly reduced survival. Not only the lymph
node involvement but also the number and the site of involved lymph nodes are of prognostic value. The number of examined, uninvolved nodes is related to better prognosis. The 20-year adjusted survival rate for axillary lymph node negative (ANN) patients was 83% whereas for the ANP patients it was only 67%.45

**Table 1.** Tumour-Node-Metastasis (TNM) classification of breast tumours according to UICC, 2002.46

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour more than 2 cm but not more than 5 cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour more than 5 cm in greatest dimension</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour of any size with direct extension to chest wall or skin only</td>
</tr>
<tr>
<td>NX</td>
<td>Regional lymph nodes can not be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastases</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in movable ipsilateral axillary lymph node(s)</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in fixed ipsilateral axillary lymph node(s) or in clinically apparent ipsilateral internal mammary lymph node(s) in the absence of clinically evident axillary lymph node metastasis</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement; or in clinically apparent ipsilateral internal mammary lymph node(s) in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement.</td>
</tr>
<tr>
<td>MX</td>
<td>Distant metastasis cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>
2.4.3 Stage

Breast cancer stage is assessed according to the TNM classification (Table 1) of the UICC, which can be assessed either pathologically (pTNM) or clinically (TNM). Stage (Table 2) is the most important prognostic factor and is the basic factor in selecting patients for different treatment strategies. The overall 5-year survival in stage I disease was 87%, in stage II 75%, and in stage III 46% \(^{51}\). The 5-year survival of stage IV patients is close to 20% \(^{55,56}\). The survival according to stage in Europe is similar to that described in the United States \(^{55}\).

Table 2. Stage according to UICC classification, 2002 \(^{46}\).

<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>I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIA</td>
<td>T0-1</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>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIIA</td>
<td>T0-2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1-2</td>
<td>M0</td>
</tr>
<tr>
<td>IIIB</td>
<td>T4</td>
<td>N0-2</td>
<td>M0</td>
</tr>
<tr>
<td>IIIC</td>
<td>T0-4</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>IV</td>
<td>T0-4</td>
<td>N0-3</td>
<td>M1</td>
</tr>
</tbody>
</table>

2.4.4 Age and menopausal status

Breast cancer is predominantly a postmenopausal disease. Appearance at young age, < 35 years, is related to aggressive disease and reduced survival \(^{57}\). The reported 5-year OS has been 64% for patients < 35 years at diagnosis \(^{58}\). Tumours in patients < 35 years are more often ductal carcinomas, poorly differentiated, and ER- and PR-negative \(^{57,59}\). Lymphovascular invasion and high proliferation activity are also characteristic of breast cancer at early age \(^{57,58,60}\). Several studies have demonstrated significantly reduced RFS and OS in the patient group < 35 years, especially in a subgroup with ER+ disease \(^{57,58}\). In general, patients diagnosed with breast cancer before age < 50 years continue to
have increased mortality \(^\text{61}\), whereas at older age, the relative breast cancer survival increases \(^\text{62,63}\).

### 2.4.5 Histopathological subtype

Breast cancers are divided into *in situ* carcinomas and invasive carcinomas depending on their capability to penetrate through the basement membrane and metastasize, which is a characteristic of invasive carcinomas \(^\text{54}\). *In situ* carcinomas arise from the terminal duct-lobular unit, expand into the involved lobules, and spread inside the ductal structures but do not invade \(^\text{64}\). Both carcinoma types can further be divided into different subtypes according to their histopathological properties (Table 3) \(^\text{64,65}\).

**Table 3.** Histopathological subtypes of breast cancer, modified from Rosen's Breast Pathology, 1997 and WHO classification for Breast Tumours \(^\text{64,65}\).

<table>
<thead>
<tr>
<th>Carcinoma type</th>
<th>Histopathological subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In situ carcinoma</em></td>
<td>Ductal (DCIS)</td>
</tr>
<tr>
<td></td>
<td>Lobular (LCIS)</td>
</tr>
<tr>
<td></td>
<td>Morbus Paget</td>
</tr>
<tr>
<td><em>Invasive carcinoma</em></td>
<td>Ductal NOS</td>
</tr>
<tr>
<td></td>
<td>Micropapillary</td>
</tr>
<tr>
<td></td>
<td>Apocrine</td>
</tr>
<tr>
<td></td>
<td>Metaplastic</td>
</tr>
<tr>
<td></td>
<td>Lipid-rich</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
</tr>
<tr>
<td></td>
<td>Adenoid Cystic</td>
</tr>
<tr>
<td></td>
<td>Glycogen-rich</td>
</tr>
</tbody>
</table>

#### 2.4.5.1 *In situ carcinoma*

The histological subtypes of *in situ* carcinoma are ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) \(^\text{64}\). LCIS is relatively uncommon and constitutes approximately 1 % to 3.8 % of all breast carcinomas. In LCIS, the cells are loosely cohesive, monomorphic, small, with uniform round nuclei, and they have distinct nucleoli, and sparse cytoplasm. Calcifications, mitoses and necrosis are infrequent but LCIS is
often multifocal and bilateral \(^{64}\). LCIS increases the risk of developing breast cancer by 1 % per year for the next 15-20 years \(^{66}\). Patients require follow-up annually after diagnosis \(^{38}\).

DCIS is a precursor lesion of invasive breast carcinoma and constitutes 15 % of breast carcinomas \(^{64}\). DCIS is graded into three categories according to cytonuclear grade and necrosis. In low grade DCIS, the cells are monotonous, uniform and round with regular nuclei and chromatin, mitoses are infrequent and necrosis is not present. In intermediate grade DCIS, the cell morphology is close to that in low grade DCIS but there may be intraluminal necrosis. High grade DCIS is usually large, composed of highly atypical cells with pleomorphic nuclei, clumped chromatin and prominent nuclei. Mitosis, necrosis and calcification occur frequently. DCIS is treated surgically with appropriate adjuvant therapies \(^{64}\). Morbus Paget is a special type of breast cancer, in which the malignant cells have spread from underlying DCIS or, more rarely, invasive carcinoma into the epidermis of the nipple or areola causing scaling, redness and finally ulceration \(^{67}\).

2.4.5.2  **Invasive carcinoma**

2.4.5.2.1  **Invasive ductal carcinoma NOS**

Invasive ductal carcinoma, not otherwise specified (NOS) is defined, according to the WHO classification, as a heterogeneous group of tumours that fail to exhibit sufficient characteristics to achieve classification as a specific histological type \(^{64}\). It is the most common histopathological subtype of breast cancer, accounting for between 68 to 85 % of all carcinomas \(^{68, 69}\). Macroscopically ductal carcinomas are often firm on palpation and they can have an irregular, stellate outline or nodular configuration \(^{64}\). Histologically ductal carcinoma can vary from well-differentiated cells forming tubule-like structures to highly atypical cells growing as solid sheets \(^{54, 64}\). Adjacent DCIS is often present \(^{64}\). The 5-year relative survival of the patients with ductal carcinoma type is 79 % \(^{68}\).

2.4.5.2.2  **Invasive lobular carcinoma**

Invasive lobular carcinoma constitutes 5-15 % of invasive carcinomas and the 5-year survival rate of the patients is 84 % \(^{64, 68}\), i.e. similar to that of ductal carcinoma. Invasive lobular carcinoma is composed of small non-cohesive cells individually dispersed or arranged in single-file linear pattern in a fibrous stroma \(^{64}\). Cells are often arranged in lines
around benign ducts in a concentric manner. Variant growth patterns of invasive lobular carcinoma have also been described. Macroscopically invasive lobular carcinomas are poorly delimited and difficult to define. Multifocal and bilateral tumours are more common than in ductal NOS carcinoma type \(^64\). Invasive lobular carcinoma metastasizes into bone, gastro-intestinal tract, uterus, meninges, ovary and serosas more often than ductal NOS carcinoma, which tend to metastasize more often to the lungs and liver \(^64\).

### 2.4.5.2.3 Other invasive carcinoma types

The survival rate of patients in other, histologically usually well differentiated subtypes which constitute approximately 10 % of breast cancers, is better compared to ductal and lobular carcinomas \(^68\)–\(^71\). The most important of the special histological subtypes are tubular, medullary and mucinous carcinoma, which have a 5-year survival rate of 95 % \(^68\). Histologically tubular carcinoma forms tubules with an open lumen surrounded by a single line of highly differentiated epithelial cells \(^64\). The histological appearance of medullary carcinoma is misleadingly aggressive with large sheets of poorly differentiated cells without any glandular structures, surrounded by abundant inflammatory cell infiltrate \(^64\). Mucinous carcinoma is characterized by clusters of small and uniform cells floating in mucus \(^64\). Some aggressive histological subtypes, such as micropapillary carcinoma, have been described \(^72\), \(^73\). The 5-year survival of inflammatory carcinoma, an aggressive form of breast cancer, is as low as 18 % \(^68\).

### 2.4.6 Histopathological grade

For invasive carcinomas, the histopathological grade (GR) is assessed according to the Scarff-Bloom-Richardson classification \(^64\), \(^74\). This scores the tumours by tubule formation (> 75 % of the tumour, 1 point; 10-75 % of the tumour, 2 points; < 10 % of the tumour, 3 points), mitotic activity (1-3 points, depends on the microscope field area) and nuclear pleomorphism (regular nuclei, nucleolus not visible, 1 point; moderate variation in nuclear size, enlarged nucleolus, 2 points; large nuclei with marked size variation, multiple nucleolus, 3 points) into three different categories, which are well differentiated (GR I, 3-5 points when totalled), moderately differentiated (GR II, 6-7 points) and poorly differentiated (GR III, 8-9 points). The best prognosis is in the grade I subgroup and the worst in the grade III subgroup \(^74\). Ten-year recurrence-free survival rate for GR I patients was 90 % versus 70 % in GR II-III subgroup \(^75\). Histological grade has been shown to
possess independent prognostic value in breast cancer. Histological grading may be biased by interobserver disagreement.

2.4.7 Hormone receptor (ER and PR) expression

Expression of oestrogen and progesterone receptors in the tumour tissue predicts a good response to hormonal therapy of breast cancer. Positive hormone receptor expression has been significantly related to better RFS and OS in several studies. In addition, hormone receptor expression predicts outcome of adjuvant endocrine therapy as well as response in the treatment of metastatic disease. To date, two different oestrogen receptors named ER-α and ER-β have been identified.

2.4.8 Blood and lymph vessel invasion

Peritumoural blood vessel invasion by malignant cells is related to disease spreading and has predicted shorter RFS and OS in the ANP patient subgroup. In the ANN patient group, it independently predicted shorter RFS and OS, especially if combined with angiogenic activity of the tumour. Invasion into lymph vessels at the peritumoural area is an accurate indicator of lymph node metastases, even in small carcinomas. Lymph vessel invasion has independently predicted shortened survival. Dermal lymphatic invasion is often related to inflammatory carcinoma but associates generally with a good prognosis when expressed alone.

2.5 Prognostic value of factors related to cell proliferation, DNA-ploidy and apoptosis

2.5.1 Mitotic indices

Evaluation of cell proliferation by counting the fraction of tumour cells undergoing mitosis has provided prognostic value in breast cancer. Several different scoring systems have been described including the mitotic figure count, the mitotic activity index (MAI), and the volume-corrected mitotic index (M/V). High mitotic activity has predicted the appearance of axillary lymph node metastases and independently predicted survival.
2.5.2 PCNA, S-phase fraction, DNA-ploidy

Proliferating cell nuclear antigen (PCNA), S-phase fraction (SPF) and DNA-ploidy are also parameters used to measure cell proliferation. In breast cancer, PCNA, SPF, and ploidy have all shown prognostic value in patient outcome. However, in some studies, only combining these parameters together has provided any prognostic information.

2.5.3 Ki-67

Recently, the most common method used to measure cell proliferation has been the assessment of the Ki-67 expression. Ki-67 is a 395 kDa sized nuclear protein, which is expressed in all active phases of the cell cycle (early G1, S, G2, and M phases) but not in quiescent cells (G0). Several studies have established the independent prognostic value of Ki-67 expression in breast cancer and it is currently used routinely in clinical practice. MIB-1 antibody towards Ki-67 can be assessed on paraffin-embedded samples making the test more practical.

2.5.4 Apoptosis

The balance between cell proliferation and programmed cell death, apoptosis, is essential for normal cellular regulation, and alterations in apoptotic activity may be used to evaluate the aggressiveness of the disease in addition to the cell proliferation rate. The proportion of cell undergoing apoptosis can be measured by counting the apoptotic cells or by assessing the expression of apoptosis related factors such as antiapoptotic bcl-2 and pro-apoptotic bax of the Bcl-2 family. A high proportion of apoptotic cells has been related to aggressive clinicopathological factors and unfavourable survival in breast cancer. Several studies have demonstrated an association between high bcl-2 expression and favourable clinicopathological factors as well as improved survival in breast cancer, which has been of independent prognostic value in some studies. Bax expression, instead, seems to have only limited prognostic value for breast cancer patients treated with chemotherapy. In some studies, however, apoptotic markers have not revealed any prognostic value.
2.6 Prognostic value of factors related to regulation of cell growth and differentiation

Uncontrolled cell proliferation due to defects in the cell-cycle progression regulating machinery, as well as imbalance between cell proliferation and cell death, are critical for development and progression of cancer\textsuperscript{119, 133}. Normally the progression of the cell cycle is strictly regulated by a large network of different factors\textsuperscript{133, 134}. When the cells receive sufficient growth factor stimulation during the first two-thirds of their G\textsubscript{1} phase, the cell cycle machinery is activated and the mitosis is completed (Fig. 1)\textsuperscript{133}. The decision of the cell cycle progression is made at the restriction point in late G\textsubscript{1}, which is the final possible time point to decide whether the cell will complete the remainder of the cell cycle, re-enter G\textsubscript{0} phase or differentiate\textsuperscript{133}. Increased expression of cell cycle promoting factors and inactivation of negative regulators or their reduction, are involved in carcinogenesis\textsuperscript{134}.

Figure 1. Schematic view of the cell cycle modified from Sandhu and Slingerland, 2000\textsuperscript{134}.

2.6.1 pRB

pRB is the protein product of the retinoblastoma tumour suppressor gene \textit{RB-1} and it is involved in negative regulation of cell cycle, induction of terminal differentiation and inhibition of apoptosis\textsuperscript{135, 136}. Inactivation of pRB or aberrant pRB pathway activity is common in carcinogenesis of human tumours\textsuperscript{137, 138}. Phosphorylation of pRB in the late G\textsubscript{1} phase of the cell cycle (Fig. 1) inactivates its growth suppression allowing the cells to
enter into the remainder of the cell cycle \(^{133, 135}\). Phosphorylation of pRB is also critical for cells to exit from \(G_0\) phase into the \(G_1\) phase \(^{139}\). In the late M phase, pRB is activated by dephosphorylation \(^{137}\). A mutation of the \(RB-1\) gene has been described in breast cancer \(^{140, 141}\) though other mechanisms as well are responsible for inactivation of pRB function \(^{137, 138}\). In breast cancer, abnormal pRB expression has not shown any prognostic value \(^{142-145}\).

