Use of proteomics for the early diagnosis of breast cancer

Annemieke van Winden

Use of proteomics for the early diagnosis of breast cancer

Thesis, Utrecht University, with a summary in Dutch

ISBN 978-90-5335-268-7
Author Annemieke van Winden
Cover design Igor Vermeer - vermeerfotografie.nl
Printed by Ridderprint, Ridderkerk, The Netherlands

Use of proteomics for the early diagnosis of breast cancer

Het gebruik van proteomics voor de diagnose van borstkanker in een vroeg stadium

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 15 april 2010 des middags te 12.45 uur

door

Adriana Wilhelmina Johanna van Winden

geboren op 19 februari 1982 te Pijnacker

Promotoren:

Prof. dr. J.H. Beijnen

Prof. dr. P.H.M. Peeters

Co-promotoren:

Dr. C.H. van Gils

Dr. R.C.H. Vermeulen

The research described in this thesis was performed at

Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, The Netherlands
&
the Department of Pharmacy & Pharmacology of the Slotervaart Hospital / The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands

Printing of this thesis was financially supported by:

Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, The Netherlands
The Netherlands Laboratory for Anticancer Drug Formulation (NLADF), Amsterdam, The Netherlands
J.E. Jurriaanse Stichting, Rotterdam, The Netherlands
Amgen B.V., Breda, The Netherlands
Pfizer B.V., Capelle aan den IJssel, The Netherlands
Roche Nederland B.V., Woerden, The Netherlands
GlaxoSmithKline B.V., Zeist, The Netherlands
Eli Lilly Nederland B.V., Houten, The Netherlands

Manuscripts based on the studies described in this thesis

Chapter 2

van Winden AWJ, Gast MCW, Beijnen JH, Rutgers EJTh, Grobbee DE, Peeters PHM, van Gils CH. Validation of previously identified serum biomarkers for breast cancer with SELDI-TOF MS: a case-control study. *BMC Med Genomics, 2009 Jan 19;2:4.*

Chapter 3

van Winden AWJ, Beijnen JH, Loof A, van Heerde WL, Vermeulen R, Peeters PHM, van Gils CH. Search for breast cancer biomarkers in fractionated serum samples by protein profiling with SELDI-TOF MS. *Submitted*

Chapter 4

van Winden AWJ, Krop EJM, Kåredal MH, Gast MCW, Lindh CH, Jeppsson MC, Jönsson BAG, Grobbee DE, Peeters PHM, Beijnen JH, van Gils CH, Vermeulen R. Searching for early breast cancer biomarkers by serum protein profiling of pre-diagnostic samples; a nested case-control study. *Submitted*

Chapter 5

van Winden AWJ, van den Broek I, Gast MCW, Engwegen JYMN, Sparidans RW, van Dulken EJ, Depla ACTM, Cats A, Schellens JHM, Peeters PHM, Beijnen JH, van Gils CH. Serum degradome markers for the detection of breast cancer. *Submitted*

Chapter 6

van Winden AWJ, Rodenburg W, Pennings JLA, van Oostrom CTM, Beijnen JH, Peeters PHM, van Gils CH, de Vries A. Bead-based multiplexed immunoassay to identify early breast cancer biomarkers in pre-diagnostic serum. *Submitted*

Chapter 7

van Winden AWJ, Vermeulen R, Peeters PHM, Beijnen JH, van Gils CH, Early diagnostic protein biomarkers for breast cancer: how far have we come? *Submitted*

Contents

Chapter 1

General introduction

Incidence of breast cancer

The incidence of female breast cancer in The Netherlands has doubled from 1950 to 1990 to around 100 per 100,000 person-years. The incidence then dropped, but increased again since 2000 to around 124 per 100,000 person-years in 2003 (1). In 2007 there were approximately 12,800 women diagnosed with primary breast cancer (2), and 3,180 women died because of the disease (3). Despite the increase in incidence, age-standardized mortality rates have been constant over time, and even decreased since the introduction of the national breast cancer screening program. Although 10-year survival drastically improved from the late 1970s to 2004, Dutch mortality rates for breast cancer still are among the highest in Europe (23.1 deaths per 100,000 during 2000-2004) (1,4,5). For comparison, only in Denmark breast cancer mortality rates are higher (25.9 deaths per 100,000), while rates are lowest in Spain with 14.5 deaths per 100,000 person-years (5).

Early diagnosis of breast cancer

To improve survival of breast cancer early detection is of vital importance. A European-wide study investigating the influence of disease stage at diagnosis on survival showed that in regions with high percentages of women with early stage disease (T1N0M0), the overall 5-year relative survival was much higher compared to the overall survival in regions with low percentages of women diagnosed in an early stage (6). For example, a region in France that showed the highest percentage of women with early stage disease (39%), also showed the highest 5-year overall survival (86%), while five-year overall survival was lowest in Estonia (66%), where the proportion of early stage disease was also the lowest (9%). Differences in survival between regions greatly reduced after adjustment for TNM stage, indicating that survival differences were mainly due to differences in stage at diagnosis (6). In The Netherlands a trend supporting these findings is observed. In the years that the relative 10-year survival increased from 53% to 75% for women aged 50-69 (between 1975 and 2004), the proportion of women diagnosed with Stage III & IV breast cancer decreased from 30% to 13%, while the proportion of women diagnosed with Stage I breast cancer increased from 25% to 39% (1). This change to a more favorable stage distribution in recent years is mainly the merit of the nation-wide breast cancer

screening program. This program uses mammography at regular intervals. However, even among screened women 1 out of 3 breast cancers occur in between two screening examinations (7). Moreover, a quarter of all breast cancers occur in women aged 49 years or younger (1), but these women are currently not included in the national breast cancer screening programs due to reduced sensitivity of mammography. Younger women have breasts with higher density compared to older women (8,9), which decreases the sensitivity of mammography to detect small breast lesions (8-10). The aforementioned groups of women would therefore benefit from early detection techniques other than mammography.

To further increase the proportion of breast tumors diagnosed in an early stage, new tests are needed. This is the reason that in recent years the search for new biomarkers, mainly in serum, received a lot of attention. Changes in the serum proteome, for example could indicate the presence of a breast tumor. These biomarkers could originate from the tumor or the tumorigenesis process, but could also be generated by processes that are a response to the presence of the tumor. If the presence of these markers can be confirmed in very early stages of breast cancer, they yield high potential as a diagnostic tool for a-symptomatic breast lesions that are currently undetectable by mammographic screening.

Methods of protein based biomarker investigation

There are two approaches for the search for new protein biomarkers for cancer. The first method is referred to as a discovery-oriented approach. This method does not need any a-priori hypothesis about biomarkers, but compares the serum protein profile of cancer cases with controls. The classical method for this is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Proteins are with this method separated in two dimensions: their isoelectric point (pI) and their mass. Although this method can measure the intensities of thousands of proteins in a sample simultaneously, it has poor reproducibility and low throughput (one sample per gel) (11,12). In recent years, major developments have been made in mass spectrometry (MS) techniques, such as surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS and matrix assisted laser desorption/ionization (MALDI) -TOF MS. With these techniques proteins are purified from a small amount

of sample, either by arrays with various chromatographic surfaces (SELDI) or with the use of reversed-phase C18 magnetic beads (MALDI). They are then mixed with a matrix solution which leads to crystallization. After irradiation of the matrix-embedded sample, molecular ions are generated of which the mass relative to the charge, and the relative amount is measured. The result is a protein profile that visualizes a large part of the proteome in the sample. By comparing these protein profiles between breast cancer patients and healthy controls, proteins can be detected that are differently expressed between these groups. These techniques are especially suitable for analysis of lower molecular weight proteins (up to about 15,000 Dalton). The advantage is that a large number of the proteins in a sample can be measured simultaneously in a large number of samples at once (96 samples per wells plate). Additionally, different isoforms and post-translational modifications of one protein can be detected with these methods. However, the dynamic range for protein detection with these techniques is much smaller (10^2) than the range in which proteins are present in serum (10^{10}) (13). Consequently, only proteins that are present in the highest concentration range can be detected, which limits the detection of lower abundant proteins. With liquid chromatography tandem MS (LC-MS/MS), a mixture of peptides that resulted from protein digestion with trypsin can be separated and peptide masses can be measured. Based on this information, a few hundred to more than a 1000 (for three-dimensional LC) proteins per sample can be directly identified and quantified (11). There is a high likelihood of false-positive results with these MS techniques, because many proteins are measured and there is no prior hypothesis. Therefore, results of such experiments have to be interpreted with caution.