### 2.6.2 p53

Inactivation of transcription factor p53 by a gene mutation or deletion is one of the most common events in human cancer \(^{146}\). Wild-type p53 functions as a tumour suppressor, and acts to maintain the cellular integrity \(^{147}\). In response to DNA damage, p53 induces a cell cycle arrest at \(G_1\)-S or the \(G_2\)-M boundary in proliferating cells to allow repair to proceed or if this is impossible, then it activates the apoptotic mechanisms of the cell \(^{148-152}\). These functions of p53 are mediated via transcriptional regulation of genes such as p21\(^{\text{WAF1}}\) \(^{151, 153}\), bel-2 and bax \(^{154}\).

Nuclear accumulation of p53 protein in tumour cells is usually a sign of an inactive protein, which is related to increased proliferation activity and higher genomic instability \(^{152}\). Overexpression of nuclear p53 has been a marker of aggressive disease in different cancers \(^{155-159}\). In breast cancer, abnormal nuclear p53 protein has been detected in 11-54 % of the tumours \(^{159}\). Overexpression of nuclear p53 has independently predicted shorter RFS and OS in most of the breast cancer studies where this has been examined though some non-significant results have also been described \(^{156}\). The cytoplasmic localization of p53 represents also inactivation of p53 tumour suppressor function \(^{160, 161}\) and has shown prognostic value in breast cancer alone and in combination with nuclear p53 expression \(^{162}\).

### 2.6.3 p21\(^{\text{WAF1}}\)

p21\(^{\text{WAF1}}\) is a member of the kinase inhibitor protein (KIP) family of three proteins, and its activity is related to the regulation of the cell cycle progression \(^{163}\). These proteins regulate cell cycle both negatively, via inhibition of cyclin D-CDK4/6, cyclin E-CDK2 and cyclin A-CDK2 complexes, and positively, via activation of cyclin D/CDK complexes (Fig. 1) \(^{163}\). Deregulation of p21\(^{\text{WAF1}}\) has been described in human cancers \(^{134}\).
The p21\textsuperscript{WAF1} protein has been identified as a mediator of p53 dependent growth inhibition \textsuperscript{152}. Several factors such as p53 and pRB induce cell cycle arrest at G\textsubscript{1}-S or G\textsubscript{2}-M transitions via upregulation of p21\textsuperscript{WAF1} expression \textsuperscript{148, 164-167}. During the cell cycle arrest, p21\textsuperscript{WAF1} interacts with the PCNA molecule allowing DNA repair \textsuperscript{168, 169}. p21\textsuperscript{WAF1} expression has also been related to terminal differentiation and cell senescence \textsuperscript{170, 171}, and recently, p21\textsuperscript{WAF1} has been documented to function as an anti-apoptotic factor as well as promoting cell proliferation \textsuperscript{172}. In breast cancer, the role and prognostic value of p21\textsuperscript{WAF1} is controversial \textsuperscript{173-177}. The recent p21\textsuperscript{WAF1} results in breast cancer are summarized in Table 4. In epithelial ovarian cancer, colorectal carcinoma, and gastric carcinoma low p21\textsuperscript{WAF1} expression has predicted decreased survival \textsuperscript{178-180}, whereas in head-and-neck squamous cell carcinoma (HNSCC), an opposite result has been obtained \textsuperscript{181}. In cutaneous malignant melanoma, p21\textsuperscript{WAF1} expression showed no prognostic value \textsuperscript{182}. Thus, the role of p21\textsuperscript{WAF1} in cancer seems to be very complex and controversial.

2.6.4 p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2}

p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2} are the two other members of the KIP family of cell cycle regulating proteins \textsuperscript{163}. Loss of p27\textsuperscript{KIP1} expression has been reported in different malignancies including breast cancer \textsuperscript{183, 184}. Expression has usually been related to aggressive clinicopathological parameters in breast cancer \textsuperscript{185, 186, 187} and low p27\textsuperscript{KIP1} expression has predicted poor survival \textsuperscript{183, 184}. However in some breast cancer studies, p27\textsuperscript{KIP1} has shown only little or no prognostic value \textsuperscript{185-187}.

To date, there are limited data concerning the p57\textsuperscript{KIP2} protein. p57\textsuperscript{KIP2} has been proposed to be a potential tumour suppressor gene \textsuperscript{188-190}. In some carcinomas, loss of p57 expression has been claimed to be an indicator of aggressive disease and decreased survival \textsuperscript{191-194}. Individuals carrying germline deletions in the gene encoding p57 have been proposed to have an increased breast cancer risk \textsuperscript{195}. To date, the expression and prognostic value of p57 in breast cancer has not been investigated.
Table 4. The recent literature on the role of $p21^{\text{WAF1}}$ in breast cancer.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>n</th>
<th>$p21^{\text{WAF1}}$ cut-off</th>
<th>High $p21^{\text{WAF1}}$ expression</th>
<th>$p21^{\text{WAF1}}$ association with p53</th>
<th>$p21^{\text{WAF1}}$ / high expression associations with clinicopathological factors</th>
<th>High $p21^{\text{WAF1}}$ expression and survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bankfálvi et al. 175</td>
<td>2000</td>
<td>120</td>
<td>10 %</td>
<td>30 %</td>
<td>+</td>
<td>NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Barbareschi et al. 174</td>
<td>1996</td>
<td>91</td>
<td>10 %</td>
<td>26 %</td>
<td>NS</td>
<td>GR III</td>
<td>RFS / ND</td>
</tr>
<tr>
<td>Bukholm et al. 196</td>
<td>1997</td>
<td>70</td>
<td>10 %</td>
<td>27 %</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Caffo et al. 197</td>
<td>1996</td>
<td>261</td>
<td>10 %</td>
<td>32 %</td>
<td>NS</td>
<td>size ↑, ANP, GR III, MAI ↑</td>
<td>RFS / NS</td>
</tr>
<tr>
<td>Ceccarelli et al. 198</td>
<td>2001</td>
<td>435</td>
<td>5 %</td>
<td>ND</td>
<td>ND</td>
<td>ANP</td>
<td>NS / ND</td>
</tr>
<tr>
<td>Diab et al. 195 a</td>
<td>1997</td>
<td>115</td>
<td>0 %</td>
<td>43 %</td>
<td>+</td>
<td>NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Domagala et al. 196</td>
<td>2001</td>
<td>222</td>
<td>0.1 %</td>
<td>30 %</td>
<td>NS</td>
<td>GR I, MIB ↓</td>
<td>ND / NS</td>
</tr>
<tr>
<td>Ellis et al. 199 b</td>
<td>1997</td>
<td>56</td>
<td>0 %</td>
<td>55 %</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Giannikaki et al. 200</td>
<td>1997</td>
<td>102</td>
<td>ND</td>
<td>37 %</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Göhring et al. 177</td>
<td>2001</td>
<td>307</td>
<td>IRS 2</td>
<td>32.2 %</td>
<td>NS</td>
<td>PCNA↑</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Jiang et al. 201</td>
<td>1997</td>
<td>106</td>
<td>25 %</td>
<td>31.9 %</td>
<td>NS</td>
<td>ANN, GR I</td>
<td>RFS / OS ↑</td>
</tr>
<tr>
<td>Koura et al. 202 a</td>
<td>2003</td>
<td>94</td>
<td>6 %</td>
<td>49 %</td>
<td>NS</td>
<td>NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Mathoulin-Poret et al. 203</td>
<td>2000</td>
<td>162</td>
<td>1 %</td>
<td>53 %</td>
<td>NS</td>
<td>Ki-67 ↑</td>
<td>NS / NS</td>
</tr>
<tr>
<td>McClelland et al. 204 b</td>
<td>1999</td>
<td>91</td>
<td>IRS 30</td>
<td>25.3 %</td>
<td>-</td>
<td>NS</td>
<td>ND / OS ↓</td>
</tr>
<tr>
<td>Michels et al. 205</td>
<td>2003</td>
<td>104</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
<td>NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>O'Hanlon et al. 206 c</td>
<td>2002</td>
<td>105</td>
<td>10 %</td>
<td>65 %</td>
<td>NS</td>
<td>NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Pellikainen et al. 207</td>
<td>2003</td>
<td>420</td>
<td>5 %</td>
<td>38 %</td>
<td>+</td>
<td>GR III, NP ↑</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Reed et al. 208 a</td>
<td>1999</td>
<td>77</td>
<td>0 %</td>
<td>68 %</td>
<td>NS</td>
<td>ER ↑</td>
<td>ND</td>
</tr>
<tr>
<td>Rey et al. 209</td>
<td>1998</td>
<td>77</td>
<td>5 %</td>
<td>57 %</td>
<td>NS</td>
<td>GR II-III, ductal, TF ↑, SPF ↑</td>
<td>ND</td>
</tr>
<tr>
<td>Sjöström et al. 210 b</td>
<td>2000</td>
<td>134</td>
<td>10 %</td>
<td>49 %</td>
<td>NS</td>
<td>NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Thor et al. 211</td>
<td>2000</td>
<td>798</td>
<td>0 %</td>
<td>91 %</td>
<td>-</td>
<td>MIB ↑, ER ↑</td>
<td>NS / OS ↓</td>
</tr>
<tr>
<td>Wakesugi et al. 212</td>
<td>1997</td>
<td>104</td>
<td>10 %</td>
<td>49 %</td>
<td>NS</td>
<td>NS</td>
<td>RFS / ND</td>
</tr>
<tr>
<td>Winters et al. 213</td>
<td>2001</td>
<td>73</td>
<td>IRS 6.95</td>
<td>ND</td>
<td>+</td>
<td>GR I-II, ANN, ER +</td>
<td>RFS / ND</td>
</tr>
<tr>
<td>Winters et al. 214</td>
<td>2003</td>
<td>73</td>
<td>IRS 6.95</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
<td>RFS / OS ↓</td>
</tr>
<tr>
<td>Xia et al. 215</td>
<td>2004</td>
<td>130</td>
<td>ND</td>
<td>26 %, 33 % d</td>
<td>ND</td>
<td>NS</td>
<td>ND / OS ↓</td>
</tr>
<tr>
<td>Yang et al. 216</td>
<td>2003</td>
<td>107</td>
<td>0 %</td>
<td>64.5 %</td>
<td>ND</td>
<td>HER2+</td>
<td>RFS / ND</td>
</tr>
</tbody>
</table>

Abbreviations used in the table are: + = positive association; - = negative association; size = tumour size; ductal = ductal carcinoma type.

Footnotes: a) ANN patients, b) Advanced carcinoma, c) ANP patients d) 26 % for nuclear, 33 % for cytoplasmic
2.6.5 AP-2

AP-2 is a DNA-binding transcription factor \(^{217}\), which forms a protein family of highly homologous members \(^{218-223}\). The three first characterized AP-2 proteins namely AP-2α, AP-2β, and AP-2γ \(^{218}\) are, to date, the best known members. AP-2 exhibits both activating and repressing properties on target genes, which contain GC-rich DNA sequences as binding sites for the homo- or heterodimerized AP-2 protein complex \(^{217, 218, 224-226}\). AP-2 proteins are cell-type specific transcription factors \(^{227}\), which show different spatial and temporal expression patterns during embryonic development \(^{228, 229}\). Despite their structural similarities AP-2 proteins appear to have quite separate functions \(^{218}\) and, indeed, AP-2α has been proposed to be tumour suppressive, whereas AP-2γ seems more likely to induce cell proliferation \(^{230-232}\). However, the regulatory effect of the AP-2 protein on transcription is modified by several mechanisms including coactivators such as PARP, repressors, PKA mediated phosphorylation, and the balance between different transcription factors \(^{231, 233-235}\). AP-2 transactivation potential is also decreased by sumolation \(^{236}\) and loss of AP-2α related to hypermethylation has been described in breast cancer \(^{237}\). AP-2 mediates transcriptional activation in response to protein kinase C and cAMP \(^{238}\), independent of AP-2 mRNA or protein synthesis \(^{239}\).

AP-2 proteins play an important role in the regulation of gene expression during development, apoptosis, cell growth and differentiation \(^{240}\), and their aberrant function has been related to growth defects \(^{241, 242}\) as well as to malignant transformation \(^{243, 244}\). Indeed, several studies have demonstrated an increase in malignancy parallel to a loss of AP-2 suggesting a tumour suppressive function for the protein \(^{245, 246}\). This growth inhibitory effect may be mediated, at least partly, via upregulation of p21\(^{\text{WAF1}}\) \(^{247}\) or by direct interaction with p53 \(^{248}\). In addition, AP-2 is involved in the transcriptional upregulation of genes essential for normal growth of benign breast epithelium, as well as for carcinogenesis, such as ER-α \(^{249, 250}\), MMP-2 \(^{251}\), VEGF \(^{252}\), HER2 \(^{218, 223, 253}\), and IGF-1 \(^{244}\) (Fig. 2). On the other hand, AP-2 expression is also capable of suppressing MMP-2 activity \(^{252}\) and VEGF transcription \(^{231}\) indicating a more complex regulation pattern. AP-2 is required for pRB induced activation of antiapoptotic bcl-2 gene \(^{256}\) (Fig. 2), which promotes morphogenesis in epithelial breast cells \(^{257}\), as well as for pRB and c-Myc induced activation of E-cadherin promoter in epithelial cells \(^{258}\). A physical interaction between AP-2 and pRB has been described both in vitro and in vivo \(^{258}\). Another study
reported that AP-2 is a negative regulator of transactivation by c-Myc\textsuperscript{224}, and is able to inhibit c-Myc induced cell-cycle progression and apoptosis\textsuperscript{240} (Fig. 2). In addition, AP-2\textalpha can be cleaved by caspases during TNF-\alpha-induced apoptosis impairing its DNA-binding activity\textsuperscript{259}. Thus in breast tissue, overexpression of AP-2\textalpha has been shown to inhibit growth and morphogenesis\textsuperscript{232}.

**Figure 2.** Schematic representation of some of the pathways related to growth regulation by AP-2.

To date, the expression and prognostic value of AP-2 has been investigated in some malignancies\textsuperscript{246, 260, 261}. However, in breast carcinoma only a few preliminary studies using small clinical breast cancer series (n = 81 and n = 86) have been published\textsuperscript{245, 262}. Turner et al. (1998) demonstrated nuclear expression of AP-2\textalpha and AP-2\textgamma in breast tissue \textit{in vivo} and upregulation of AP-2\textgamma in carcinomas supporting their role in the control of cell growth and differentiation\textsuperscript{262}. Gee et al. (1999) reported a loss of nuclear AP-2 expression during disease progression from benign breast to carcinoma using an antibody specific for both AP-2\textalpha and AP-2\textbeta, pointing to a tumour suppressive role for AP-2\textsuperscript{245}. In both studies, AP-2 expression associated with ER-positivity but did not show any statistically significant relationship to survival\textsuperscript{245, 262}. 
2.6.6  *EGFR*-family

Human epidermal growth factor receptor (EGFR) family of subclass I receptor tyrosine kinases consists of four members namely EGFR (ErbB-1) \(^{263}\), HER2 (ErbB2) \(^{264-266}\), HER3 (ErbB3) \(^{267, 268}\), and HER4 (ErbB4) \(^{269}\). These receptors are transmembrane glycoproteins with an intracellular tyrosine kinase activity \(^{270}\). The isolated receptor itself is inactive but upon ligand stimulation the receptors form active homo- or heterodimers, which are involved in various cell regulation processes including cell proliferation, survival, migration and differentiation \(^{270, 271}\). To date several ligands such as EGF-like molecules and TGF-\(\alpha\) have been documented \(^{270}\). HER2 has no known ligand but it is an important mediator of lateral signalling in co-operation with other EGFR members \(^{272}\). Indeed, HER2 potentiates the signalling by evading inactivation processes \(^{273}\) and is the favoured dimerization partner \(^{272}\).

Because of their central role in the regulation of cellular processes, aberrant functions of EGFR family members are often associated with malignancy \(^{271}\). Especially, breast cancer development and progression are often related to the deregulation of EGFR family members \(^{274}\). To date, the prognostic role of EGFR and HER2 in breast cancer has been intensively studied. Overexpression of EGFR has been reported in 14-56 % of breast carcinomas \(^{275}\), and it has been associated with aggressive disease and reduced survival \(^{276-278}\). However, in some investigations the prognostic value of EGFR has been limited \(^{279, 280}\). Overexpression of EGFR has also been related to failure of endocrine therapy \(^{281, 282}\), as well as to successful response to endocrine therapy \(^{275}\).

Pathological membranous overexpression of the HER2 receptor is present in 10-34 % of breast cancers \(^{283}\), and this is related to aggressive clinicopathological parameters as well as to resistance to adjuvant therapy, and it independently predicts reduced survival \(^6, 283\). Recently, HER2 has become a target of monoclonal antibody therapy (Herceptin®, trastuzumab) \(^{284}\). The mechanisms by which overexpression of HER2 increases malignancy include enhancement of several properties such as angiogenesis \(^{285, 286}\), invasion \(^{287, 289}\), and anchorage-independent growth \(^{290}\). The pathological HER2 overexpression is related to HER2 gene amplification and increased transcriptional activity \(^{291-293}\). Members of two transcription factor families, AP-2 and Ets, have shown increased binding to the HER2 promoter in HER2 overexpressing cells \(^{218, 292}\). Indeed,
AP-2 is required to achieve maximal HER2 transcriptional activity. The results of the studies concerning the relationship between HER2 and AP-2 in clinical breast cancer series have been controversial. Turner et al. (1998) showed a positive association between AP-2α and HER2 expression, which was even stronger when investigated in relation to expressions of both AP-2α and AP-2γ, whereas Gee et al. (1999), found an inverse relationship between AP-2 and HER2 expression in HER2-positive tumours using AP-2 α/β antibody.

There is much less known about the two other members of the EGFR family, HER3 and HER4, in breast cancer. Both HER3 and HER4 are expressed in benign and malignant breast tissues. Overexpression of HER3 has been reported in 13-35% of breast tumours. The relation of HER3 to clinicopathological data has been controversial and HER3 expression has usually lacked prognostic value. However, in the study of Pawlowski et al. (2000), positive HER3 expression predicted a better overall survival. Different expression patterns of HER4 have been described in the literature, and the results concerning the prognostic value of HER4 in breast cancer have been controversial. Indeed, two studies have associated high HER4 expression with decreased survival but one found an association with increased survival. Accordingly, the relationship of HER3 and HER4 to breast cancer prognosis remains to be investigated.

2.7 Prognostic value of factors related to cell-matrix interactions, invasion and angiogenesis

Interactions between stromal and epithelial cells are essential for normal growth and function of the epithelial cells including development, differentiation, and cell proliferation. These cell-matrix interactions play also a central role in carcinogenesis. If malignant cells wish to invade surrounding tissue, they need a capacity to pass through biological barriers such as the basement membrane, and have to be able to unleash unlimited growth potential independent of the cellular environment. In addition, the growth and spread of tumour cells is enhanced by alterations in the extracellular environment, which include induction of angiogenesis and modulation of the extracellular matrix (ECM) by protein degradation.
2.7.1 Matrix metalloproteinases

The family of matrix metalloproteinases comprises of more than 25 enzymes which possess the ability to degrade the components of the ECM and the basement membrane including type IV collagen, laminin, entactin, proteoglycans, and glycosaminoglycans. Based on their substrate specificity and domain structure, the members of the MMP family have been divided into four subgroups: collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMP). MMPs play an important role in carcinogenesis by creating a microenvironment that facilitates tumour growth and invasion (Fig. 3). This includes regulation of cell growth and apoptosis, induction of angiogenesis, and degradation of biological barriers such as the basement membrane.

Figure 3. Steps involving MMPs in tumour progression (arrows), modified from Nelson et al., 2000.

![Figure 3](image)

The gelatinase subgroup of MMPs is of particular interest because of its ability to degrade the components of the basement membrane and thus facilitate invasion as well as metastasis. The gelatinase subgroup comprises of two enzymes, namely MMP-2 and MMP-9. Different gelatinase expression patterns have been described in the literature though it is increasingly believed that gelatinase transcription takes place mainly in stromal cells, whereas its storage and activation occurs in carcinoma cells. These enzymes are secreted as inactive zymogens, which following the appropriate stimulus, are cleaved into their active forms, a process regulated by several mechanisms including tissue inhibitors of metalloproteinases (TIMPs) and MT1-MMP.
Other signalling pathways, including those related to AP-2 and HER2 participate in the regulation of gelatinase expression and activity. Indeed, MMP-2 promoter has AP-2 binding sites \(^{326, 327}\), and AP-2 is required for MMP-2 transcription \(^{321, 328}\). In addition, AP-2α has been shown to participate in transcriptional activation of MMP-9 \(^{329}\). In SB-2 melanoma cells, inactivation of wild type AP-2 by a dominant negative AP-2 displayed an increase in MMP-2 expression, microvessel density, angiogenesis, and invasiveness \textit{in vivo} \(^{255}\). AP-2 is also required for the regulation of MMP activity controlling factors such as TIMPs \(^{251, 330}\), which may be reflected in the gelatinase function. Thus, AP-2 may play an essential role in the regulation of gelatinase expression.

Overexpression of HER2 enhances carcinogenesis and is related to increased cell migration \(^{288}\), increased invasive capacity and metastatic potential of the cells \(^{287, 289}\). These properties are closely associated with an increase in gelatinase expression and activity, which is detected in HER2 overexpressing cells \(^{289, 331, 332}\). In addition, several EGF-like ligands are known to upregulate gelatinase expression \(^{333, 334}\). Therefore, the aggressive malignant behaviour seen in HER2 overexpressing breast cancers may be related to increased gelatinase activity induced by deregulated EGFR signalling pathways \(^{335}\).

Although the expression of gelatinases has been intensively studied in breast cancer, no consensus has been reached \(^{336-339}\). Furthermore, the prognostic value of the gelatinases in breast cancer is controversial. Recent papers concerning the prognostic value of gelatinases in breast cancer are summarized in Table 5. To date, few studies have investigated the gelatinases using large clinical breast cancer series \(^{339-342}\). Tetu et al. (1998) investigated MMP-2 expression using \textit{in situ} hybridization in a series of 575 breast cancer patients and did not find any relation to survival \(^{342}\). Talvensaari-Mattila et al. (1998 and 2003) reported decreased survival in an MMP-2 positive patient group in two large breast cancer series using immunohistochemistry \(^{339, 341}\). MMP-9 expression has been studied only in one large breast cancer series of 210 patients, in which immunohistochemically detected high MMP-9 expression in carcinoma cells predicted better survival in the ANN group \(^{340}\).
Table 5. Recent publications concerning the expression and prognostic value of MMP-2 and MMP-9 in breast cancer detected by immunohistochemistry or *in situ* hybridization.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>N</th>
<th>Cut-off</th>
<th>MMP expression in stromal cells</th>
<th>MMP expression in cancer cells</th>
<th>Relation of high MMP expression to clinico-pathological data</th>
<th>Survival MMPs/high expression, (univariate) RFS / OS</th>
<th>Survival MMPs/high expression, (multivariate) RFS / OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidone et al.</td>
<td>1991</td>
<td>187</td>
<td>60 %</td>
<td>ND</td>
<td>cytoplasmic, cell surface</td>
<td>NS</td>
<td>NS / NS</td>
<td>ND</td>
</tr>
<tr>
<td>Hirvonen et al.</td>
<td>2003</td>
<td>137</td>
<td>1 % &amp; 50 %</td>
<td>ND</td>
<td>cytoplasmic</td>
<td>NS</td>
<td>NS / NS</td>
<td>ND</td>
</tr>
<tr>
<td>Nakopoulou et al.</td>
<td>2003</td>
<td>135</td>
<td>10 % &amp; 30 % cancer, 10 % stroma</td>
<td>27.4 % +</td>
<td>cytoplasmic 83 % +, 53 % ++</td>
<td>cancer: size ↑, stroma: Ki67↓, TIMP ↑ stage III-IV</td>
<td>NS / NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Talvensaari-Mattila et al.</td>
<td>1998</td>
<td>177</td>
<td>1 % &amp; 50 % occasionally</td>
<td>cytoplasmic 84 % +, 22 % ++</td>
<td>ND</td>
<td>ND</td>
<td>ND / OS ↓</td>
<td>ND / OS ↓</td>
</tr>
<tr>
<td>Talvensaari-Mattila et al.</td>
<td>2001</td>
<td>100</td>
<td>1 % &amp; 50 %</td>
<td>ND</td>
<td>cytoplasmic 69 % +, 36 % ++</td>
<td>hematogenous metastasis ↑</td>
<td>RFS ↓ / ND</td>
<td>ND</td>
</tr>
<tr>
<td>Talvensaari-Mattila et al.</td>
<td>1999</td>
<td>108</td>
<td>1 % &amp; 50 %</td>
<td>ND</td>
<td>cytoplasmic 76 % +, 48 % ++</td>
<td>ND</td>
<td>RFS ↓ / OS ↓</td>
<td>ND</td>
</tr>
<tr>
<td>Talvensaari-Mattila et al.</td>
<td>2003</td>
<td>453</td>
<td>1 % &amp; 50 %</td>
<td>ND</td>
<td>cytoplasmic 78 % +, 50 % ++ none</td>
<td>NS</td>
<td>RFS ↓ / OS ↓</td>
<td>ND / OS ↓</td>
</tr>
<tr>
<td>Tetu et al.</td>
<td>1998</td>
<td>575</td>
<td>10 % Score, 48th percentile</td>
<td>77.7 % + cytoplasmic in inflammatory and vascular cells</td>
<td>cytoplasmic 58 % +, occasionally membranous</td>
<td>HER2 +, ductal size ↓, non-ductal, PR +, stage I-II, age &gt; 55 years</td>
<td>NS / NS</td>
<td>NS / NS</td>
</tr>
<tr>
<td>Scorilas et al.</td>
<td>2001</td>
<td>210</td>
<td></td>
<td>77.7 % + cytoplasmic in inflammatory and vascular cells</td>
<td>cytoplasmic 58 % +, occasionally membranous</td>
<td>HER2 +, ductal size ↓, non-ductal, PR +, stage I-II, age &gt; 55 years</td>
<td>NS / NS</td>
<td>NS / NS</td>
</tr>
</tbody>
</table>

a) ANN patients, b) Postmenopausal, ANP patients, c) Premenopausal, ANP patients
2.7.2 **Tissue inhibitors of metalloproteinases (TIMP)**

Tissue inhibitors of metalloproteinases (TIMPs) form the major subgroup of MMP inhibitors. To date, four TIMPs (TIMPs 1-4) have been characterized, showing varying efficacy against all different MMPs with different expression and regulatory patterns. The MMP activity is inhibited by the formation of high-affinity 1:1 stoichiometric, noncovalent complexes between TIMPs and active MMPs. In addition, TIMP-2 is necessary for activation of pro-MMP-2 at the cell membrane, and furthermore, TIMP-2, -3 and -4 can bind to pro-MMP-2, whereas TIMP-1 and -3 are capable of binding pro-MMP-9. Interestingly, TIMPs have both stimulating and inhibiting effects on cell proliferation, angiogenesis, apoptosis, and cancer progression. Overexpression of TIMP-1, -2, and -3 has been shown to reduce tumour cell growth, whereas in malignancies, TIMP-2 has been shown to predict poor outcome, which is also the case for TIMP-1 and TIMP-2 in most breast cancer studies. In some studies, TIMP-1 and -2 positivities have shown a favourable effect on survival in breast cancer, whereas TIMP-3 expression has been related to better response to adjuvant therapy.

2.7.3 **Other factors related to cell-matrix interactions**

2.7.3.1 **Hyaluronan (HA)**

Hyaluronan (HA) is a one of the main components of the extracellular matrix and is classified as a glycosaminoglycan. HA is expressed in connective, epithelial and neural tissues, where it is synthesized at cell membranes by hyaluronan synthase (HAS), and it is actively involved in tissue remodelling during morphogenesis and wound healing. HA is capable of inducing cell proliferation and migration, as well as increasing neovascularization and metastatic properties. These functions inevitably link HA with the development and progression in pathological conditions and, accordingly, elevated HA levels have been observed in different malignancies. In breast cancer, high HA expression both in peritumoural stroma and in cancer cells has been associated with adverse clinicopathological factors including poor differentiation and axillary lymph node positivity. High HA expression has also been shown independently to predict poor OS in breast cancer. Similarly, in other carcinomas, high HA expression has been related to unfavourable outcome of the disease.
2.7.3.2 CD44
The major cell surface hyaluronan receptor, CD44 is a transmembrane glycoprotein which forms a large family of receptor isoforms. Similarly to HA, CD44 is also involved in the regulation of tumour promoting events. In breast cancer cells, CD44 variants have increased the cell motility, invasiveness, angiogenesis, and reduced HA mediated cell adhesion in vitro. CD44 shedding underpins the anti-invasive and antiangiogenic properties of breast myoepithelial cells. Carcinomas expressing high levels of CD44 seem to be more malignant than those with a low expression level. CD44 can also bind to tyrosine kinases HER2 and c-Src further increasing malignancy.

The function of CD44 is closely related to MMPs and in breast cancer cells, an association between CD44 and activated MMP-9 on the plasma membrane has been detected in the "invadopodia" of the tumour cells potentially increasing cancer progression. In clinical breast cancer series, high CD44 expression has been shown to be adversely associated with outcome, though opposite and nonsignificant results have been reported.