The second approach in biomarker research is the candidate-based method. This method is based on an a-priori hypothesis about breast cancer biomarkers. These studies use for instance antibody-based assays, such as ELISA (enzyme-linked immunosorbent assay), and the relatively new multiplex bead-based immunoassay. With ELISA the amount of material needed per marker to be measured is in the order of 50-100 µl. A major advantage of the multiplex bead-based immunoassay is its ability to simultaneously measure multiple specific proteins of interest, in much

smaller sample volumes (10-50 µl) than ELISA (14). This technique is therefore very suitable to evaluate a panel of candidate markers.

Objective of this thesis

The objective of this thesis is to identify biomarkers that indicate the presence of a breast lesion in an early stage.

Discovery-oriented approaches

In the first part of this thesis we present the results of discovery-based methods. We use SELDI-TOF MS and two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry (2D-nanoLC-MS/SM) to discover new breast cancer biomarkers.

Studies among symptomatic and/or mammographically detected breast cancer

First, we study the serum proteome using SELDI-TOF MS in samples of patients with symptomatic and/or mammographically detected breast cancer. In this study we try to replicate the results for three putative biomarkers detected in previous studies (**Chapter 2**). In **Chapter 3** we study the protein profiles of a second set of symptomatic patients to find new markers. In this study, we fractionate serum samples before protein profiling with SELDI-TOF MS to enhance discovery of low abundant breast cancer markers. These studies in symptomatic and/or mammographically detected breast cancer patients could potentially render new biomarkers that are differentially expressed among cases and controls. However, their utility for *early* detection remains unclear. To address this question studies using pre-diagnostic serum samples are needed.

Studies among asymptomatic breast cancer cases using pre-diagnostic serum samples

In **Chapter 4** we use both SELDI-TOF MS and 2D-nanoLC-MS/MS for protein profiling of pre-diagnostic serum samples of breast cancer cases. These cases were diagnosed within three years after enrollment in the Prospect-EPIC (European Prospective

Investigation into Cancer and nutrition) cohort. This study population is important for finding biomarkers that are able to detect cancer in an asymptomatic phase. The other side of the coin is that responses are likely to be less pronounced than in studies using symptomatic and/or mammographically detected breast cancer cases, and therefore harder to detect.

Candidate-based approaches

In the second part of this thesis we focus on the quantitative measurement of several candidate biomarkers. In **Chapter 5** we use a validated liquid chromatography-tandem mass spectrometric assay (LC-MS/MS) specifically developed to quantitatively measure the concentration of six potential breast cancer biomarkers (15). We determine the discriminative value of these markers in symptomatic and/or mammographically detected breast cancer patients. We also investigate the effect of surgical removal of the tumor on these biomarker concentrations to further address causality between the tumor or tumor-microenvironment and the biomarker. In **Chapter 6** we study the diagnostic capacity of a panel of ten candidate markers in asymptomatic stages of breast cancer. Candidate breast cancer markers are measured with a bead-based multiplexed immunoassay in pre-diagnostic serum samples of the same Prospect-EPIC participants as included in the study described in Chapter 3.

Finally, in **Chapter 7** I present an overview of potential breast cancer biomarkers detected until date and discuss methods of protein profiling for detection of breast cancer biomarkers. I conclude with suggestions for future biomarker research.

References

1. Louwman WJ, Voogd AC, van Dijck JA, Nieuwenhuijzen GA, Ribot J, Pruijt JF et al. On the rising trends of incidence and prognosis for breast cancer patients diagnosed 1975-2004: a long-term population-based study in southeastern Netherlands. ***Cancer Causes Control*** *2008;* 19(1): 97-106.
2. Kennisnetwerk Integrale Kankercentra. http://www.ikcnet.nl/uploaded/docs/Landelijk/cijfers/incidentie%202007/A01_NL.xls *Retrieved January 2010*.
3. Kennisnetwerk Integrale Kankercentra. http://www.ikcnet.nl/uploaded/docs/Landelijk/cijfers/incidentie%202007/B1_NL.xls *Retrieved January 2010*.
4. Botha JL, Bray F, Sankila R, Parkin DM. Breast cancer incidence and mortality trends in 16 European countries. ***Eur. J. Cancer*** *2003;* 39(12): 1718-1729.
5. La Vecchia C, Bosetti C, Lucchini F, Bertuccio P, Negri E, Boyle P et al. Cancer mortality in Europe, 2000-2004, and an overview of trends since 1975. ***Ann. Oncol.*** *2009; Epub ahead of print*.
6. Sant M, Allemani C, Capocaccia R, Hakulinen T, Aareleid T, Coebergh JW et al. Stage at diagnosis is a key explanation of differences in breast cancer survival across Europe. ***Int. J. Cancer*** *2003;* 106(3): 416-422.
7. National evaluation of breast cancer screening in the Netherlands 1990-2007. Twelfth evaluation report *2010*.
8. Boyd NF, Guo H, Martin LJ, Sun L, Stone J, Fishell E et al. Mammographic density and the risk and detection of breast cancer. ***N. Engl. J. Med.*** *2007;* 356(3): 227-236.
9. Mandelson MT, Oestreicher N, Porter PL, White D, Finder CA, Taplin SH et al. Breast density as a predictor of mammographic detection: comparison of interval- and screen-detected cancers. ***J. Natl. Cancer Inst.*** *2000;* 92(13): 1081-1087.
10. Kolb TM, Lichy J, Newhouse JH. Comparison of the performance of screening mammography, physical examination, and breast US and evaluation of factors that influence them: an analysis of 27,825 patient evaluations. ***Radiology*** *2002;* 225(1): 165-175.
11. Engwegen JY, Gast MC, Schellens JH, Beijnen JH. Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. ***Trends Pharmacol. Sci.*** *2006;* 27(5): 251-259.
12. Lilley KS, Razzaq A, Dupree P. Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. ***Curr. Opin. Chem. Biol.*** *2002;* 6(1): 46-50.
13. Hoffman SA, Joo WA, Echan LA, Speicher DW. Higher dimensional (Hi-D) separation strategies dramatically improve the potential for cancer biomarker detection in serum and plasma. ***J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*** *2007;* 849(1-2): 43-52.
14. Schwenk JM, Gry M, Rimini R, Uhlen M, Nilsson P. Antibody suspension bead arrays within serum proteomics. ***J. Proteome. Res.*** *2008;* 7(8): 3168-3179.
15. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. ***J. Clin. Invest.*** *2006;* 116(1): 271-284.

Part 1

Discovery by protein profiling

Chapter 2

Validation of previously identified serum biomarkers for breast cancer with SELDI-TOF MS: a case-control study

Abstract

Serum protein profiling seems promising for early detection of breast cancer. However, the approach is also criticized, partly because of difficulties in validating discriminatory proteins. This study's aim is to validate three proteins previously reported to be discriminative between breast cancer cases and healthy controls. These proteins had been identified as a fragment of inter-alpha trypsin inhibitor heavy chain H4 (4.3 kDa), C-terminal-truncated form of C3a des-arginine anaphylatoxin (8.1 kDa) and C3a des-arginine anaphylatoxin (8.9 kDa). Serum protein profiles of 48 breast cancer patients and 48 healthy controls were analyzed with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). Differences in protein intensity between breast cancer cases and controls were measured with the Mann-Whitney U test and adjusted for confounders in a multivariate logistic regression model. Four peaks, with mass-to-charge ratio (*m/z*) 4276, 4292, 8129 and 8941 were found that were assumed to represent the previously reported proteins. *M*/z 4276 and 4292 were statistically significantly lower in breast cancer cases compared to healthy controls ($p<0.001$). *M/z* 8941 was lower in breast cancer cases ($p<0.001$) and *m/z* 8129 was not related with breast cancer ($p=0.87$). Adjustment for sample preparation day, sample storage duration and age did not substantially alter results. *M/z* 4276 and 4292 both represented the previously reported 4.3 kDa protein and were both lower in breast cancer patients, which is in accordance with the results of most previous studies. *M/z* 8129 was in contrast with previous studies not related with breast cancer. Remarkably, *m/z* 8941 was lower in breast cancer cases whereas in previous studies it was higher. Differences in patient populations and pre-analytical sample handling could have contributed to discrepancies. Further research is needed before we can conclude on the relevance of these proteins as breast cancer biomarkers.

Introduction

In the search for new breast cancer biomarkers several studies have been performed comparing serum protein profiles of breast cancer cases with those of healthy controls using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) (1-5). In these studies several proteins have been found to be linked with the presence of a breast tumor. However, only occasionally the same proteins are found to be associated with the disease state (1,4,5). This is likely to be caused by the use of different protocols for sample handling, sample preparation and sample storage as well as the use of different ProteinChip arrays and binding- and wash buffers. However, even in validation studies using identical protocols, it has not been possible to replicate the results of previous studies entirely (2,4). Differences in results may be caused by chance; simply because a large number of peaks is tested some proteins will be found to discriminate between breast cancer cases and healthy controls. Also differences between the patient populations (like in age) and in characteristics of the tumors may have led to different results.