2.7.3.3 Tenascin and versican
Tenascin and versican are large extracellular matrix components, which have been shown to possess anti-cell-adhesive properties. Tumour associated versican and tenasin secretion by both stromal and epithelial cells has been reported. Tenascin exists as multiple isoforms which possess different effects. Tenascin and versican bind specifically to each other as well as to other extracellular matrix components. Both molecules are related to increased cell motility and tenasin-C can also induce cell proliferation, as well as angiogenesis and expression of MMPs. Tenasin-C expression has been shown to increase in the breast stroma around pre-invasive and invasive lesions. Similarly, high stromal versican expression has been reported to localize at the most invasive tumour areas in breast tissue. In some studies, stromal tenasin and versican expressions have provided prognostic value in breast cancer, with high expression being a marker of poor survival.

2.7.4 Angiogenesis
Angiogenesis i.e. formation of new blood vessels is essential for tumour cell growth and metastasis. A solid tumour cannot grow larger than 1 mm³ in the absence of
neovascularization. In addition, neovascularization serves as a route for highly invasive cells to disseminate into distant organs, in which the cells again need to establish their own vascular network to grow. The growth stimulating effect of angiogenesis is not only due to increased blood supply but also to increased secretion of growth promoting factors related to induction of neovascularization and extracellular matrix remodelling. To date, several factors secreted by tumour, endothelial, inflammatory and stromal cells have been identified as being involved in the positive regulation of angiogenesis. These include vascular endothelial growth factor (VEGF), its receptors VEGFRs, fibroblast growth factor (FGF), TNF-α, MMPs, TIMPs, HA and CD44. TIMPs may have also a negative effect on angiogenesis. The angiogenic activity can be measured by evaluating the intratumoural microvessel density (IMD or MVD) or the expression of angiogenesis stimulating molecules. A high angiogenic activity has been associated with decreased survival in breast cancer as well as in other malignancies.
3. AIMS OF THE STUDY

The general aim of the present study was to evaluate the value of new molecular markers in association with the traditional clinicopathological factors with respect to the clinical outcome of breast cancer patients. In addition, the purpose of this study was to enlarge the current knowledge of breast cancer biology, with a special focus on molecular biology related to disease progression. The specific aims of the present study were:

I. To investigate the expression and prognostic value of transcription factor AP-2 in breast cancer.

II. To evaluate the expression and prognostic value of tumour suppressor p21\(^{\text{WAF1}}\) in breast cancer, and its relation to cell proliferation measured by Ki-67, as well as the role of AP-2 and p53 in the regulation of p21\(^{\text{WAF1}}\) expression.

III. To investigate the expression of HER2 in the present breast cancer series, the relation of HER2 overexpression to AP-2 and HER2 gene amplification as well as the prognostic value of HER2.

IV. To analyze the expression and prognostic value of MMP-2 and MMP-9 in breast cancer as well as their association with AP-2 and HER2 expression.
4. MATERIAL AND METHODS

4.1 Patients

The present study is part of the Kuopio Breast Cancer Project, a prospective long term clinical study involving 520 breast cancer patients diagnosed among the 2500 women who were referred to Kuopio University Hospital because of a clinical breast lump, suspicious mammographic finding, or a breast symptom (e.g. pain, nipple discharge) between April 1990 and December 1995. Women willing to participate in the project were interviewed and examined by a trained study nurse before any diagnostic procedures. The participation rate of the patients with diagnosed breast cancer was 98%. Thus, the patient series represents unselected typical breast cancer cases from the University Hospital catchment area. Altogether, 479 invasive and 41 non-invasive carcinomas were diagnosed. The stage was assessed according to the UICC classification. Patients were routinely followed-up according to the study protocol until five years or death. None of the patients were lost to follow-up. Patients with non-invasive carcinomas (n = 41), metastatic disease at diagnosis (n = 19), presence of distant metastases not assessed (TNM classification MX, n = 3), or insufficient tumour material (n = 32-37) were excluded from the analyses. Thus, altogether 420-425 patients were left for analyses (Table 6).

4.2 Treatment

Patients were treated with radical surgery, usually with modified mastectomy of the breast or with local tumour resection, most often wide resection or quadrantectomy in combination with axillary lymph node evacuation when appropriate, depending on the stage of the disease. Following surgical treatment, the patients were offered adjuvant chemo- and/or hormonal therapy and radiotherapy depending on the mode of the surgery, the patient’s menopausal status and the stage of the disease, according to the national guidelines. In brief, postoperative radiotherapy was given to all patients treated with breast conserving surgery and to all ANP patients irrespective of the mode of surgery. All premenopausal patients with ANP and some with ANN status presenting with other adverse prognostic factors, such as oestrogen/progesterone receptor negative or poorly differentiated tumours, were given adjuvant chemotherapy (i.e. Cyclophosphamide-Mitoxanthrone-Methotrexate-5-Fluorouracil) for six cycles, which was the standard therapy at the time. All postmenopausal women with ER and/or PR positive tumours were
Table 6. Clinicopathological data of the patients in each publication (I-IV).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Number of patients</td>
<td>420</td>
<td>100</td>
<td>420</td>
<td>100</td>
</tr>
<tr>
<td>Follow-up time, months</td>
<td>55.0</td>
<td>54.9</td>
<td>54.8</td>
<td>55.0</td>
</tr>
<tr>
<td>Mean (Range)</td>
<td>(1.2 – 115.1)</td>
<td>(1.2 – 115.1)</td>
<td>(1.2 – 115.1)</td>
<td>(1.2 – 115.1)</td>
</tr>
<tr>
<td>Age, years</td>
<td>59.1</td>
<td>59.0</td>
<td>59.1</td>
<td>59.2</td>
</tr>
<tr>
<td>Mean (Range)</td>
<td>(23.3-91.6)</td>
<td>(23.3-91.6)</td>
<td>(23.3-91.6)</td>
<td>(23.3-91.6)</td>
</tr>
<tr>
<td>Tumour size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>218</td>
<td>52</td>
<td>221</td>
<td>53</td>
</tr>
<tr>
<td>T2</td>
<td>168</td>
<td>40</td>
<td>166</td>
<td>39</td>
</tr>
<tr>
<td>T3</td>
<td>24</td>
<td>6</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>T4</td>
<td>10</td>
<td>2</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>244</td>
<td>58</td>
<td>247</td>
<td>59</td>
</tr>
<tr>
<td>Positive</td>
<td>168</td>
<td>40</td>
<td>166</td>
<td>39</td>
</tr>
<tr>
<td>Unknown</td>
<td>8</td>
<td>2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>160</td>
<td>38</td>
<td>163</td>
<td>39</td>
</tr>
<tr>
<td>II</td>
<td>213</td>
<td>51</td>
<td>212</td>
<td>50</td>
</tr>
<tr>
<td>III</td>
<td>39</td>
<td>9</td>
<td>38</td>
<td>9</td>
</tr>
<tr>
<td>Unknown</td>
<td>8</td>
<td>2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>270</td>
<td>64</td>
<td>273</td>
<td>65</td>
</tr>
<tr>
<td>Lobular</td>
<td>68</td>
<td>16</td>
<td>65</td>
<td>15</td>
</tr>
<tr>
<td>Other</td>
<td>82</td>
<td>20</td>
<td>82</td>
<td>20</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>110</td>
<td>26</td>
<td>108</td>
<td>26</td>
</tr>
<tr>
<td>II</td>
<td>192</td>
<td>46</td>
<td>192</td>
<td>46</td>
</tr>
<tr>
<td>III</td>
<td>118</td>
<td>28</td>
<td>120</td>
<td>28</td>
</tr>
<tr>
<td>ER-status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>326</td>
<td>78</td>
<td>323</td>
<td>77</td>
</tr>
<tr>
<td>Negative</td>
<td>93</td>
<td>22</td>
<td>95</td>
<td>22</td>
</tr>
<tr>
<td>No data</td>
<td>1</td>
<td>&lt;1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>PR-status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>261</td>
<td>62</td>
<td>261</td>
<td>62</td>
</tr>
<tr>
<td>Negative</td>
<td>157</td>
<td>37</td>
<td>157</td>
<td>37</td>
</tr>
<tr>
<td>No data</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-</td>
<td>131</td>
<td>31</td>
<td>132</td>
<td>31</td>
</tr>
<tr>
<td>Post-</td>
<td>289</td>
<td>69</td>
<td>288</td>
<td>69</td>
</tr>
<tr>
<td>Recurrence at 5 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>344</td>
<td>82</td>
<td>344</td>
<td>82</td>
</tr>
<tr>
<td>Yes</td>
<td>76</td>
<td>18</td>
<td>76</td>
<td>18</td>
</tr>
<tr>
<td>Cause of death at 5 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>333</td>
<td>79</td>
<td>334</td>
<td>79</td>
</tr>
<tr>
<td>Breast ca</td>
<td>50</td>
<td>12</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td>Other</td>
<td>37</td>
<td>9</td>
<td>36</td>
<td>9</td>
</tr>
</tbody>
</table>
adjuvantly treated with either tamoxifen or toremifene for three years within another study protocol. Thus, within a stage, the postoperative treatment was uniform, with only a few exceptions.

4.3 Histology
The tumour samples were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin. The histological diagnosis was confirmed by reviewing 1 to 4 original sections of the primary tumour. All tumours were simultaneously re-evaluated for histological type and grade by two senior pathologists, who were unaware of the clinical data. The most representative blocks were selected for cutting into new 5-μm thick sections for immunohistochemical and chromogenic in situ hybridization analyses.

4.4 Immunohistochemical staining of AP-2, p21<sup>WAF1</sup>, and p53 (1-11)
The immunohistochemical staining proceeded according to the following protocol, which was modified depending on the antibody in use (Table 7). In brief, the sections were deparaffinized in xylene, rehydrated in ethanol and washed twice with distilled water. To achieve better antigen retrieval, the samples were boiled in a microwave oven in a citrate buffer (pH 6.0). Endogenous peroxidases were blocked by 5% hydrogen peroxidase treatment for 5 min. The samples were washed with phosphate-buffered saline (PBS, pH 7.2) and incubated in 1.5% normal serum to prevent non-specific antigen binding. The samples were incubated with the primary antibody overnight at 4°C.

Before applying the secondary antibody, the samples were washed twice with PBS. The slides were incubated for 35 min with the biotinylated secondary antibody, followed by a wash and 45 min incubation in an avidin-biotinylated peroxidase complex reagent (Vectastain Rabbit ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Expressions were visualized with a 5 min diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) treatment. The slides were counterstained with Mayer’s hematoxylin, dehydrated and mounted with DePex (BDH Ltd, Poole, UK). A routinely processed breast cancer section without the primary antibody served as a negative control in each staining series. The positive control for AP-2 was melanoma. In addition, strongly stained inflammatory cells served as internal controls in the breast tumour samples. Known p21<sup>WAF1</sup> positive colorectal carcinoma and p53 positive larynx carcinoma samples
were used as positive controls for p21\textsuperscript{WAF1}, and p53, respectively. Control samples stained as expected.

Table 7. Summary of the antibodies used in the studies.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Study</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Specificity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-2</td>
<td>I-IV</td>
<td>C-18</td>
<td>1:2000</td>
<td>Polyclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>p21\textsuperscript{WAF1}</td>
<td>II</td>
<td>NCL-WAF-1, clone 4D10</td>
<td>1:20</td>
<td>Monoclonal</td>
<td>Novocastra Laboratories Ltd.</td>
</tr>
<tr>
<td>p53</td>
<td>II</td>
<td>DO-7</td>
<td>1:1000</td>
<td>Monoclonal</td>
<td>DAKO</td>
</tr>
<tr>
<td>Ki-67</td>
<td>II, III</td>
<td>MIB-1</td>
<td>1:600</td>
<td>Monoclonal</td>
<td>Immunotech</td>
</tr>
<tr>
<td>HER2</td>
<td>III, IV</td>
<td>NCL-CB11</td>
<td>1:100</td>
<td>Monoclonal</td>
<td>Novocastra Laboratories Ltd.</td>
</tr>
<tr>
<td>MMP-2</td>
<td>IV</td>
<td>Ab-1, clone CA-4001</td>
<td>1:25</td>
<td>Monoclonal</td>
<td>Neomarkers</td>
</tr>
<tr>
<td>MMP-9</td>
<td>IV</td>
<td>MAB3309</td>
<td>1:2500</td>
<td>Monoclonal</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>

4.5 Immunohistochemical staining of Ki-67, MMP-2, and MMP-9 (II, IV)
Ki-67, MMP-2 and MMP-9 staining was demonstrated using Sequenza\textsuperscript{TM} Immunostaining Center (Shandon Scientific Limited, Astmoor, UK). Therefore, the basic staining procedure was as described in the previous chapter except that the incubation periods were longer: 35 min for normal serum, 40 min (Ki-67) and 45 min (MMPs) for secondary antibody, and 50 min for ABC-reagent. Positive control was lung carcinoma specimen for Ki-67, and placenta as well as breast carcinoma for MMP-2 and MMP-9.

4.6 Immunohistochemical staining of HER2 (III)
The HER2 immunohistochemistry was performed according to a routine staining method by using the TechMate 500-staining automat (DAKO, Golstrup, Denmark) in Kuopio University Hospital. The samples were processed as described previously. Normal serum treatment was not performed, and the samples were incubated in the primary antibody at room temperature. HER2 expression was visualized by the labeled streptavidin-biotin system (LSAB, ChemMate\textsuperscript{TM} Detection Kit, DAKO), which included a biotinylated secondary antibody, streptavidin peroxidase (HRP), diaminobenzidine solution (DAB), and HRP substrate buffer. LSAB detection kit was used according to the instructions of
the manufacturer. The slides were counterstained with Mayer’s hematoxylin, dehydrated and mounted with Pertex (Histolab Products AB, Göteborg, Sweden). A HER2 positive breast carcinoma specimen with known amplification served as a positive control.

4.7 Western blot (I)

During surgery, fresh breast cancer specimens were obtained from some of the patients. Immediately after resection, the samples were covered with OCT, cooled in liquid isopentane and liquid nitrogen and stored at -70°C until analysis. Four tumour samples with high AP-2 expression in immunohistochemical staining and one benign sample were selected for Western blot analyses. The same antibody as in IHC was used to detect AP-2.

The frozen tissue samples were homogenized, incubated on ice for 30 min and centrifuged at 13000 rpm for 15 min at 4°C. Homogenization was performed using lysis homogenization buffer (0.02M Tris-HCl, 0.002M EDTA, 0.1M NaCl, pH 8.0) with protease inhibitors (1mmol VO₄, 10µg/ml Aprotin, 10µg/ml Leupeptin, 1mmol PMSF). Following centrifugation, the samples were either refrigerated and stored at -70°C until analysis or prepared immediately for gel transfer. In the latter case, samples were heated at 100°C for 5 min with an equal amount of SDS sample buffer (0.125M Tris-HCl, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, pH 6.8). Next the centrifuged samples were separated on an SDS-polyacrylamide gel (100V for 3 hours at room temperature) and transferred onto a nitrocellulose membrane (Hypond ECL Nitrocellulose membrane, Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK) by electrophoresis (100V for 2 hours at 4°C). The filters were blocked with 0.1 % soy buffer solution (0.137M NaCl, 1.47mM KH₂PO₄, 8.1mM Na₂HPO₄, 2.7mM KCl, 0.1 % Tween, 0.1 % soy, pH 7.4) and then incubated with the primary antibody diluted 1:5000 for 30min. After a wash with PBS-Tween buffer, the horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied onto the filter diluted in 1:12000 and incubated for 45min. After a wash, the signals were visualized using a commercial substrate kit (Supersignal West Pico Trial Kit, Pierce, Perbio Science Deutschland GmbH, Bonn, Germany). A prestained marker (Biorad Laboratories, California, USA) and Trial Mix –marker (Novagen, CN Bioscience,
Ltd., Beeston, Nottingham, UK) were run in parallel to the samples. A benign breast tissue sample served as a positive control.

4.8 Chromogenic in situ hybridization (CISH) (III)
HER2-positive cases (IHC 2-3+) having sufficient tumour material (n = 51) as well as 20 negatively stained samples were included in the analysis. For CISH, the paraffin sections were first heated at 37°C for 1h and at 60°C for 2½h followed by deparaffinization in xylene and rehydration in ethanol, and air-drying. The samples were heated in CISH pretreatment buffer (Spot-Light™ Tissue Pretreatment Kit, Zymed Laboratories Inc, San Francisco, CA, USA) at 93°C for 10min in the microwave oven and cooled for 15min at room temperature. Then the samples were incubated with 100μl of the pretreatment enzyme (Spot-Light™ Tissue Pretreatment Kit, Zymed) in a humid chamber for 5min at 38-40°C or alternatively for 7min at room temperature, dehydrated with graded ethanol and air-dried. The DIG-labeled HER2 probe (Spot-Light™ HER2 probe, Zymed) was applied onto the slides (25-30μl) and a coverslip was placed on the sections. The edges of the coverslip were sealed with rubber cement (Sanford Corporation, Bellwood, USA). The specimens were denatured for 3min at 94°C on a heat block (GENOtronic, Biozym, Holland) and incubated in a humid chamber for 17h at 38-40°C. The samples were washed with 7.5mM tri-Na-citrate buffer for 5min on a heat block at 80°C, and with 0.025 % Tween-PBS. Endogenous peroxidase activity was blocked in 3 % H₂O₂ diluted with methanol for 10min. The visualization of HER2 CISH was performed by using a commercial Spot-Light™ CISH™ Detection Kit (Zymed). The non-specific staining was blocked by CAS-block for 10min following incubation with FITC-conjugated sheep anti-DIG antibody for 40min. After a wash in Tween-PBS, the HRP-conjugated goat anti-FITC antibody was applied onto the slides for 40min. DAB chromogen for 24min was applied to visualize HER2 expression. The slides were counterstained with hematoxylin, dehydrated with graded ethanol and cleared in xylene, and mounted with Histomount (Zymed). Specimens negative and lightly as well as strongly positive in HER2 immunohistochemical staining were included in the series. A negative control slide was used in each staining batch.
4.9 Evaluation of the staining

All slides were evaluated using a light microscope. The specimens were analyzed by one or two observers, who were unaware of the patients' clinical outcome and reviewed by a senior pathologist. Discrepancies between the observers were found in less than 10% of the slides examined, and consensus was reached on a further review.

4.9.1 AP-2, p21\textsuperscript{WAF1}, p53, Ki-67, MMP-2 and MMP-9 (I-IV)

Both nuclear and cytoplasmic AP-2 expression was recorded, whereas the expression of p21\textsuperscript{WAF1}, p53 and Ki-67 was nuclear. Cytoplasmic MMP expressions were recorded separately for malignant and stromal cells. For statistical analyses, the percent distribution of stained tumour cell nuclei in the samples (AP-2, p21\textsuperscript{WAF1}, p53, and Ki-67) or cytoplasmic expression in the cancer cells (MMPs) was divided into two expression groups using the median as the cut-off value. The expression groups for AP-2 were low (< 80%) and high (≥ 80%), for p21\textsuperscript{WAF1} low (< 5%) and high (≥ 5%), for p53 low (< 10%) and high (≥ 10%), and for Ki-67 low (< 20%) and high (≥ 20%). The median value has been used previously as a cut-off for AP-2. Ki-67 expression data have been categorized using 20% as a cut-off also in previous studies. Cytoplasmic AP-2 expression was considered positive if > 10% of cells in the tumour area were stained. The categories for MMP-2 in cancer cells were reduced (< 80%) and high (≥ 80%), and for MMP-9 reduced (≤ 85%) and high (> 85%). Stromal expression of MMPs was divided into a positive and a negative expression group according a 20% cut-off for positively stained stromal cells.

4.9.2 HER2 (III)

The specimens were analysed according to the scoring proposed by the HercepTest (DAKO). Samples having a weak, moderate or intense staining of the entire membrane in more than 10% of the tumour cells (2+ and 3+) were considered as HER2 overexpressing. Other staining patterns were considered as negative (0 and 1+). Three observers evaluated HER2 amplification analysed by CISH. The gene encoding HER2 was considered amplified if >2 brown-coloured dots were present at least in 10% of the tumour cell nuclei.
4.10 Statistical analyses
The statistical analyses were carried out by using the SPSS for Windows 9.0 programme (SPSS Inc., Chicago, IL, USA). Differences between continuous variables were investigated using non-parametric Mann-Whitney-U or Spearman correlation -tests. The interrelationships between categorized IHC variables and their associations with clinicopathological parameters were tested with contingency tables and a \( \chi^2 \)-test. The univariate survival analyses were performed using the Kaplan Meier’s log-rank analysis and the independent prognostic value of variables was further examined with Cox’s regression analysis. Probability values \( \leq 0.05 \) were considered as significant in the analyses. In the Cox’s multivariate analysis, the Enter-method was used with an additional removal limit of \( p < 0.10 \). Both breast cancer-related survival (BCRS) and recurrence-free survival (RFS) were examined. Patients who died of reasons other than breast cancer were censored at the time of death. The recurrence-free time was defined as a time between the diagnosis and the date of the first local recurrence or a distant metastasis, whichever one appeared first. Patients who remained healthy or died without breast cancer during the follow-up were censored at the time of the last control examination or death.

4.11 Ethical aspects
This study plan was approved by the Ethical Committee of the Kuopio University and Kuopio University Hospital.
5. RESULTS

5.1 Clinicopathological data (I-IV)

The clinicopathological data used in the separate studies (I-IV) are summarized in Table 6. The mean follow-up time of the patients included in the analyses of this thesis (n = 425) was 54.8 months and the median 57.2 months (range 1.2 – 115.1 months). The mean age of the patients was 59.1 years and the median 56.7 years (range 23.3 – 91.6 years). During the first five years of follow-up, 76 patients (18 %) had a recurrence, 50 patients (12 %) died of breast cancer and 37 patients (9 %) died of other causes. The overall 5-year survival rate was 77 %. The 5-year recurrence-free survival rate of the patients was 79 % and the breast cancer-related survival 85 %. The 5-year survival of the excluded stage IV patients (n = 19) was 21 %.

5.2 Expressions of biological factors

5.2.1 Expression of AP-2 (I-IV)

Benign breast epithelium adjacent to carcinomas showed a high nuclear AP-2 expression pattern. High nuclear AP-2 expression (n = 420) was seen in 50 % of the tumours (the median 80 %, range 0-100 %) and cytoplasmic expression was present in 47 % of the cases (Fig. 4A-B). The nuclear AP-2 expression was significantly reduced in carcinomas when compared to the expression observed in benign breast epithelium (Mann Whitney U, p < 0.001). In a Western blot analysis, the primary antibody recognized a protein sized about 50 kDa, which has been reported to be the size of AP-2α.

5.2.2 Expression of p21WAF1 and p53 (II)

The adjacent benign tissue was mainly negative for p21WAF1 though some scattered positive cells were invariably expressed. The expression of p21WAF1 in carcinomas (n = 420) was predominantly nuclear but faint cytoplasmic staining was often present. The general expression of p21WAF1 in carcinomas was low (the median 2.5 %, range 0-70 %), and the fraction of samples considered as high expressers was 38 %. The nuclear expression of p53 in carcinomas (n = 418) was considered as high in 46 % of the tumours (the median 7.5 %, range 0-100 %).
5.2.3 Cell proliferation measured by Ki-67 expression (II-III)
Cell proliferation parameter measured by nuclear Ki-67 showed constantly some positively stained benign breast epithelial cells adjacent to carcinomas. In benign epithelial cells the rate was of cell proliferation always considered as low. Ki-67 data were available for 420 tumours. The proportion of tumours considered as highly proliferating was 44% (median 17.5%, range 0 – 97.5%).

5.2.4 Expression of HER2 (III-IV)
Adjacent benign breast epithelium was negative for HER2. In carcinomas (n = 425) membranous HER2 expression was recorded as being positive in the IHC, and the proportion of HER2 overexpression was 13%. Gene amplification was observed in 78% of the HER2 IHC-positive tumours (n = 51). The amplification rate was 100% in 3+ stained tumours and 61% in 2+ stained tumours. Two of the HER2 IHC-negative samples (n = 20) showed a positive signal.

5.2.5 Expression of MMP-2 and MMP-9 (IV)
Benign breast epithelium and vascular endothelium stained positively for MMPs. Both MMP-2 (n = 421) and MMP-9 (n = 415) were expressed in the cytoplasm of tumour cells (Fig. 4C-E) as well as in stromal fibroblasts (Fig. 4E) and inflammatory cells. The general expression levels of MMP2 and MMP-9 were high (the median 80% for MMP-2 and 85% for MMP-9, range 0-100%). In cancer cells, MMP-2 expression was considered as high in 54%, and MMP-9 in 52% of the cases. Stromal MMP-2 positivity was observed in 46%, and MMP-9 positivity in 38% of the cases.

5.3 Relation of biological factors to clinicopathological data

5.3.1 Relation of AP-2 expression to clinicopathological data (I)
Low nuclear AP-2 expression was associated with advanced stage, axillary lymph node positivity, large tumour size, ductal or other non-lobular carcinoma type, poor differentiation of the tumour, and high recurrence rate. Cytoplasmic AP-2 positivity associated with ER- and PR-negative tumours, ductal or other non-lobular carcinoma type, and poor differentiation. The results are summarized in Table 8.
Figure 4. A) Ductal carcinoma, GR II, with strong nuclear AP-2 expression (arrows). B) Ductal carcinoma, GR III, with strong nuclear (arrow) and cytoplasmic (asterisk) AP-2 expression. C) Positive cytoplasmic MMP-2 expression in ductal carcinoma cells (asterisk), GR II. D) Positive cytoplasmic MMP-9 expression in cancer cells (asterisk), ductal carcinoma GR II. E) Positive cytoplasmic MMP-9 expression in cancer cells (asterisk) and in stromal cells (arrows), ductal carcinoma GR II. F) Negative control slide of the carcinoma sample in the figure A. Bar = 40 μm.
5.3.2 Relation of $p21^{\text{WAF1}}$ and p53 expression to clinicopathological data (II)
Both high $p21^{\text{WAF1}}$ and high p53 expression associated with poor differentiation. In addition, high p53 expression was related to ER-negativity. The expression of $p21^{\text{WAF1}}$ was lowest in lobular carcinomas. The results are summarized in Table 8.

5.3.3 Relation of cell proliferation (Ki-67) to clinicopathological data
A high fraction of proliferating cells was associated with lymph node positivity ($\chi^2$, $p = 0.006$), and advanced stage, large tumour size, poor differentiation, ER- and PR-negativity, ductal carcinoma type, and recurrent disease ($\chi^2$, $p < 0.001$ for all). Cell proliferation did not associate with menopausal status.

5.3.4 Relation of HER2 expression to clinicopathological data (III)
HER2-positivity was associated with advanced stage, large tumour size, poor differentiation, ductal or other non-lobular carcinoma type, ER- and PR-negativity, premenopausal status, and recurrent disease in the whole patient group. The results are summarized in Table 8.

5.3.5 Relation of MMP-2 and MMP-9 expression to clinicopathological data (IV)
A high MMP-2 expression in carcinoma cells was associated with stage I disease. Stromal MMP-2 positivity was related to poor differentiation, ER-negativity, and ductal or other non-lobular carcinoma types. The high MMP-9 expression in carcinoma cells was associated with small tumour size and lower recurrence rate. Positive stromal MMP-9 expression associated with poor differentiation and ductal carcinoma type. The results are summarized in Table 8.

5.4 Interrelationships between biological factors

5.4.1 Relation of AP-2 to $p21^{\text{WAF1}}$ and p53 expression (II)
The expression of AP-2 was not related to $p21^{\text{WAF1}}$ or p53 in the whole patient group or in the nodal subgroups. In the whole patient group, $p21^{\text{WAF1}}$-positive tumours were more often p53-positive ($\chi^2$, $p = 0.041$). AP-2 expression was not related to $p21^{\text{WAF1}}$ expression in p53-positive tumours or to combined $p21^{\text{WAF1}}$/p53 expression.
Table 8. Relation of biological factors to clinicopathological parameters and cell proliferation measured by Ki-67 expression ($\chi^2$-test).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>p = 0.002*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>p = 0.017</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>p = 0.012*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Tumour size</td>
<td>p = 0.003*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>p = 0.011</td>
<td>ns</td>
<td>ns</td>
<td>p = 0.007*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Histological type**</td>
<td>p = 0.048</td>
<td>p = 0.005</td>
<td>p = 0.025</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>p &lt; 0.001</td>
<td>ns</td>
<td>p = 0.018</td>
<td>ns</td>
</tr>
<tr>
<td>Histological grade</td>
<td>p = 0.001*</td>
<td>p = 0.012</td>
<td>p = 0.017</td>
<td>p = 0.008</td>
<td>p &lt; 0.001</td>
<td>ns</td>
<td>p &lt; 0.001</td>
<td>ns</td>
<td>p = 0.013</td>
<td>ns</td>
</tr>
<tr>
<td>ER-status</td>
<td>ns</td>
<td>p = 0.005*</td>
<td>ns</td>
<td>p = 0.002*</td>
<td>p &lt; 0.001*</td>
<td>ns</td>
<td>p &lt; 0.001*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PR-status</td>
<td>ns</td>
<td>p = 0.043*</td>
<td>ns</td>
<td>ns</td>
<td>p &lt; 0.001*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Menopausal status</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>p = 0.004*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Recurrence at 5 years</td>
<td>p = 0.003*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>p &lt; 0.001</td>
<td>ns</td>
<td>ns</td>
<td>p = 0.036*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>p &lt; 0.001*</td>
<td>ns</td>
<td>ns</td>
<td>p = 0.026</td>
<td>p &lt; 0.001</td>
<td>p = 0.003*</td>
<td>p &lt; 0.001</td>
<td>p = 0.021*</td>
<td>p &lt; 0.001</td>
<td>ns</td>
</tr>
</tbody>
</table>

** = Ductal vs lobular vs other histological type, * = inverse association
5.4.2 Relation of AP-2 to HER2 expression (III)

A high nuclear AP-2 expression was associated with HER2 overexpression ($\chi^2$, p = 0.007) in the whole patient group as well as in the ANN, ANP and ER- but not ER+ subgroups. Association between high cytoplasmic AP-2 and HER2 overexpression was observed in the ANP and ER- subgroups. Co-expression of nuclear and cytoplasmic AP-2 was seen in 48 % of HER2 overexpressing tumours ($\chi^2$, p < 0.0001). This association was also seen in the ANP subgroup. High nuclear AP-2 expression and HER2 amplification co-expressed in 55 % of the HER2+ cases. HER2 amplification alone was observed in 23 % of the HER2 overexpressing tumours, and 12 % were positive for nuclear AP-2 alone. In 10 % of the immunohistochemically HER2 overexpressing tumours neither amplification nor AP-2 expression were observed.