For instance, in 2002 Li et al. (3) performed a study to identify serum biomarkers for invasive breast cancer. In this study three proteins with masses of 4.3 kDa, 8.1 kDa and 8.9 kDa were found that were together able to discriminate best between breast cancer cases and non-cancer controls. The non-cancer control group in this study consisted of both women with benign breast disease and healthy controls. The 4.3 kDa protein was lower in breast cancer cases compared to non-cancer controls, the 8.1 and 8.9 kDa proteins were both higher (3).

In 2005, Mathelin et al. (2) performed a study to validate the results of Li et al. (3) with different samples in a different laboratory using the same assay. In this study five peaks were found which masses corresponded to those of the proteins found by Li et al. (2,3). Two peaks probably representing the 4.3 kDa protein and its oxidized form (mass-to-charge ratio (m/z) of 4286 and 4302) were both statistically significantly lower in breast cancer cases compared to non-cancer controls (women with benign breast disease and healthy controls). Two peaks that could possibly represent the 8.9 kDa protein (m/z of 8919 and 8961) were both statistically

significantly higher in breast cancer cases compared to non-cancer controls (2). Above mentioned findings are in accordance with those of Li et al. (3). Contrary, the intensity of the peak likely to represent the 8.1 kDa protein (*m/z* of 8129) was not different between breast cancer cases and non-cancer controls in this study (2).

At the same time, Li also performed a validation study (4) of the candidate biomarkers previously reported by their group. They used different samples but analyzed them in the same laboratory using the same assay. In this study women with benign breast disease were included as cases, together with women with ductal carcinoma in situ (DCIS) or Stage I, II or III breast cancer. Three peaks with masses similar to those of the previously reported proteins were found to be discriminative between cases and controls. Strikingly, in this study the 4.3 kDa peak was higher in cases compared to controls, instead of lower. The 4.3 kDa peak was identified in this study as a fragment of inter-alpha trypsin inhibitor heavy chain H4 (ITIH4). The 8.1 and 8.9 kDa peaks were again both higher in cases compared to controls and were identified in this study as a C-terminal-truncated form of C3a des-arginine anaphylatoxin ($C3a_{desArg\Delta8}$) and C3a des-arginine anaphylatoxin ($C3a_{desArg}$), respectively (4).

Potential limitations of these studies include differences between cases and controls in storage duration (3) and age (2,3). In the present study we analyzed samples of cases and controls, who were frequency matched for age at intake and storage duration of their serum sample, with the same protocol. With this study we aimed to determine whether the three previously reported proteins (2-4) are truly discriminative between breast cancer cases and healthy controls, after adjustment for any differences in age and storage duration.

Material and Methods

Study population

We performed a case-control study with 48 women (aged 25 to 88 years) diagnosed with primary invasive breast cancer and 48 healthy controls (healthy female relatives or friends of the patients who accompanied them to the hospital). Cases and controls were frequency matched regarding their age and the storage duration of their serum sample as much as possible. Age and menopausal status of the cases

were obtained through examination of the medical records. Tumor type, tumor size, tumor differentiation, estrogen receptor (ER) and progesterone receptor (PR) status and HER2/neu and p53 expression were determined by pathological examination. Lymph node involvement and the presence of metastasis were also examined.

Serum samples of the cases and controls, which were collected between January 2003 and June 2005, were obtained from a serum bank at the Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands. These serum samples were collected after receiving individuals' informed consent under approval of the Institutional Review Board Control. Serum samples of the cases were obtained after diagnosis of breast cancer and before surgery or any other kind of treatment. Blood collection, processing and storage of the serum samples was performed under strictly defined conditions which were the same for cases and controls. All serum samples were collected with the use of BD Vacutainer SST plastic serum tubes with clot activator and gel (Becton-Dickinson, Franklin Lakes, NJ, USA). After collection, blood samples were allowed to clot for 30 minutes at room temperature and were subsequently centrifuged for 15 minutes at 3000 rpm at room temperature. Thereafter, samples were aliquotted and stored at -30°C.

Protein profiling

We executed the same sample preparation protocol and used the same ProteinChip arrays (IMAC30 activated with nickel; Bio-Rad Labs, Hercules, Ca, USA) and wash- and binding buffers as described by Li et al. (3). A minor difference is that in this study unsaturated instead of saturated sinapinic acid (SPA; Bio-Rad Labs) was used, prepared according to manufacturer's instruction.

Samples of breast cancer cases and controls were alternately and in duplicate manually applied to the arrays. Half of the case samples and half of the control samples were prepared and applied to the arrays on day 1 and the other half of the case and control samples were prepared and applied to the arrays on day 2. Detection of the proteins bound to the arrays was performed with SELDI-TOF MS for all arrays on the same day. For this we used the newest SELDI-TOF MS instrument, the PCS 4000 ProteinChip Reader, the enterprise edition (Bio-Rad Labs). Contrary to the first generation instruments, the PCS 4000 has an increased dynamic range of

the detector. This means that it has no fixed maximal signal and therefore saturation of the detector is less likely to occur. Furthermore, instead of using arbitrary units, peak intensities are scaled in μA, corresponding to the real electric current generated by the impact of ions onto the detector (6).

At the ProteinChip Reader, 10 shots with an intensity of 4500 nJ were fired on every fourth position of the entire spot. The detector attenuation was set to 1000 Da and masses up to 200,000 Da were detected with a focus at 8000 Da. The *m/z* was calibrated externally with an All-in-1 standard peptide mixture containing vasopression (1084.3 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH 1-24 (2933.5 Da), bovine insulin beta-chain (3495.9 Da), human insulin (5807.7 Da) and recombinant hirudin (6963.5 Da) (Bio-Rad Labs). Data were analyzed with the ProteinChip Software package, version 3.1 (Bio-Rad Labs). Baseline subtraction was applied to the spectra and intensities of masses between 3,000 and 200,000 Da were normalized to the average total ion current (TIC) of all spectra. Ion noise from the matrix passed through in the spectra up to 3,000 Da and therefore this region was excluded from the analysis. Spectra with a very high or low TIC were eliminated from the analysis. This was the case if the normalization factor (NF) of a spectrum deviated more than two standard deviations (SD) from the mean NF.

With the Biomarker Wizard (BMW) software application (Bio-Rad Labs), peaks with a signal-to-noise ratio greater than 5 and which were present in at least 20% of the spectra were auto-detected. Peak clusters were completed with peaks with a signal-to-noise ratio greater than 2, within a 0.3% mass window of the detected peaks. In the spectra with no peak in a detected peak cluster, a mark was placed at the average *m/z* of that peak cluster. In the duplicate spectra of a subject, the intensities of the peaks with the same mass were averaged. To estimate the reproducibility of these duplicates the median coefficient of variance (CV) and the inter-quartile range (IQR) was calculated per *m/z* in cases and controls together.

Data analysis

The Mann-Whitney U test was used to test if the median intensities of the detected peaks were statistically significantly different (p-value<0.01) between breast cancer

cases and healthy controls. The area under the ROC (Receiver Operating Characteristic) curve (AUC) was estimated per peak to evaluate the performance of a peak to classify samples into the two groups.

The peaks with an *m/z* most similar to the mass of the previously reported proteins (2-4) were selected and all analyses described below were performed on these peaks. These peaks were most likely to represent the previously reported proteins (2-4), also because these peaks were detected under the same conditions as were used to detect the previously reported proteins (2-4). We analyzed the same matrix (serum) on the same array type using the same protocol. By doing this we did not only select those proteins with the same mass, but also those binding under the same conditions to the chip, indicating similarity in isoelectric point (pI).

In the control group we investigated whether age, the duration of sample storage, and day of sample preparation influenced peak intensity. For this, we compared the median intensities of the peaks between different categories of these variables with the Kruskal-Wallis test (if >2 categories) or with the Mann-Whitney U test (if 2 categories). To this end, controls were categorized according to tertiles of age; <49.3 years, 49.3-57.5 years or >57.5 years and were also categorized according to quartiles of sample storage duration; <12 months, 12-17 months, 18-31 months or >31 months. Controls were also divided in two groups according to day of sample preparation; day 1 or day 2. Subsequently, the Jonckheere-Terpstra test was used to test if there was a trend in the median peak intensities between the different categories of age and sample storage duration.

To investigate whether any relationship between the intensity of the peaks and the presence of a breast tumor could be explained by above-mentioned variables, a logistic regression analysis was performed, estimating the crude odds ratio (OR) and the OR adjusted for these variables. For this purpose cases and controls were categorized by tertiles of peak intensity (low, intermediate or high intensity), based on the distribution in the control group. Because the range in peak intensities strongly differed between samples prepared on day 1 and those prepared on day 2, this was done separately for the set of cases and controls prepared on day 1 and the set prepared on day 2. Afterwards, the subjects in the same category in the two sets were combined. We also performed a backward logistic regression

analysis in which we simultaneously included the peaks assumed to represent the previously reported proteins (2-4) (continuous). Peaks were removed from the model if they did not statistically significantly contribute to the discrimination of cases and controls.

To investigate whether the intensities of the peaks were related to stage of disease, we tested if the median peak intensities were different between categories of TNM stage, tumor size, lymph node involvement and tumor differentiation. We also tested if the peak intensities were related to menopause status, hormone receptor status and HER2/neu and p53 expression. The relation between menopause status and peak intensities was investigated in cases only, since no information about this variable was available for the controls. To test these relations we used the Kruskal-Wallis test or the Mann-Whitney U test, dependent on number of categories. TNM stage was categorized as: IIA (*n*=26), IIB (*n*=11) or III (= IIIA + IIIC, *n*=11) and tumor size as: ≤2 cm (*n*=18) or >2 cm (*n*=30). Lymph node involvement was categorized as: no regional lymph node metastasis (*n*=9) or positive axillary lymph nodes (*n*=39). Tumor differentiation was classified into high and intermediate differentiation (*n*=25) or low differentiation (*n*=22) (1 missing value). Menopause status was categorized as pre- (*n*=15) or postmenopausal (*n*=30) (2 missing values). ER and PR status were categorized as ER- (*n*=12) or ER+ (*n*=35) and PR- (*n*=19) or PR+ (*n*=28). HER2/neu and p53 expression was categorized as HER2/neu- (*n*=35) or HER2/neu+ (*n*=12) and p53- (n=18) or p53+ (*n*=23) (6 missing values). The Jonckheere-Terpstra test was used to test if there was a trend in the median peak intensities between the different categories of TNM stage. For above mentioned statistical analyses SPSS 12.0.1 was used and p-values <0.05 were considered statistically significant.

Results

Study population

The characteristics of the breast cancer cases and healthy controls are presented in **Table 1**. The average age at time of blood collection was 58 years for the cases and 53 years for the controls. Sixty-five percent of the cases were postmenopausal and 35% was premenopausal. The menopausal status of two women was not reported in

Table 1 - Characteristics of the breast cancer cases, the healthy controls and their serum samples

	Breast cancer cases (*n*=48)	Healthy controls (*n*=48)
Age at diagnosis (years)		
Mean (SD)	58 (14)	53 (9)
Menopause status, *n* (%)		
Pre	16 (34.8)	-
Post	30 (65.2)	-
Missing	2	48 (100)
Sample storage duration (months)		
Median (IQR)	16 (11-35)	17 (11-31)
Time from diagnosis to blood sampling (days)		
Median (IQR)	7 (0-20)	
TNM stage, *n* (%)		
IIA	26 (54.2)	
IIB	11 (22.9)	
IIIA	6 (12.5)	
IIIC	5 (10.4)	
Tumor size, *n* (%)		
>0.5-1 cm	2 (4.2)	
>1-2 cm	16 (33.3)	
>2-5 cm	28 (58.3)	
>5 cm	2 (4.2)	
Lymph node involvement, *n* (%)		
No	9 (18.8)	
1-3	29 (60.4)	
> 3	10 (20.8)	
Differentiation, *n* (%)		
High	6 (12.8)	
Intermediate	19 (40.4)	
Low	22 (46.8)	
Missing	1	
ER status, *n* (%)		
Negative	13 (27.1)	
Positive	35 (72.9)	
PR status, *n* (%)		
Negative	20 (41.7)	
Positive	28 (58.3)	
HER2/neu expression, *n* (%)		
Negative	36 (75.0)	
Positive	12 (25.0)	
P53 expression, *n* (%)		
Negative	18 (42.9)	
Positive	24 (57.1)	
Missing	6	

SD: standard deviation; IQR: inter-quartile range; ER: estrogen receptor; PR: progesterone receptor

their medical record. No information about menopause status was available for the controls. For cases, the median duration between diagnosis and sample collection was 7 days and all samples were collected before the start of treatment. The median duration of sample storage until analysis was almost equal for the samples of the cases (16 months) and those of the controls (17 months).

More than half of the cases were affected with Stage IIA breast cancer and nearly a quarter of the cases were diagnosed with Stage IIB breast cancer. Stage III breast cancer was diagnosed in the other cases. More than 60% of the tumors were larger than 2 cm and in more than 80% of the cases the tumor had spread to the axillary lymph nodes. Eighty-seven percent of the tumors showed a low or intermediate degree of differentiation (1 missing value). None of the cases was diagnosed with distant metastases. The majority of the tumors were ER+ (73%) and PR+ (58%). A quarter of the tumors showed an overexpression of HER2/neu and half of the tumors had an overexpression of p53. For 6 cases, p53 status was unknown. These subjects were diagnosed in the Netherlands Cancer Institute (NKI), however their surgery was performed in another hospital. In those hospitals, the p53 status of the tumor was not determined.

Peak detection

After normalization, 17 of the 192 spectra (48 cases and 48 controls in duplicate) were eliminated from the analysis because their NF deviated more than two SD from the mean NF. These spectra belonged to 10 controls and 6 cases. Of one case both spectra (both duplicates) had to be eliminated. With the BMW software application, 45 peak clusters were auto-detected in the 175 left spectra, in the mass-region between 3,000 Da and 200,000 Da. Subsequently, in the duplicate spectra of a subject, the intensities of the detected peaks with the same mass were averaged. For the subjects with one spectrum left, only peak intensities in that spectrum were used for analysis. A Mann-Whitney U test performed on these (averaged) peak intensities showed that the intensities of 20 of the 45 peaks were statistically significantly different between breast cancer cases and healthy controls (p-value <0.01). These discriminatory peaks are listed in a table in the **Appendix** in order of *m/z*.

Four peaks were detected which we assumed to represent the three previously reported proteins (2-4) based on similarities in mass and detection under the same conditions. The median intensity of these peaks, as well as the p-value of the Mann-Whitney U test and the AUCs are listed in **Table 2** in order of the *m/z* of the peaks. Two peaks were detected that both were assumed to represent the previously reported protein of 4.3 kDa (2-4). This protein was identified by Li et al. (4) as a fragment of ITIH4 and has a theoretical molecular weight (Mw) of 4285 Da. We based this mass on the amino acid sequence reported by Song et al. (7) and calculated it using ExPASy Proteomic Server (8). The two peaks found in our study had an *m/z* which was almost similar to that Mw, namely 4276 and 4292. The mass difference between these two peaks is 16 Da, the exact mass of an oxygen-atom. The intensities of these two peaks were also highly correlated (Pearson R^2=0.834; p<0.001 (in the control group)), indicating that they were present in about the same ratio in every spectra. Consequently, we assumed *m/z* 4276 to be the 4.3 kDa ITIH4 fragment and m/z 4292 to be the oxidized form of this protein. Both these peaks should therefore be considered for the comparison with the previously reported 4.3 kDa protein (2-4). The median intensity of both peaks was statistically significantly lower in cases compared to controls (11.04 (IQR:2.10-29.31) versus 39.48 (IQR:14.17-77.58); p<0.0001 and 14.54 (IQR:8.52-29.54) versus 42.07 (IQR:29.67-63.39); p<0.0001, respectively).

Table 2 - Intensities in cases and controls of the peaks most likely representing the previously reported proteins (2-4) in order of *m/z*

	Breast cancer cases (*n*=47)		Healthy controls (*n*=48)			Mann-Whitney U test	ROC-curve	
M/z	*Median intensity*	*IQR*	*Median intensity*	*IQR*	*Intensity in cases vs. controls*	*p-value*	*AUC*	*95%CI*
4276	11.04	2.10-29.31	39.48	14.17-77.58	Lower	<.0001	0.72	0.61-0.82
4292	14.54	8.52-29.54	42.07	29.67-63.39	Lower	<.0001	0.77	0.67-0.87
8129	25.92	20.48-28.19	26.05	21.06-28.08	-	.870	0.51	0.39-0.63
8941	31.27	24.07-44.88	73.47	48.69-86.72	Lower	<.0001	0.83	0.75-0.91

M/z: mass-to-charge ratio; IQR: inter-quartile range; ROC-curve: Receiver Operating Characteristic curve; AUC: area under the curve; 95%CI: 95% confidence interval

One peak was found in this study that was assumed to represent the previously reported protein of 8.1 kDa (2-4). This protein was identified by Li et al. (4) as a C-terminal-truncated form of $C3a_{desArg.}$ Its theoretical Mw is 8133 Da, based on the amino acid sequence reported by Li et al. (4) and calculated using ExPASy Proteomic Server (8). The *m/z* of the peak found in our study was almost identical to that Mw, namely 8129. However, no difference in the intensity of this peak was observed between cases and controls (p=0.87).