5.4.3 Relation of AP-2 to MMP-2 and MMP-9 expression (IV)

Both high nuclear and cytoplasmic AP-2 expressions were associated with high MMP-2 expression in carcinoma cells ($\chi^2$, p = 0.002 for nuclear, p < 0.001 for cytoplasmic) in the whole patient group, as well as in the nodal subgroups. AP-2 did not associate with stromal MMP-2 expression. The high nuclear AP-2 expression was associated with high MMP-9 expression in cancer cells ($\chi^2$, p = 0.004) in the whole patient group and in the ANN subgroup. The high cytoplasmic AP-2 expression was related to high MMP-9 expression in cancer cells only in the ANP subgroup. AP-2 and stromal MMP-9 expressions were not statistically significantly related to each other.

5.4.4 Relation of HER2 to p21\textsuperscript{WAFI} and p53 expression

HER2 overexpression was related to high p21\textsuperscript{WAFI} expression in the ER+ patient group ($\chi^2$, p = 0.001). No association was observed in the whole patient group or nodal subgroups. High p21\textsuperscript{WAFI} expression in HER2 positive cases was independent of AP-2 and p53. HER2 and p53 expressions were not associated with each other.

5.4.5 Relation of HER2 to MMP-2 and MMP-9 expression (IV)

Stromal MMP-2 positivity was associated with HER2 overexpression in the whole patient group ($\chi^2$, p = 0.016), and in the ANN subgroup. Expression of MMP-2 in the carcinoma cells was not associated with HER2 expression. High MMP-9 expression in carcinoma cells was associated with HER2 overexpression in the ANN subgroup but not in the whole
patient group or in the ANP subgroup. Positive stromal MMP-9 expression was related to HER2 overexpression in ER-positive tumours ($\chi^2$, $p = 0.036$) but not in the other investigated subgroups.

5.5 Relation of biological factors to cell proliferation (II-III)

High nuclear AP-2 expression was associated with low cell proliferation rate in the whole patient group (Table 8) as well as in the ANP subgroup. High $p21^{WAF1}$ expression was related to high cell proliferation in the ER+ subgroup ($\chi^2$, $p = 0.001$) but not in any of the other groups investigated (Table 8). p53 high expressing tumours were more often highly proliferating (Table 8) in the whole patient group but in the nodal subgroups p53 was not associated with cell proliferation. Combined $p21^{WAF1}$/p53 expression was not related to cell proliferation, whereas HER2 overexpressing tumours, and those with positive stromal gelatinase expression exhibited a high proliferation rate (Table 8). Those tumours with HER2-/AP-2+ expression showed significantly higher proportion (73 %) of low proliferating cells compared to that in HER2-/AP-2- (50 %), HER2+/AP-2+ (25 %), and HER2+/AP-2- (22 %) in the whole patient group ($\chi^2$, $p < 0.001$) and in the nodal subgroups. High MMP-2 and MMP-9 expression in cancer cells associated with low cell proliferation rate (Table 8) in the whole patient group and additionally, in the ANN subgroup (MMP-2), and in the ANP subgroup (MMP-9).

5.6 Prognostic factors for breast cancer

5.6.1 Clinicopathological factors (I-IV)

The prognostic value of clinicopathological factors as well as biological parameters was investigated in relation to recurrence-free and breast cancer related survival. In the whole patient group, advanced stage, axillary lymph node positivity, large tumour size, and poor differentiation predicted both shorter RFS and BCRS (Table 9). A shorter BCRS was, in addition, predicted by ER- and PR-negativity (Table 9).

In the ANN subgroup ER-negativity was the only prognostic factor associated with poor BCRS ($p = 0.0039$). None of the traditional prognostic factors predicted RFS due to low number of events hindering the statistical analyses in this subgroup.
Table 9. Summary of clinicopathological parameters in the univariate survival analysis of the whole patient group. p-values are of Log Rank analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>BCRS</th>
<th>RFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>417</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>417</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tumour size</td>
<td>425</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Histological grade</td>
<td>425</td>
<td>0.0306</td>
<td>0.0441</td>
</tr>
<tr>
<td>Histological type</td>
<td>425</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>ER-status</td>
<td>423</td>
<td>&lt;0.0001</td>
<td>ns</td>
</tr>
<tr>
<td>PR-status</td>
<td>422</td>
<td>0.0011</td>
<td>ns</td>
</tr>
<tr>
<td>Menopausal status</td>
<td>425</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

In the ANP subgroup shorter BCRS was predicted by ER- and PR-negativity (p = 0.0042, and p = 0.0039, respectively), large tumour size (p = 0.0039), and advanced stage p < 0.0001). Shorter RFS was predicted by advanced stage (p = 0.0068).

5.6.2 Biological factors

5.6.2.1 Relation of AP-2 expression to survival (I)

In the univariate analyses, low nuclear AP-2 expression was a significant predictor of shorter RFS (p = 0.0028) in the whole patient group (Table 10) and in the ANP subgroup (p = 0.0108), see figure 3 in original publication I. AP-2 expression was not related to BCRS (Table 10). Cytoplasmic involvement was associated with poorer survival compared to high strictly nuclear AP-2 expression in the whole patient group (p = 0.0314 for BCRS, and p = 0.0050 for RFS, Fig. 4A in I) and in the ANP subgroup (p = 0.0411 for BCRS, and p = 0.0030 for RFS, Fig. 4B in I). Cytoplasmic AP-2 expression alone did not have any prognostic value. In the multivariate analysis, low nuclear AP-2 expression predicted shorter RFS in the whole patient group (p = 0.0292) and in the ANP subgroup (p = 0.0151).

5.6.2.2 Relation of p21^{WAF1} and p53 expressions to survival (II)

Expressions of p21^{WAF1} and p53 did not have any prognostic significance for survival in the whole patient group (Table 10) or in the nodal subgroups. In the subset of patients
treated with adjuvant hormonal therapy, high $p21^{\text{WAF1}}$ expression predicted longer BCRS ($p = 0.0179$) and RFS ($p = 0.0433$). Combined $p21^{\text{WAF1}}/p53$ expression did not reach statistical significance in the survival analysis.

**Table 10.** Summary of biological factors in the univariate survival analysis of the whole patient group. $p$-values of Log Rank analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>BCRS</th>
<th>RFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-2 (nuclear)</td>
<td>420</td>
<td>ns</td>
<td>0.0028</td>
</tr>
<tr>
<td>AP-2 (cytoplasmic)</td>
<td>420</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>p21 expression</td>
<td>420</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>p53 expression</td>
<td>418</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Cell proliferation (Ki-67)</td>
<td>420</td>
<td>$&lt; 0.0001$</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>HER2 expression</td>
<td>425</td>
<td>0.0063</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>MMP-2 in cancer cells</td>
<td>421</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>MMP-2 in stroma</td>
<td>421</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>MMP-9 in cancer cells</td>
<td>415</td>
<td>ns</td>
<td>0.0351</td>
</tr>
<tr>
<td>MMP-9 in stroma</td>
<td>415</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

### 5.6.2.3 Relation of cell proliferation (Ki-67) to survival (II-III)

A high fraction of proliferating cells predicted both shorter RFS and BCRS in the whole patient group (Table 10) as well as in the ANN subgroup ($p = 0.0019$ for BCRS, and $p < 0.0001$ for RFS), and in the ANP subgroup ($p = 0.0006$ for BCRS, and $p = 0.0006$ for RFS). The cell proliferation rate independently predicted BCRS and RFS in the whole patient group and in the nodal subgroups.

### 5.6.2.4 Relation of HER2 expression to survival (III-IV)

HER2 overexpression predicted shorter BCRS ($p = 0.0063$) and RFS ($p < 0.0001$, Fig. 2 in III) in the whole patient group (Table 10), and in the ANN subgroup ($p = 0.0342$ for BCRS, and $p = 0.0456$ for RFS). In the ANP subgroup, HER2 overexpression predicted only shorter RFS ($p = 0.0002$). Combining of HER2 and AP-2 expressions separated four different prognostic groups, of which the best prognosis was in the HER2+/AP-2+ subgroup and the worst in the HER2+/AP-2- subgroup ($p = 0.0103$ for BCRS, and...
p < 0.0001 for RFS (Fig. 3 in III) in the whole patient group. Combined expression of HER2 and AP-2 predicted RFS also in the ANP subgroup. In the multivariate analysis, HER2 overexpression predicted shorter RFS in the whole patient group and in the ANP subgroup.

5.6.2.5 Relation of MMP-2 and MMP-9 expressions to survival (IV)

MMP-2 expression neither in carcinoma cells nor in stromal cells possessed any prognostic value in the whole patient group (Table 10) or in the nodal subgroups. Instead, a high MMP-9 expression in cancer cells predicted better RFS (p = 0.0351, Fig. 1 in IV) in the whole patient group but not in the BCRS analysis (Table 10) or in the nodal subgroups. Positive stromal MMP-9 expression predicted shorter RFS and BCRS in ER-positive disease (p = 0.0389 for RFS and p = 0.0081 for BCRS), especially in a subgroup of T1 tumours (p = 0.0031 for RFS, Fig. 2 in IV, and p = 0.0089 for BCRS). Stromal MMP-9 expression did not have any prognostic value in the whole patient group (Table 10) or in the nodal subgroups. In multivariate analysis, reduced MMP-9 expression in carcinoma cells (p = 0.0248) independently predicted shorter RFS in the whole patient group, and stromal MMP-9 positivity shorter BCRS and RFS in the ER+ T1 subgroup.

5.6.3 Multivariate survival analysis of all cases

The multivariate analysis included all variables which showed prognostic significance in the univariate survival analysis. Stage was used instead of lymph node status and tumour size. All cases with complete set of data available were included in the Cox’s model. In the whole patient group, BCRS (n = 402) was independently predicted by stage, cell proliferation and histological grade, and RFS (n = 395) by cell proliferation, stage, HER2 expression, histological grade and nuclear AP-2 expression (Table 11). The independent prognostic factors in the nodal subgroups are presented in table 12.
Table 11. Independent predictors in the Cox’s multivariate analysis for BCRS and RFS in the whole patient group.

<table>
<thead>
<tr>
<th>Category</th>
<th>Hazards ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breast cancer-related survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole patient group (n = 402)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Reference</td>
<td>2.24</td>
<td>0.99-5.04</td>
</tr>
<tr>
<td>II</td>
<td>7.65</td>
<td>3.17-18.49</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cell proliferation (Ki-67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Reference</td>
<td>3.65</td>
<td>1.72-7.71</td>
</tr>
<tr>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Reference</td>
<td>1.58</td>
<td>0.63-4.00</td>
</tr>
<tr>
<td>II</td>
<td>0.62</td>
<td>0.21-1.84</td>
<td>0.3857</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Recurrence-free survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole patient group (n = 395)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell proliferation (Ki-67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Reference</td>
<td>4.04</td>
<td>2.26-7.22</td>
</tr>
<tr>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Reference</td>
<td>1.38</td>
<td>0.77-2.49</td>
</tr>
<tr>
<td>II</td>
<td>3.87</td>
<td>1.88-7.97</td>
<td>0.0002</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Reference</td>
<td>2.66</td>
<td>1.53-4.63</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Reference</td>
<td>1.09</td>
<td>0.55-2.16</td>
</tr>
<tr>
<td>II</td>
<td>0.47</td>
<td>0.21-1.03</td>
<td>0.0592</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-2 (nuclear)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1.76</td>
<td>1.07-2.92</td>
<td>0.0269</td>
</tr>
<tr>
<td>High</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 12. Independent predictors in the Cox’s multivariate analysis for BCRS and RFS in the nodal subgroups.

<table>
<thead>
<tr>
<th>Category</th>
<th>Hazards ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breast cancer-related survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ANN patient group (n = 222)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell proliferation (Ki-67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Reference</td>
<td></td>
<td>0.0257</td>
</tr>
<tr>
<td>High</td>
<td>4.45</td>
<td>1.20-16.54</td>
<td></td>
</tr>
<tr>
<td><em>ANP patient group (n = 165)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>0.0014</td>
</tr>
<tr>
<td>II</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3.03</td>
<td>1.53-5.99</td>
<td></td>
</tr>
<tr>
<td>Cell proliferation (Ki-67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Reference</td>
<td></td>
<td>0.0213</td>
</tr>
<tr>
<td>High</td>
<td>2.74</td>
<td>1.16-6.46</td>
<td></td>
</tr>
<tr>
<td><strong>Recurrence-free survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ANN patient group (n = 242)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell proliferation (Ki-67)</td>
<td></td>
<td></td>
<td>0.0002</td>
</tr>
<tr>
<td>Low</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>5.80</td>
<td>2.31-14.56</td>
<td></td>
</tr>
<tr>
<td><em>ANP patient group (n = 165)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 expression</td>
<td></td>
<td></td>
<td>0.0025</td>
</tr>
<tr>
<td>Negative</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>2.67</td>
<td>1.41-5.03</td>
<td></td>
</tr>
<tr>
<td>AP-2 (nuclear)</td>
<td></td>
<td></td>
<td>0.0102</td>
</tr>
<tr>
<td>Low</td>
<td>2.37</td>
<td>1.23-4.59</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell proliferation (Ki-67)</td>
<td></td>
<td></td>
<td>0.0387</td>
</tr>
<tr>
<td>Low</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1.97</td>
<td>1.04-3.75</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>0.0403</td>
</tr>
<tr>
<td>II</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1.87</td>
<td>1.03-3.43</td>
<td></td>
</tr>
</tbody>
</table>
6. DISCUSSION

6.1 Evaluation of the study material
The present large, prospective series is a representative cohort of typical breast cancer patients at Kuopio University Hospital area during the years 1990-1995 and forms part of a larger Kuopio Breast Cancer Project. There was a 98% participation rate of the patients diagnosed with breast cancer at that time. The original study population consisted of 520 unselected breast cancer patients, of which the patients with non-invasive tumour, metastatic disease, unknown metastatic data (TNM classification MX) or insufficient tumour material were excluded. The clinicopathological data, however, used in the present study do not differ from the data of the original invasive carcinoma cohort. The prospective nature of the present study eliminates most of the biases related to retrospective study design, such as missing data or differences in treatment. In the present study, diagnosis, treatment and follow-up of the patients have been uniform and none of the patients have been lost to follow-up. In the present series only 2% of the patients had missing data. Therefore, the clinicopathological data are considered as very representative. The overall 5-year breast cancer survival rate in the present study was 77%, which is comparable with that in Scandinavia and Europe. The 5-year survival of excluded stage IV patients (21%) was also close to the relative survival described elsewhere in Europe. In the present series, the mean age of the patients was 59.1 years, which is close to that reported in Finland and in other parts of Europe. In addition, the clinicopathological data are in line with that usually reported in the literature.