Also one peak was found that was assumed to represent the previously reported protein of 8.9 kDa (2-4). This protein was identified by Li et al. (4) as $C3a_{desArg}$ and has a theoretical Mw of 8938 Da. We based this mass on the amino acid sequence reported by Li et al. (4) and calculated it using ExPASy Proteomic Server (8). The *m/z* of the peak found in our study was almost identical to that Mw, namely 8941. The intensity of this peak was statistically significantly lower in cases compared to controls (31.27 (IQR:24.07-44.88) versus 73.47 (IQR:48.69-86.72); p<0.0001). The small mass differences between the theoretical masses of the previously found proteins and the *m/z* of the peaks found in our study could be due to the mass calibration of the ProteinChip Reader used. Representative spectra from breast cancer cases and healthy controls showing the four peaks found in this study are presented in **Figure 1**.

The median CV's per peak in cases and controls were 31% (IQR:15-62), 17% (IQR:7-47), 11% (IQR:5-22) and 13% (IQR:5-23) for *m/z* 4276, 4292, 8129 and 8941, respectively. The duplicate analyses were also investigated separately and the results for the four peaks in the two analyses were similar to each other and to the results of the total analysis, i.e. the difference in intensity between cases and controls was similar with comparable significance levels (data not shown).

Influence of day of sample preparation, sample storage duration and age, on peak intensities

The relations between peak intensities and day of sample preparation, sample storage duration and age were estimated in the control group. The results are presented in **Table 3**. A clear relation between peak intensity and day of sample preparation was found for the peaks with an *m/z* of 4276, 4292 and 8129. The

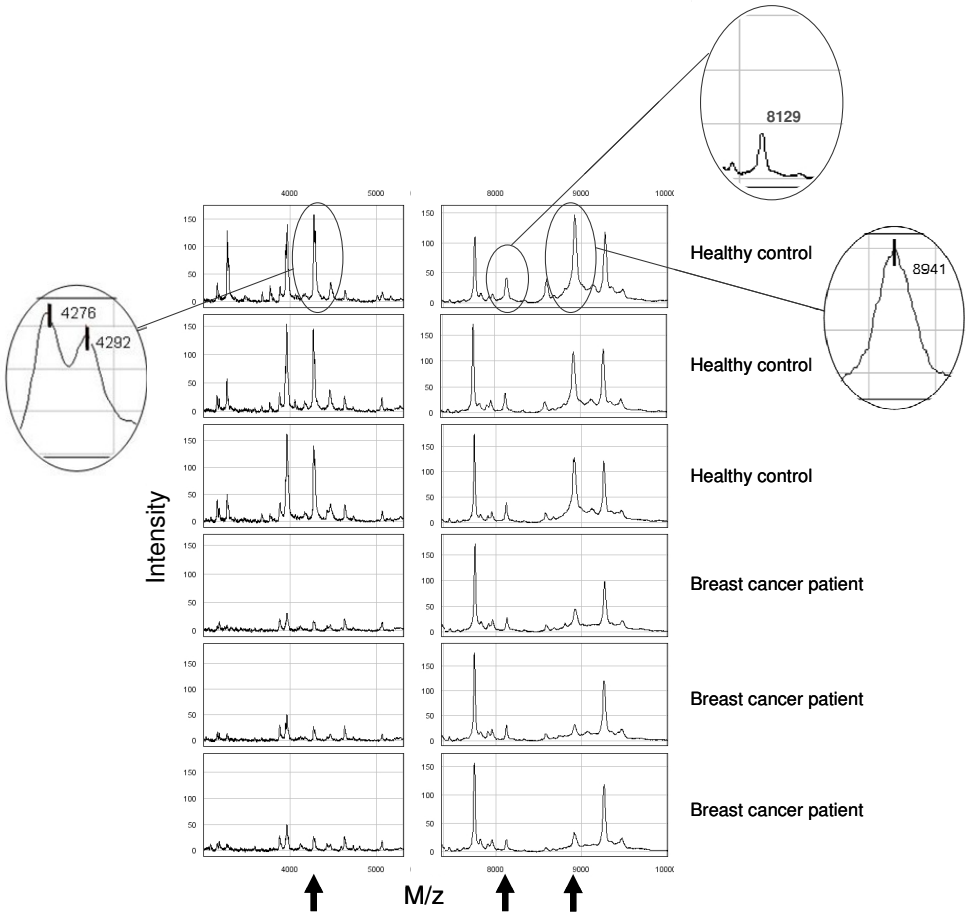

Figure 1 - Representative SELDI-TOF MS spectra showing the intensity of the peaks with an *m/z* of 4276, 4292, 8129 and 8941 in breast cancer cases and healthy controls

intensities of these peaks were statistically significantly higher in control samples prepared on day 1 than those prepared on day 2. We also found a statistically significant trend in intensity over the four storage duration categories for these three peaks, with lower intensities in samples that were stored for a longer time. Unintentionally, samples stored for less than 18 months were all prepared on day 1 and samples stored for 18 months or more on day 2. Therefore, it is difficult to disentangle the effects of day of preparation and storage duration. Age was not significantly related to the intensity of any of the peaks as shown in **Table 3**.

Table 3 - Intensities of the peaks per day of sample preparation, category of sample storage duration and age group in healthy controls

		M/z 4276	*M/z* 4292	*M/z* 8129	*M/z* 8941
	n	*Median intensity (IQR)*	*Median intensity (IQR)*	*Median intensity (IQR)*	*Median intensity (IQR)*
Day of sample preparation					
Day 1	24	74.04 (47.56-103.27)	62.78 (45.17-79.91)	27.39 (22.79-29.62)	78.94 (45.96-84.49)
Day 2	24	24.83 (9.58-38.24)	34.61 (28.54-39.32)	22.65 (18.02-26.89)	72.85 (50.43-105.05)
p-value*	48	<.001	.001	.008	.853
Sample storage duration (months)					
≤ 11	13	75.81 (33.59-118.06)	63.48 (37.10-79.58)	27.54 (21.94-31.63)	57.48 (43.11-79.66)
12-17	11	72.28 (50.60-87.56)	62.11 (46.25-83.88)	27.24 (24.70-29.87)	81.30 (52.43-99.14)
18-31	12	32.33 (12.37-40.50)	33.99 (25.21-45.65)	23.66 (15.58-27.08)	62.97 (42.67-111.06)
≥ 32	12	16.43 (8.38-35.85)	34.77 (29.84-37.99)	22.18 (20.46-26.89)	73.47 (52.76-105.05)
p-value#	48	.001	.011	.063	.620
p-trend†	48	<.001	.003	.032	.423
Age (yrs)					
< 49.3	16	45.26 (19.00-77.58)	46.38 (31.02-62.95)	25.15 (21.29-27.55)	73.84 (49.50-84.49)
49.3-57.5	16	19.79 (8.38-62.11)	35.78 (28.52-63.14)	24.90 (21.98-27.62)	69.16 (39.17-85.8)
> 57.5	16	45.90 (23.58-85.81)	40.01 (30.47-72.05)	27.12 (20.43-33.59)	76.15 (56.15-101.64)
p-value#	48	.346	.680	.753	.850
p-trend†	48	.583	.940	.438	.880

M/z: mass-to-charge ratio; IQR: inter-quartile range; * Mann-Whitney U Test ; # Kruskal Wallis Test; † Jonckheere-Terpstra Test

Relationships between peak intensities and the presence of breast cancer

The relationships between the intensities of the four peaks and the presence of breast cancer, before and after adjustment for day of sample preparation, sample storage duration and age are listed in **Table 4**. For the peaks with an *m/z* of 4276, 4292 and 8941, women with low peak intensity were statistically significantly more

often affected with breast cancer than women with high peak intensity (reference group). The crude OR's with 95% confidence interval (95%CI) for these three peaks were 6.0 (95%CI:2.0-18.2), 7.6 (95%CI:2.4-24.3) and 13.0 (95%CI:3.3-50.8), respectively. Women with intermediate peak intensity for any of these three peaks were not more likely to be affected with breast cancer than women in the reference group. The intensity of the peak with an *m/z* of 8129 was not related to the presence of breast cancer. After adjustment for day of sample preparation, sample storage duration and age results remained essentially the same.

Table 4 - The relationships between peak intensities and the presence of breast cancer before and after adjustment for sample characteristics and subject age

	n			
	cases	*controls*	*Crude OR (95%CI)*	*Adjusted OR* (95%CI)*
***M/z* 4276**				
Low intensity	36	16	6.0 (2.0-18.2)	5.3 (1.7-17.0)
Intermediate intensity	5	16	0.8 (0.2-3.3)	0.9 (0.2-3.7)
High intensity	6	16	1.0 (referent)	1.0 (referent)
***M/z* 4292**				
Low intensity	38	16	7.6 (2.4-24.3)	6.4 (1.8-22.3)
Intermediate intensity	4	16	0.8 (0.2-3.5)	0.7 (0.2-3.1)
High intensity	5	16	1.0 (referent)	1.0 (referent)
***M/z* 8129**				
Low intensity	13	16	0.8 (0.3-2.3)	0.7 (0.2-2.0)
Intermediate intensity	19	17	1.1 (0.4-2.9)	1.0 (0.4-2.8)
High intensity	15	15	1.0 (referent)	1.0 (referent)
***M/z* 8941**				
Low intensity	39	16	13.0 (3.3-50.8)	13.3 (3.2-55.0)
Intermediate intensity	5	16	1.7 (0.3-8.2)	1.8 (0.4-9.1)
High intensity	3	16	1.0 (referent)	1.0 (referent)

OR: odds ratio; 95%CI: 95% confidence interval; *M/z:* mass-to-charge ratio; * OR's were adjusted for the following variables: day of preparation (day 1 or day 2), age (continuous) and storage duration (continuous); Tertiles of the intensities per peak determined in the controls, separately for day of preparation, were applied to cases prepared on the same day. Subjects were afterwards combined in three categories.

Subsequently, we performed a backward logistic regression analysis in which we simultaneously included the four peaks (continuous). *M/z* 8129 did not statistically significantly contribute to the model. *M/z* 4276, *m/z* 4292 and *m/z* 8941

all statistically significantly contributed to the discrimination of cases and controls (data not shown).

Relationships between peak intensities and tumor characteristics and menopause status

The relations between the intensities of the four peaks and tumor stage, tumor size, lymph node involvement and tumor differentiation are shown in **Table 5**. The median intensities of the peaks with an *m/z* of 4276 and 4292 in cases without lymph node involvement were higher than in cases with lymph node involvement (*m/z* 4276: 24.34 (IQR:5.99-72.28) versus 8.66 (IQR:1.87-24.37) and *m/z* 4292: 25.69 (IQR:9.91-54.35) versus 13.13 (IQR:5.97-23.74), although not statistically significantly (p=0.14 and p=0.14). No statistically significant relations were observed between the intensities of the four peaks and any of the other tumor characteristics.

The relations between the intensities of the four peaks and menopause status, hormone receptor status and HER2/neu and p53 expression are listed in **Table 6**. For *m/z* 8129, a statistically significant lower intensity was observed in postmenopausal cases compared to premenopausal cases (25.3 (IQR:17.8-26.6) versus 27.6 (IQR:22.2-32.7); p=0.03). In cases with p53- tumors compared to cases with p53+ tumors, the lower intensity for *m/z* 8129 was borderline statistically significant (23.7 (IQR:17.6-26.8) versus 26.5 (IQR:24.8-30.1); p=0.07). No other statistically significant relation was found.

Table 5 - Intensities of the peaks for different categories of the tumor characteristics

		M/z 4276	*M/z* 4292	*M/z* 8129	*M/z* 8941
	n	*Median intensity (IQR)*	*Median intensity (IQR)*	*Median intensity (IQR)*	*Median intensity (IQR)*
TNM stage					
IIA	26	13.00 (1.79-83.10)	19.51 (8.07-54.23)	26.27 (23.48-30.07)	33.56 (24.09-50.43)
IIB	10	4.97 (1.33-17.24)	11.44 (4.83-15.79)	25.44 (16.56-27.75)	30.49 (20.16-34.59)
III	11	11.88 (4.89-24.69)	15.86 (9.88-22.87)	25.70 (15.68-26.54)	37.55 (25.26-47.29)
p-value*	47	.340	.211	.203	.473
p-trend#	47	.553	.368	.075	.667
Tumor size					
0.5-2 cm	18	10.24 (1.36-85.58)	18.44 (5.44-63.09)	26.53 (22.86-30.08)	30.48 (23.24-61.21)
> 2 cm	29	11.88 (3.97-24.51)	12.21 (8.71-24.28)	25.90 (19.08-27.45)	32.90 (24.04-44.16)
p-value†	47	.844	.526	.204	.896
Lymph node involvement					
No	9	24.34 (5.99-72.28)	25.69 (9.91-54.35)	26.48 (23.33-30.29)	38.92 (27.68-53.99)
Yes	38	8.66 (1.87-24.37)	13.13 (5.97-23.74)	25.81 (18.87-27.65)	30.49 (23.30-43.31)
p-value†	47	.137	.144	.224	.224
Differentiation					
High-Intermediate	25	11.04 (2.99-24.47)	14.06 (7.86-22.52)	25.72 (19.84-27.45)	31.27 (24.13-42.65)
Low	21	19.90 (2.98-60.12)	17.06 (8.71-44.18)	26.54 (19.68-29.79)	28.99 (23.34-45.88)
p-value†	46	.635	.384	.360	.903

M/z: mass-to-charge ratio; IQR: inter-quartile range; * Kruskal Wallis Test; # Jonckheere-Terpstra Test; † Mann-Whitney U Test

Table 6 - Intensities of the peaks for different categories of hormone-receptor status, Her2/neu and P53 expression and menopause status

		M/z 4276	*M/z* 4292	*M/z* 8129	*M/z* 8941
	n	*Median intensity (IQR)*	*Median intensity (IQR)*	*Median intensity (IQR)*	*Median intensity (IQR)*
ER status					
Negative	12	15.2 (0.7-70.6)	18.3 (6.2-48.1)	26.3 (25.2-28.5)	39.9 (24.3-57.5)
Positive	35	11.0 (3.9-24.3)	14.5 (8.9-22.9)	25.7 (17.9-28.2)	30.5 (24.1-43.4)
p-value*	47	.961	.696	.421	.380
PR status					
Negative	19	7.1 (4.0-29.3)	10.9 (8.5-30.8)	26.0 (21.7-27.8)	31.3 (24.0-43.3)
Positive	28	14.6 (1.8-28.2)	15.4 (6.8-28.7)	25.8 (19.5-29.6)	32.1 (24.1-46.7)
p-value*	47	.649	.696	.762	.588
HER2/neu expression					
Negative	35	11.0 (1.9-29.3)	15.9 (5.6-29.5)	25.9 (20.5-27.8)	32.9 (24.1-46.9)
Positive	12	9.5 (4.3-66.2)	11.1 (9.1-43.2)	26.0 (16.2-29.3)	29.7 (23.3-41.7)
p-value*	47	.961	.826	.751	.575
P53 expression					
Negative	18	6.5 (1.6-24.3)	12.7 (5.3-31.3)	23.7 (17.6-26.8)	27.1 (17.7-42.6)
Positive	23	11.0 (4.7-29.4)	14.9 (8.9-29.5)	26.5 (24.8-30.1)	32.9 (27.8-60.8)
p-value*	41	.599	.546	.070	.172
Menopause status					
Premenopausal	15	7.9 (1.9- 84.3)	12.2 (10.1-69.2)	27.6 (22.2-32.7)	29.0 (22.7-43.3)
Postmenopausal	30	10.2 (2.0-25.6)	14.3 (5.5-29.8)	25.3 (17.8-26.6)	32.1 (24.2-45.4)
p-value*	45	.942	.580	.030	.563

M/z: mass-to-charge ratio; IQR: inter-quartile range; ER: estrogen receptor; PR: progesterone receptor; *Mann-Whitney U Test

Protein identity

The assumption that the proteins found in this study indeed represent the previously reported proteins (2-4) was based on similarities in the mass as well as on the conditions under which these proteins were detected. However, there is also another indication to assume that *m/z* 4276 and *m/z* 4292 indeed represent the 4.3 kDa fragment of the ITIH4 protein and its oxidized form. We found three other peaks in this study which masses (*m/z* 3156, *m/z* 3270 and *m/z* 3965) highly corresponded with the theoretical masses of three other fragments of ITIH4 previously described by Villanueva et al. (9) and Song et al. (7) (3158 Da, 3274 Da and 3972 Da). We based these theoretical masses on the amino acid sequences reported by Villanueva et al. (9) and Song et al. (7) and calculated them using ExPASy Proteomic Server (8). The intensities of *m/z* 3270 and *m/z* 3965 were highly correlated with the intensities of *m/z* 4276 and m/z 4292 (*m/z* 3270 and *m/z* 4276: Pearson R^2=0.812, *m/z* 3270 and *m/z* 4292: R^2=0.722, *m/z* 3965 and *m/z* 4276: R^2=0.821 and *m/z* 3965 and *m/z* 4292: R^2=0.756 ($p<0.001$ for all)). This high correlation in intensity is only expected when all these proteins originated from the same protein. Since masses of all these proteins have very high resemblances with the theoretical masses of the previously reported ITIH4 fragments and these proteins were all detected under the same conditions, we can assume that all these peaks represent fragments of ITIH4.