6.2 Evaluation of the study methods
The histological diagnosis was confirmed by reviewing one to four original sections of the primary tumour, and the tumours were re-evaluated for histological type and grade by senior pathologists, who were unaware of the clinical data. Accordingly, the histopathological data are uniform reducing interobserver dependent bias. Immunohistochemical staining is a relatively inexpensive, fast and easy method for detecting biological factors, though several technical factors such as differences in tissue pretreatment, antibody specificity, and scoring of the staining may affect the quality of the results. A standardized IHC staining method was used in this study, and all slides were evaluated by one or two observers and reviewed by a senior pathologist to reach a
consensus. HER2 amplification in HER2 overexpressing samples was confirmed using a CISH method, which has recently been demonstrated to be a good alternative to FISH. The results of CISH presented here are comparable with those reported previously with regard to the oncogene amplification rate in IHC overexpressing tumours. Western blot using frozen fresh tumour samples was performed to analyse the specificity of the AP-2 antibody. In all staining series and in the molecule biological methods, the positive controls stained as expected and the negative controls remained negative. For statistical analyses, the continuous variables derived from immunohistochemical analyses were dichotomized into two expression groups using the median value as the cut-off, which can be used without introducing statistical bias. In addition, it divides the material into two equally sized groups and is often used as a cut-off. However, disadvantages of dichotomizing continuous variables concerning biological parameters may be artificiality and loss of information.

6.3 Clinicopathological prognostic factors in breast cancer

In the present study, stage and histological grade were the independent traditional prognostic factors that related to BCRS and RFS in the whole patient group. In addition, lymph node status, tumour size, and ER- and PR- expressions predicted patient outcome in univariate survival analysis. Previously, stage or lymph node status and tumour size have been the most important prognosticators in breast cancer. Histological grade has also a strong value in assessment of breast cancer prognosis, as also demonstrated here. Hormone receptor status has been related to breast cancer outcome and has been shown to be an independent prognostic value in some studies, but not in all studies. In this study ER or PR expressions did not reach statistical significance in multivariate analysis. The high number of small T1 carcinomas (> 50 %) and stage I diseases (39 %) in the present series may affect the value of prognostic factors. Indeed, lymph node staging and histological grade have been the only independent prognosticators of small ≤ 1 cm primary breast cancer. In the subgroup of ANN patients, the traditional prognostic factors proved to have insufficient statistical power, and similarly, in previous studies their value has been limited. Accordingly, the present series is comparable with most previous breast cancer materials indicating that the obtained results can be generalized also to other similar population based breast cancer cohorts.
6.4 AP-2 – expression and breast cancer

6.4.1 Expression of AP-2 and its relation to clinicopathological data

The family of AP-2 transcription factors is crucially involved in regulation of genes related to breast tissue growth and morphogenesis as well as breast cancer. In the present study, both nuclear and cytoplasmic AP-2 expression in carcinomas was observed similarly to that described in epithelial ovarian cancer, cutaneous malignant melanoma, prostate cancer, cervical intraepithelial neoplasia, childhood medulloblastoma, and colorectal carcinoma. In breast carcinoma, AP-2 localization has been predominantly nuclear. Differences in the expression patterns may be partly due to different specificities of the antibodies in use. In addition, the expression of AP-2 transcription factors seems to be cell-type specific.

In the present series, nuclear AP-2 expression was reduced in carcinomas compared to highly positive benign breast epithelium as also shown in the study of Gee et al. (1999). Reduced nuclear AP-2 expression in carcinomas associated with clinicopathological parameters related to aggressive disease and thereby indirectly indicated an increased metastatic potential of the tumour cells. Furthermore, AP-2 expression shifted from a nuclear to a cytoplasmic form along with increasing atypia of carcinoma specimens. Previously, high AP-2 expression has been associated with high differentiation in breast cancer as well as in other carcinomas, supporting these results. Indeed, overexpression of AP-2α has been reported to be growth inhibitory in breast tissue. These results support a tumour suppressive role for AP-2 in breast cancer as has been proposed by Gee et al. (1999). Based on the current knowledge, it seems that the tumour suppressive function of AP-2 may be mediated via transcriptional upregulation of p21WAF1, direct interaction with p53, or by yet unknown mechanisms.

6.4.2 Role of AP-2 in the regulation of cell proliferation, p21WAF1 and p53

In this study, high nuclear AP-2 expression in carcinomas was related to a low cell proliferation rate, which is supported by Gee et al. (1999), who reported lower cell proliferation rate measured by Ki-67 antibody and mitotic activity counts in AP-2 high expressing breast tumours. Similarly, a reduction of actively cycling cells was seen in the mammary gland overexpressing AP-2α. Cytoplasmic AP-2 expression was not
related to cell proliferation, though in prostate cancer a low cytoplasmic AP-2 expression has been statistically related to increased cell proliferation. Overexpression of AP-2α has been shown to result in cell cycle arrest in the G1 phase via induction of p21WAF1 and the appearance of hypophosphorylated pRb, induction of apoptosis and inhibition of growth in cancer cells. On the other hand, opposite results linking high AP-2α expression to increased cell proliferation and reduced differentiation have been published.

A potential role of AP-2 in the upregulation of p21WAF1 expression has been proposed (Fig. 2). However, in the present series, these two factors were not related to each other as was also described for epithelial ovarian cancer. In addition, in prostate cancer p21WAF1 has been expressed independently of AP-2. Previously, in breast cancer, a positive association between AP-2 and p21WAF1 has been demonstrated in a study using a small number of breast cancer patients indicating that AP-2 mediated p21WAF1 expression. Similar results have been reported in cutaneous malignant melanoma and colorectal cancer. Accordingly, in breast cancer, the role of AP-2 in p21WAF1 regulation may not be as straightforward as previously assumed. McPherson et al. (2002) showed that AP-2α targets the p53 protein at p53-regulated promoters, yielding p21WAF1 induction as a result of a direct interaction between AP-2α and wild-type p53. In the present study, however, AP-2 and p21WAF1 were not statistically associated with each other even when investigated with respect to p53 status. The upregulation of p21WAF1 seems to be a net effect of several different pathways, and thus reduces the value of single factors.

6.4.3 Relation of AP-2 to HER2 and MMPs
This was the first study to investigate the relationship between AP-2 and HER2 in a large clinical breast cancer series. To date, AP-2 proteins have been associated with HER2 overexpression in vitro, and both positive and negative associations between these two factors have been reported in vivo. In the current study, 67% of HER2-positive tumours showed high nuclear AP-2 expression and 48% both high nuclear and cytoplasmic AP-2 expression supporting the results of Turner et al. (1998). Similarly, Gilbertson et al. (1997) with the same AP-2 antibody as used here identified a positive association between AP-2 protein and HER2, HER3 and HER4 expression levels in
childhood medulloblastomas. In the present series AP-2 expression and/or HER2 gene amplification were present in 90% of the HER2 overexpressing tumours strengthening the theory that AP-2 may be crucially involved in HER2 regulation in vivo. Other mechanisms such as activation by Ets may explain the increase in HER2 transcription in the remaining 10% of the cases.

The role of AP-2 in relation to HER2 was further examined by comparing the cell proliferation rate to HER2/AP-2 co-expression. A significantly higher proportion of low proliferating cells was observed in the HER2-/AP-2+ subgroup compared to the others. In the HER2+ subgroups, the cell proliferation activity was high and not significantly altered by the extend of AP-2 expression, suggesting that AP-2 may be insufficient to halt cell proliferation or inactive in HER2 overexpressing cases (Fig. 2). This is also supported by the high cytoplasmic AP-2 expression observed in the HER2-positive tumours, which may indicate inappropriate function of AP-2. Further support is derived from the prognostic advantage the high nuclear AP-2 expression offered. Accordingly, these results point to a tumour suppressive role for AP-2 in breast cancer, even though AP-2 proteins are positively related to HER2 overexpression and required for maximal HER2 gene transcription in breast cancer cells.

The regulation of AP-2 proteins, HER2 and ER form a complex interplay in breast cancer. AP-2α and AP-2γ are involved in the transcriptional upregulation of ER, whereas HER2 is related to deregulation of the ER-pathway. In the present study nuclear AP-2 and ER were not related to each other, though in the breast cancer literature a positive association between AP-2α and ER has been described. Both high nuclear and cytoplasmic AP-2 expression associated with HER2 expression in the ER- subgroup, which, however, was not seen in the ER+ subgroup. Interestingly, it has been suggested that oestrogen-activated ER can suppress HER2 expression via AP-2. Constitutive expression of AP-2β and AP-2γ but not AP-2α abrogates the oestrogenic repression of HER2 promoter. In addition, AP-2 expression is under oestrogenic regulation as AP-2α expression is reduced in the presence of oestrogen, whereas oestrogen induces AP-2γ expression in ER-positive breast cancer cells. It has been also shown that ER extinguishes AP-2α transactivating properties suggesting that ER and AP-2α may be
competing for limiting cofactors. Furthermore, AP-2γ, but not other AP-2 proteins, activate HER3 promoter.

The role of AP-2 in gelatinase regulation has not been studied previously in breast cancer. In this study, high AP-2 expression, both nuclear and cytoplasmic, showed an association with MMP expression in cancer cells but not in stromal cells. AP-2 has been reported to be an important transcriptional activator of MMP-2 and a transcriptional cofactor of MMP-9. Accordingly, AP-2 may be partly involved in the regulation of MMP transcription in cancer cells. AP-2 has also revealed opposite properties as wild-type AP-2 has negatively regulated MMP-2 transcription. In SB-2 melanoma cells, inactivation of AP-2 by a dominant negative AP-2 resulted in an increase in MMP-2 mRNA expression and activity as well as invasiveness. Sp1 and Sp3 transcription factors are also involved in the transcriptional activation of MMP-2 and the concentration between AP-2 and these factors may affect the gene expression. Indeed, in cell line studies, a balance between AP-2 and Sp1 or Sp3 seems to be essential and the reduced ratio between AP-2 and Sp1 or Sp3 expression via loss of AP-2 has been correlated with PAR-1 overexpression or activation of VEGF transcription. A similar mechanism may also be involved in the regulation of MMP-2 in breast cancer. In addition, AP-2 is involved in the regulation of TIMP-1 and TIMP-2, which inhibit the function of activated MMPs. Though AP-2 is required for MMP transcription, the overall regulation may be more complex and, for example, balanced by TIMP expression (Fig. 2).

6.4.4 Prognostic value of AP-2

This study demonstrated for the first time the independent prognostic value of nuclear AP-2 expression in breast cancer. In the final multivariate model, reduced nuclear AP-2 expression predicted shorter RFS in the whole patient group and in the ANP subgroup. In breast cancer, previously, nonsignificant trends towards better survival with high nuclear AP-2 expression have been demonstrated. However, in stage I cutaneous malignant melanoma, low nuclear AP-2 expression has been shown to be an independent predictor of shorter RFS. Furthermore, in survival analysis of the present material combining nuclear AP-2 and HER2 expressions yielded four different groups, of which the best prognosis was seen in HER2-/AP-2+ subgroup and the worst in HER2+/AP-2-. In the 5-
year RFS analysis, the probability of recurrence-free survival in these subgroups was 89% and 39%, respectively. These results strongly indicate that the nuclear AP-2 in breast cancer is tumour suppressive and may be required for the maintenance of cellular integrity rather than to potentiate the oncogenic effect of HER2 which seems to be highly malignant.

In the present series, cytoplasmic expression of AP-2 was an indicator of poorer survival, when compared to high, strictly nuclear AP-2 expression. Cytoplasmic expression alone did not show any prognostic value. Recently, we demonstrated a high proportion of HER2 overexpression cases in the subgroup of high nuclear and cytoplasmic AP-2 expression, which may partly explain the reduced survival related to cytoplasmic AP-2 expression. In epithelial ovarian cancer, high cytoplasmic AP-2 expression independently predicted better survival 200, a finding which may be explained by cell type specificities. In colorectal cancer, cytoplasmic AP-2 expression was not related to prognosis 180. Indeed, an aberrant localization of cellular markers has been associated with decreased survival in cancers 458. Accordingly, the increased cytoplasmic AP-2 expression reported here may indicate less active or inactivated AP-2, resulting in increased growth of the breast cancer cells.

As a conclusion, the role of AP-2 in breast cancer seems to be tumour suppressive. AP-2 proteins, as well as other factors, may have a dual role in transcriptional regulation depending on the intracellular concentration of the factors. However, the expression and function of AP-2 seem to be cell type specific and the role of separate AP-2 proteins remains unclear. AP-2 expression seems to add information on breast cancer biology and prognosis and may potentially be used in clinical practice. However, further studies are required to analyse the separate roles of different AP-2 proteins as well as their impact on prognosis. Combining AP-2 and HER2 expressions may add information on breast cancer prognosis, which is an interesting prospect needing to be evaluated in future studies.

6.5 Expression of 21WAF1 and its relation to p53, clinicopathological factors and prognosis in breast cancer

The expression of p21WAF1 in breast cancer has been investigated in several studies though with variation within the results (Table 4). Similar to the results reported here, the nuclear
expression of p21\textsuperscript{WAF1} has been relatively low with a median value of 3-5\% in the majority of the breast cancer studies to date\textsuperscript{174, 197}. The proportion of p21\textsuperscript{WAF1} high expressing tumours has varied from 25\% to 91\% depending on the scoring and cut-off in use (Table 4). Here the proportion was 38\%, which is close to that described in several other studies (Table 4). An occasional cytoplasmic expression of p21\textsuperscript{WAF1} has been described in some breast cancer studies\textsuperscript{174, 177, 204}, whereas some studies concerning predominantly cytoplasmic p21\textsuperscript{WAF1} expression have been published recently\textsuperscript{213-215}. In the present series, the cytoplasmic staining was so faint that it was left unevaluated. Differences in the results may originate from the different antibodies and cut-offs used in the studies (Table 4). In the present study, the proportion of p53 high expressing tumours was 46\%. Previously the reported p53 overexpression rate in breast cancer has varied from 15\%\textsuperscript{174} to 48\%\textsuperscript{210} depending on the patient material, antibodies and cut-offs used in the studies.