The peak with an *m/z* of 8941 was actually previously identified in our laboratory in a breast cancer serum sample as $C3a_{desArg}$. The method of protein identification was similar to that performed in the validation study by Li et al. (4). The 8.9 kDa protein was purified using QhyperD fractionation (Biosepra Inc., Malborough, MA, USA) and concentrated on YM50 spin concentrators (Millipore, Billerica, MA, USA). Subsequently, the eluate with the 8.9 kDa protein was de-salted on RP18 beads (Bio-Rad Labs). This purification process was monitored by profiling each fraction on IMAC30 Ni arrays and NP20 arrays (a non-selective, silica chromatographic surface) (Bio-Rad Labs). The de-salted eluate containing the 8.9 kDa protein was subsequently subjected to SDS-PAGE analysis. Gel electrophoresis was performed on Novex NuPage gels (18% Tris-Glycine gel; Invitrogen, San Diego, CA, USA). After staining, the band in the 8.9 kDa region was excised and subjected to passive elution followed by tryptic digestion of the eluate. Profiling of the gel-

eluate on a NP20 array confirmed the presence of the 8.9 kDa protein. Peptide mapping of the tryptic digest identified it as complement component 3 precursor (estimated Z-score 1.57, 4% sequence coverage). Amino acid sequencing of 6 peptides in the tryptic digest by tandem MS on a Q-TOF identified the protein as C3a des-arginine anaphylatoxin ($C3a_{desArg}$, 61% sequence coverage), a protein with theoretical mass 8939.46 Da and pI 9.54. This identity was confirmed by an immunoassay, for which ProteinA beads were loaded with a C3a polyclonal antibody (Abcam Ltd, Cambridge, UK).

Discussion

In this study including 48 breast cancer cases and 48 healthy controls we discovered four peaks with an *m/z* (4276, 4292, 8129 and 8941) within the mass range of the three previously reported proteins (4.3 kDa, 8.1 kDa and 8.9 kDa) (2-4) using a similar analysis protocol (3). Three of these four peaks were found to be discriminative between breast cancer cases and healthy controls. The peaks with an *m/z* of 4276, 4292 and 8941 were all statistically significantly lower in cases compared to controls. The intensity of the peak with an *m/z* of 8129 was not different between cases and controls. After splitting our data of the two duplicates into two groups, results were similar. This together with CV's within an acceptable range indicates that our results are robust.

The peaks with an *m/z* of 4276 and 4292 were assumed to represent a fragment of ITIH4 (the 4.3 kDa protein) and its oxidized form. The lower intensity of the 4.3 kDa protein in breast cancer cases reported in the study by Li et al. (3) and in that by Mathelin et al. (2) was replicated in our study. The higher intensity of the 4.3 kDa protein found in the validation study by Li et al. (4) appears to be an exception.

The 8.1 kDa protein, previously identified as $C3a_{desArg\Delta8}$ (4) was higher in breast cancer cases in both studies by Li et al. (3,4). This could not be replicated in our study. In our study, no difference in intensity between breast cancer cases and controls was found for this protein, neither as it was in the study by Mathelin et al. (2).

In the three previous studies (2-4), the 8.9 kDa protein, identified as $C3a_{desArg}$ was higher in breast cancer cases. This could not be replicated in our study. In our study, this protein was lower in cases compared to controls, which thus appears to be an exception. Remarkably, a lower intensity of this protein was also found by our group in a breast cancer sample-set that consisted the subjects investigated in the current study, but which was extended with about 100 cases and 80 controls (10). Furthermore, it is also remarkable that this protein was the best discriminating protein between breast cancer cases and healthy controls found in this study. The 8941 *m/z* peak found in our study was previously identified by our group as $C3a_{desArg}$, which is thus in agreement with the identity of the 8.9 kDa protein found in the validation study by Li et al. (4). A summary of the results of the several studies is presented in **Table 7**.

Table 7 - Description of the peaks found in the different studies

		Protein 1		Protein 2	Protein 3	
Li et al. (2002) (3)	kDa	4.3 ↓		8.1 ↑	8.9 ↑	
	AUC	0.846		0.795	0.934	
	p-value	-		-	-	
Mathelin et al. (2005) (2)	*M/z*	4286 ↓	4302 ↓	8129 –	8919 ↑	8961 ↑
	AUC	-	-	-	-	-
	p-value[#]	<.000	<.001	.51	<.02	<.001
Li et al. (2005) (4)	Da	± 4300* ↑		8116 ↑	8926 ↑	
	AUC	-		0.65	0.71	
	p-value	-		-	-	
Current study	*M/z*	4276 ↓	4292 ↓	8129 –	8941 ↓	
	AUC	0.716	0.770	0.510	0.830	
	p-value[†]	<.0001	<.0001	.870	<.0001	

M/z: mass-to-charge ratio; ↓ : Lower intensity in cases compared to controls; ↑ : Higher intenstity in cases compared to controls; – : No significant relation; * Exact mass not reported; [#] Used test not reported; [†] Mann-Whitney U Test

Li et al. (4) suggested that the inconsistency in regulation of the 4.3 kDa peak (ITIH4 fragment) between their two studies is caused by the instability of this protein. In their first study (3), serum samples from breast cancer patients were collected during a longer time interval than the control samples, whereas samples in the validation study (4) were all collected within the same 2-year window. If the

instability of this protein causes further truncation during prolonged storage, this would explain why the intensity of this protein is lower in cases than in controls in Li's first study (3). In our study, however, as well as in that by Mathelin et al. (2), storage duration of samples did not differ between cases and controls and still in both studies a lower intensity was found in cases compared to controls. Therefore, the higher intensity of the 4.3 kDa protein found in the validation study by Li et al. (4) cannot be explained by this factor. Also differences in discriminatory power and/or direction of the relation between the several studies for the 8.1 and the 8.9 kDa protein cannot be explained by above mentioned factor.

Another factor that is important for storage is the temperature at which samples are stored. Although in several studies no major differences in serum protein profiles were observed after a storage period of 1-3 months at -20°C, -80°C or liquid nitrogen (11-13), Engwegen et al. (14) found that after a storage duration of 5 months at -20°C compared to -70°C several peaks were significantly increased in intensity. This shows the importance of storing samples at the lowest possible temperature when samples are stored for a prolonged time. Samples analyzed in the studies by Li et al. (3) and Mathelin et al. (2) were stored at -80 °C and -20 °C, respectively for an unknown time. Samples analyzed in the validation study by Li et al. (4) and in our study were stored at -30 °C. Samples of both cases and controls analyzed in the validation study by Li et al. (4) were collected from 2000 on (publication of results in 2005). In our study, samples of both the cases and the controls were stored for less than three and a half years. The exact influence of the differences in storage temperature in combination with storage duration on peak intensities cannot easily be predicted. This is because the influence of these factors on the several proteins is very diverse (15,16). Over time the intensity of some proteins will decrease because of fragmentation, while the intensities of the fragments of these proteins will increase. However, when these fragments are also very unstable, their intensities could also decrease after a prolonged time. On top of this, some proteins are more vulnerable to degradation than others (15,16). It is therefore difficult to predict whether these factors have influenced the results of the several studies.

Another, possibly more likely explanation for the discrepant results found in the several studies is differences in pre-analytical sample handling. Several studies have shown that the time between venipuncture and centrifugation, as well as the temperature at which samples are held meanwhile, are of major influence on protein profiles (11,13-17). When samples were held for a prolonged time (more than 60 minutes) at room temperature, low mass peaks were generated. These peaks were not formed as long as samples were kept on ice (16). Previous studies also revealed that some proteins are more vulnerable to sample handling than others (15,16). Amongst others, fragments of ITIH4 and C3a were found to be increased more than 1.5-fold within 2 hours after venipuncture in samples that were allowed to clot on room temperature (16). Samples of both cases and controls analyzed in our study were pre-analytically handled identically following a standard protocol. Samples were allowed to clot for 30 minutes at room temperature after which they were centrifuged for 15 minutes at 3000 rpm at room temperature. All blood samples analyzed in the study by Mathelin et al. (2) were allowed to clot at room temperature for a variable duration (30-60 min) after which they were centrifuged for 10 min at 3000 rpm at an unknown temperature. For the studies by Li et al. (3,4) no information about clotting and centrifugation conditions is available. It is also unknown whether samples of cases and controls were pre-analytically handled identically in these studies (3,4). Therefore we cannot exclude that differences in the sample handling protocols and differences in handling samples of cases and controls in other studies have caused the inconsistent results.

In this study we also examined the relationships between a number of patient and tumor characteristics and peak intensities in order to find possible explanations for the inconsistencies in the literature. We found a relation between the intensity of one of the peaks and menopause status. The previous studies did not provide information on menopause status (3,4) or did not investigate this relation (2).