Both p53-dependent and independent induction of p21\textsuperscript{WAF1} mediated cell cycle arrest has been described in the literature\textsuperscript{148, 164, 166, 459, 460}. In the present series, a positive association between p21\textsuperscript{WAF1} and p53 expressions was observed in line with the studies of Bankfalvi et al. (2000)\textsuperscript{173} and Diab et al. (1997)\textsuperscript{175}, suggesting p53-independent expression of p21\textsuperscript{WAF1}. Winters et al. (2001)\textsuperscript{215} reported a positive association between cytoplasmic p21\textsuperscript{WAF1} and p53 expressions. In most studies, however, the association has been negative or nonsignificant (Table 4). Accordingly, these results support the p53-independent regulation of p21\textsuperscript{WAF1} in breast cancer\textsuperscript{177, 209}, though it is possible that under some conditions, p53 is partly responsible for p21\textsuperscript{WAF1} induction in breast tissue.

In the present series high p21\textsuperscript{WAF1} expression associated with poor differentiation and non-lobular carcinoma type. Previously, a link between high p21\textsuperscript{WAF1} expression and poor differentiation has been reported but also conflicting results have been published (Table 4). The association of p21\textsuperscript{WAF1} with histological type has been rare (Table 4) and only Rey et al. (1998) have found significantly higher p21\textsuperscript{WAF1} expression in ductal carcinomas compared to other subtypes\textsuperscript{209}. In univariate survival analysis of the present series, high nuclear p21\textsuperscript{WAF1} expression predicted the response to hormonal therapy. A response to adjuvant therapy has been documented previously in studies using p21\textsuperscript{WAF1}/p53 combination\textsuperscript{176, 197, 203}, which may be explained by oestrogenic
regulation. Indeed, in ER+ breast cancer cells, the p21WAF1 gene has been shown to be more sensitive to hyperacetylationg agents and has been expressed at higher levels.

In this study, nuclear p21WAF1 expression did not possess any prognostic value. Previously, different results concerning the prognostic value of p21WAF1 expression have been published (Table 4). Lack of prognostic value of nuclear p21WAF1 has been reported in a large breast cancer series using the same p21WAF1 antibody as here, supporting the results of this as well as some other studies. Recently, an association between high cytoplasmic p21WAF1 expression and reduced survival in breast cancer has been published. Combining p21WAF1/p53 expressions did not provide additional prognostic information in the current study, in contrast to that reported in some studies. In this series, high p53 expression associated with poor differentiation and ER-negativity but failed to show any prognostic value. In line with the current results, the prognostic value of p53 has been limited in some previous breast cancer studies even though the accumulation of p53 is an indicator of a mutated protein and often related to more aggressive disease including poor differentiation and reduced survival in breast cancer.

Taken together, the prognostic value of p21WAF1 and p53 seems to be limited in breast cancer and may be greatly affected by tissue processing, antibodies, evaluation of the staining and cut-offs in use.

### 6.6 Cell proliferation and breast cancer

In the present series, the fraction of high proliferating cells was 44%, which falls within the range reported in the previous studies. High cell proliferation activity associated with aggressive clinicopathological factors in line with the literature. Cell proliferation was associated with high p21WAF1 expression in the ER+ subgroup but not in any other group investigated. Combined p21WAF1/p53 expression was not related to cell proliferation in the whole patient group or in the nodal subgroups. Both low and high cell proliferation rates have been related to high p21WAF1 expression, but nonsignificant results also can be found in the breast cancer literature. The high degree of cell proliferation in tumours with large p21WAF1 expressing capacities may indicate that cell proliferation promoting mechanisms overcome p21WAF1 induced cell cycle arrest or
that the p21WAF1 pathway may be activated by ER-related factors such as oestradiol. Increased cell proliferation was observed in p53-positive tumours suggesting an inappropriate tumour suppressive function of mutated p53 as has been demonstrated previously. Highly increased cell proliferation associated with HER2 overexpression reflecting its highly malignant potential. Indeed, hyperproliferation in the HER2 overexpressing cells is a consequence of shortening of the G1 phase and early S phase entry. HER2 overexpression also results in downregulation of p21WAF1 and p27KIP1 expression and activation of factors promoting cell proliferation. In the present series, low cell proliferation instead was related to MMP expression in carcinoma cells, which may reflect the inactivated or pro-MMP form. Increased cell proliferation was related to positive stromal MMP-2 and MMP-9 expression. Previously, active MMPs have been shown to induce cell proliferation by releasing membrane-bound growth factor precursors, by increasing the bioavailability of some growth factors and indirectly through integrins.

The high cell proliferation measured by Ki-67 was strongly related to poorer survival in all subgroups investigated and in the final multivariate model it was an independent prognostic factor of both RFS and BCRS in the whole patient group and in the nodal subgroups. Earlier, the prognostic value of Ki-67 has been demonstrated in several breast cancer studies. Accordingly, cell proliferation measured by Ki-67 is a strong prognostic factor in breast cancer and a useful tool in selecting those patients requiring more intensive treatment.

6.7 HER2 expression, relation to p21WAF1, p53, MMPs and survival in breast cancer

In the present series, the expression of HER2 was comparable to that previously reported in the literature. HER2 overexpression was detected using IHC in 13% of the cases, which falls within the described 10-34% incidence in breast cancer. The relatively low overexpression rate may be explained by the large proportion of stage I tumours (nearly 40%) in this material, a subgroup in which the overexpression occurs less frequently. Amplification detected using CISH was present in 78% of the HER2 overexpressing tumours, and in IHC 3+ stained tumours the rate was 100%, in accordance
with the literature. Overexpression was related to adverse clinicopathological factors as previously demonstrated in several studies.

HER2 overexpression has been shown to induce chemo-resistance via p21WAF1 upregulation. In addition, high nuclear and cytoplasmic p21WAF1 expressions have been associated with HER2 overexpression. In the present study, high nuclear p21WAF1 expression associated with HER2 overexpression in the ER+ subgroup, in which high p21WAF1 expression was related to a high cell proliferation rate. The mechanism, however, remains to be clarified. Recently, increased cytoplasmic p21WAF1 expression reflecting its reduced activity has been related to HER2 overexpression and poor prognosis. Similarly, p53 positivity has been associated with HER2 overexpression in some studies which, however, was not observed here. Cells expressing wild-type p53 have been shown growth inhibition and apoptosis after HER2 transfection and to limit the survival of HER2 overexpressing cells. In addition, an impaired function of both HER2 and p53 has been reported to be a strong negative prognostic factor in breast cancer.

HER2 overexpression increases the invasive capacity of the tumour cells, at least partly, by up-regulating gelatinase expression and activity. Tumour cells overexpressing HER2 more often show expression of metastasis associated proteins such as MMP-2 and CD44, and HER2 ligands are capable of regulating MMP expression. In the present study, HER2 overexpressing tumours showed a significant positive stromal MMP-2 and MMP-9 expression. Previously, stromal MMP-2 at mRNA level has been related to HER2 overexpression in breast cancer. MMP expression is induced coincident with invasion and, interestingly, in this study positive stromal MMP-2 expression was related HER2 overexpression in the early phase of carcinogenesis (such as in ANN disease) suggesting that HER2 may be involved in the upregulation of MMP activity prior to metastasis. Stromal MMP-9 positivity associated with HER2 overexpression in the ER-positive patient subgroup, in which MMP-9 predicted shorter survival. An autocrine loop or some other effect of overexpression, such as increased EMMRIN activity, may represent the foundations of the molecular events by which HER2 induces gelatinase expression and activity during an early phase of breast cancer, and further promotes disease progression and metastases.
The relation of pathological membranous HER2 overexpression to poor prognosis in breast cancer has been well documented and recently, a monoclonal antibody towards HER2 has been adopted in the treatment of metastasized breast cancer. The present study, in addition, confirms the prognostic value of HER2 overexpression in breast cancer. In the final multivariate model, HER2 overexpression independently predicted shorter RFS in the whole patient group and in the ANP subgroup. Accordingly, HER2 expression seems to be a valuable prognostic factor in breast cancer.

6.8 Expression of MMP-2 and MMP-9, relation to clinicopathological factors and prognosis in breast cancer

It has been hypothesized that most of the MMP production occurs in stromal cells and that the protein is being stored in malignant cells. In the present study, cytoplasmic MMP-2 and MMP-9 expressions were observed both in stromal and in cancer cells in accordance with previous studies. Opposing results, on the other hand, exist. In addition, benign epithelium showed positive staining which has been less common in the literature. However, positive MMP-2 and MMP-9 expression both at the mRNA and at the protein level in benign breast epithelium has been described. In studies using zymography both gelatinases have been expressed in benign breast tissue though the activity has been less than that detected in tumour tissue. In the present study, the antibodies recognized pro-MMP-2 and both pro- and active MMP-9 forms which may explain the differences in the results.

In the present series, high MMP-2 expression in carcinoma cells associated with early disease, whereas positive stromal enzyme was related to aggressive clinicopathological parameters. Previously, MMP-2 expression in cancer cells has been related to inverse clinicopathological factors or shown no association with outcome. The results concerning stromal MMP-2 expression are in line with that described in the literature. The results of MMP-9 expression, both in stromal and in cancer cells, and its relation to clinicopathological data showed a similar pattern to that reported for MMP-2. Reduced MMP-9 expression in carcinoma cells has been previously associated with aggressive breast cancer, supporting the results of this study. It is possible, that gelatinase expression in carcinoma cells may be related to pro- or otherwise inactive enzyme and that the stromal protein seems to be more active.
In the current study, MMP-2 expression neither in cancer nor in stromal cells possessed any prognostic value. Previously, several studies have reported similar results in breast cancer\textsuperscript{342, 352, 489}, whereas in some studies high MMP-2 expression in carcinoma cells has been an indicator of reduced survival (Table 5). This may be explained by the fact that the function of MMP-2 is regulated at different levels and the balance between MMP-2 and regulating factors, such as MT1-MMP and TIMP-2, may have a greater impact on prognosis\textsuperscript{337}. The antibody used here detects the pro-MMP-2 protein expression and it is possible that the levels of the active enzyme may be better related to disease outcome.

In the present study positive stromal MMP-9 expression predicted shorter RFS and BCRS in hormone sensitive ER+ disease, especially in the subgroup of small T1 tumours. Reduced survival due to stromal MMP-9 positivity among these patients with a usually very good prognosis may reflect an increase in the active MMP-9 enzyme form enhancing malignant properties such as increased angiogenic activity\textsuperscript{491-493}. In addition, MMP-9 expression may, at least partly, be hormonally regulated as oestrogen has been shown to be capable of activating MMP-9 expression via ER-\alpha, resulting in tumour progression\textsuperscript{494}. High MMP-9 expression in carcinoma cells, instead, favoured RFS in univariate survival analysis. Similarly to that reported here, MMP-9 expression in cancer cells has previously predicted improved survival in breast cancer\textsuperscript{340}, although nonsignificant results have also been published\textsuperscript{352, 489}. In the final multivariate model, however, MMP-9 expression in cancer cells did not reach an independent prognostic value.

As a conclusion, these results emphasize the importance of the crosstalk between stromal and epithelial cells\textsuperscript{369, 325}. Evaluation of stromal MMP-9 expression may be valuable in assessing the prognosis in early-stage breast cancer, although other studies are needed to verify these results. MMP-9 expression in carcinoma cells had some prognostic value but it was insufficient to independently predict breast cancer outcome. MMP-2 expression did not possess any prognostic value.
7. SUMMARY AND CONCLUSIONS

The present prospective study was performed in a large, uniformly diagnosed and treated series of breast cancer patients with well-documented and representative clinicopathological data as well as data on follow-up. With respect to the established prognostic factors and treatment results in breast cancer, the present cohort is well comparable with most of the previously published breast cancer studies. In the present study, the prognostic value of factors (AP-2, p21\textsuperscript{WAF1}, p53, Ki-67, HER2, MMP-2, and MMP-9) related to regulation of cell growth and cell proliferation, differentiation and cell-matrix interactions in breast cancer were analysed.

This study demonstrated that:

1. Reduced nuclear expression of transcription factor AP-2 was associated with disease progression and increased metastatic capability in breast cancer, and it was a novel independent prognostic factor of shorter recurrence-free survival in the whole patient group as well as in the ANP subgroup. A shift from a nuclear to a cytoplasmic expression pattern reduced survival indicating a possible inactivation of AP-2. The results support a tumour suppressive role for AP-2 in breast cancer.

2. High p21\textsuperscript{WAF1} expression predicted the response to adjuvant hormonal therapy but did not provide prognostic information on breast cancer survival. The regulation of p21\textsuperscript{WAF1} seems to be extremely complex and occurs independently of AP-2 and p53 in breast cancer. p53 did not possess any prognostic value in the present breast cancer series. Cell proliferation measured by Ki-67 was the strongest predictor of disease outcome.

3. HER2 overexpression was related to high AP-2 expression and gene amplification in breast cancer. Combining HER2 and AP-2 expressions may provide additional prognostic information for breast cancer patients. In addition, HER2 overexpression independently predicted shorter recurrence-free survival in breast cancer.

4. Expression of gelatinases seems to be partly related to expression of AP-2 and HER2. Immunohistochemically detected positive stromal MMP-9 expression adds
information on breast cancer prognosis in early carcinogenesis. High MMP-9 expression in carcinoma cells predicted a better survival but was not an optimal prognostic factor in the present study. MMP-2 expression did not have any prognostic value.

In conclusion, this study confirmed the value of traditional prognostic factors such as stage, histological grade, HER2 expression and cell proliferation (Ki-67) in breast cancer. A novel prognostic factor, AP-2, give valuable additional information on breast cancer prognosis in predicting survival, alone and in combination with HER2 expression. High stromal MMP-9 expression may be a new potential predictor of poor outcome in early breast cancer.
8. REFERENCES


37. Klijn JG, Blamey RW, Boccia R, Tominaga T, Duchateau L, Sylvestor R. Combined tamoxifen and luteinizing hormone-releasing hormone (LHRH) agonist versus LHRH agonist alone in


48. Rosen PP, Groshen S, Saigo PE, Kinne DW, Hellman S. Pathological prognostic factors in stage I (T1N0M0) and stage II (T1N1M0) breast carcinoma: a study of 644 patients with median follow-up of 18 years. J Clin Oncol 7:1229-1251, 1989.


272. Graus-Porta D, Beerril RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 16:1647-1655, 1997.


374. Underhill CB, Green SJ, Comoglio PM, Tarone G. The hyaluronate receptor is identical to a glycoprotein of Mr 85,000 (gp85) as shown by a monoclonal antibody that interferes with binding activity. J Biol Chem 262:13142-13146, 1987.


110


9. ORIGINAL PUBLICATIONS