We also investigated the relations between the intensities of the several peaks and HER2/neu and p53 expression and ER and PR status. Since breast cancer is such a heterogeneous disease, factors causing this heterogeneity, like HER2/neu and p53 expression and ER and PR status (18), should be investigated when searching for

potential biomarkers for breast cancer. It is possible that different biomarkers are needed to distinguish different molecular subtypes of breast cancer. No relations between peak intensities and ER/PR status were observed in the studies by Mathelin et al. (2) and by Li et al. (4), neither they were in our study. However, we did find a borderline statistically significant relation between p53 expression and *m/z* 8129. It might be possible that differences in ratio of the several molecular subtypes of breast cancer between the studies have led to inconsistencies in the results. However, no information about p53 expression was available in the previous studies (2-4).

In conclusion, in this study we were able to detect of all three previously reported proteins (2-4). Most remarkably, two out of the three proteins seemed to be discriminatory but not always in the same direction as in the previous studies. We did not find an immediate explanation for these inconsistencies, but it probably illustrates the susceptibility of proteins to external circumstances. For future studies, more effort should be put into the collection of blood samples of cases and controls with the use of standardized and high quality procedures. Also, a distinction should be made between molecular subtypes of breast cancer in the search for specific tumor markers. The proteins investigated in this study have not yet been proven to be reliable markers for breast cancer.

Acknowledgements

This study was financially supported by a grant from the board of the University Medical Center Utrecht and Julius Center for Health Sciences and Primary Care ('Strategische Impuls') and by the Netherlands Laboratory for Anticancer Drug Formulation (NLADF), Amsterdam, The Netherlands. The authors gratefully acknowledge the patients and healthy volunteers for donating a blood sample. We thank the Department of Clinical Chemistry of the Netherlands Cancer Institute, Amsterdam for collecting the blood samples. Ciphergen Biosystems is greatly acknowledged for the use of the PCS 4000 SELDI-TOF MS.

References

1. Vlahou A, Laronga C, Wilson L, Gregory B, Fournier K, McGaughey D et al. A novel approach toward development of a rapid blood test for breast cancer. ***Clin. Breast Cancer***. *2003;* 4(3): 203-209.
2. Mathelin C, Cromer A, Wendling C, Tomasetto C, Rio MC. Serum biomarkers for detection of breast cancers: a prospective study. ***Breast Cancer Res. Treat***. *2006;* 96(1): 83-90.
3. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. ***Clin. Chem***. *2002;* 48(8): 1296-1304.
4. Li J, Orlandi R, White CN, Rosenzweig J, Zhao J, Seregni E et al. Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. ***Clin. Chem***. *2005;* 51(12): 2229-2235.
5. Hu Y, Zhang S, Yu J, Liu J, Zheng S. SELDI-TOF-MS: the proteomics and bioinformatics approaches in the diagnosis of breast cancer. ***Breast***. *2005;* 14(4): 250-255.
6. Gast MC, Engwegen JY, Schellens JH, Beijnen JH. Comparing the old and new generation SELDI-TOF MS: implications for serum protein profiling. ***BMC. Med. Genomics*** *2008;* 1: 4.
7. Song J, Patel M, Rosenzweig CN, Chan-Li Y, Sokoll LJ, Fung ET et al. Quantification of fragments of human serum inter-alpha-trypsin inhibitor heavy chain 4 by a surface-enhanced laser desorption/ionization-based immunoassay. ***Clin. Chem***. *2006;* 52(6): 1045-1053.
8. ExPASy Proteomic Server. Compute pI/Mw tool [Computer software]. Retrieved from http://www.expasy.org/tools/pi_tool.html *October 2007*.
9. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. ***J. Clin. Invest***. *2006;* 116(1): 271-284.
10. Gast MC, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ et al. Serum protein profiling for diagnosis of breast cancer using SELDI-TOF MS. ***Oncol. Rep***. *2009;* 22(1): 205-213.
11. Hsieh SY, Chen RK, Pan YH, Lee HL. Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. ***Proteomics***. *2006;* 6(10): 3189-3198.
12. Rai AJ, Gelfand CA, Haywood BC, Warunek DJ, Yi J, Schuchard MD et al. HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. ***Proteomics***. *2005;* 5(13): 3262-3277.
13. West-Nielsen M, Hogdall EV, Marchiori E, Hogdall CK, Schou C, Heegaard NH. Sample handling for mass spectrometric proteomic investigations of human sera. ***Anal. Chem***. *2005;* 77(16): 5114-5123.
14. Engwegen JY, Alberts M, Knol JC, Jimenez CR, Depla AC, Tuynman H et al. Influence of variations in sample handling on SELDI-TOF MS serum protein profiles for colorectal cancer. ***Proteomics Clin. Appl***. *2008;* 2: 936-945.

15. Banks RE, Stanley AJ, Cairns DA, Barrett JH, Clarke P, Thompson D et al. Influences of blood sample processing on low-molecular-weight proteome identified by surface-enhanced laser desorption/ionization mass spectrometry. ***Clin. Chem.*** *2005;* 51(9): 1637-1649.
16. Timms JF, Arslan-Low E, Gentry-Maharaj A, Luo Z, T'Jampens D, Podust VN et al. Preanalytic influence of sample handling on SELDI-TOF serum protein profiles. ***Clin. Chem.*** *2007;* 53(4): 645-656.
17. Villanueva J, Philip J, Chaparro CA, Li Y, Toledo-Crow R, DeNoyer L et al. Correcting common errors in identifying cancer-specific serum peptide signatures. ***J. Proteome. Res.*** *2005;* 4(4): 1060-1072.
18. Anderson WF, Matsuno R. Breast cancer heterogeneity: a mixture of at least two main types? ***J. Natl. Cancer Inst.*** *2006;* 98(14): 948-951.

Appendix

Peaks with statistically significant different intensities (p<0.01) in cases compared to controls in order of *m/z*

	Breast cancer cases (*n*=47)	Healthy controls (*n*=48)		Mann-Whitney U test	ROC-curve
M/z	*Median intensity (IQR)*	*Median intensity (IQR)*	*Intensity in cases vs. controls*	*p-value*	*AUC (95% CI)*
3156	11.32 (7.14-22.94)	23.11 (14.03-61.23)	Lower	<.0001	0.75 (0.66-0.85)
3270	4.33 (0.84-10.06)	13.46 (5.03-21.02)	Lower	.002	0.69 (0.58-0.79)
3965	22.13 (10.42-54.69)	63.32 (26.17-80.70)	Lower	.002	0.68 (0.57-0.79)
4276	11.04 (2.10-29.31)	39.48 (14.17-77.58)	Lower	<.001	0.72 (0.61-0.82)
4292	14.54 (8.52-29.54)	42.07 (29.67-63.39)	Lower	<.0001	0.77 (0.67-0.87)
4472	11.60 (7.53-15.63)	16.49 (12.18-19.92)	Lower	<.001	0.73 (0.63-0.83)
5330	28.53 (21.89-50.90)	20.95 (11.03-33.15)	Higher	.003	0.68 (0.57-0.79)
5896	120.51 (74.47-177.88)	65.60 (36.54-107.16)	Higher	<.001	0.74 (0.64-0.84)
6102	15.22 (8.28-21.20)	8.56 (4.18-13.08)	Higher	<.001	0.73 (0.63-0.83)
7457	5.86 (4.50-6.64)	4.21 (2.58-6.31)	Higher	.006	0.66 (0.55-0.77)
7754	159.15 (120.82-175.30)	116.73 (94.10-145.66)	Higher	.002	0.69 (0.58-0.79)
7959	16.27 (12.32-17.73)	12.17 (9.44-15.67)	Higher	.002	0.69 (0.58-0.80)
8601	11.73 (7.42-15.99)	20.35 (12.40-25.34)	Lower	<.001	0.73 (0.63-0.83)
8941	31.27 (24.07-44.88)	73.47 (48.69-86.72)	Lower	<.0001	0.83 (0.75-0.91)
9142	2.41 (0.91-3.68)	5.35 (3.39-7.29)	Lower	<.0001	0.82 (0.74-0.91)
9280	76.29 (56.37-95.74)	56.03 (31.70-75.94)	Higher	<.001	0.71 (0.60-0.81)
9474	10.28 (7.84-11.94)	7.50 (4.02-9.85)	Higher	<.001	0.71 (0.61-0.81)
10,054	1.42 (0.71-2.50)	2.47 (1.64-4.07)	Lower	.003	0.68 (0.57-0.79)
11,720	1.66 (1.30-2.33)	1.26 (0.76-1.87)	Higher	.002	0.68 (0.58-0.79)
54,214	0.16 (0.13-0.21)	0.11 (0.08-0.14)	Higher	<.001	0.73 (0.63-0.84)

M/z: mass-to-charge ratio; IQR: inter-quartile range; ROC-curve: Receiver Operating Characteristic curve; AUC: area under the curve; 95% CI: 95% confidence interval

Chapter 3

Search for breast cancer biomarkers in fractionated serum samples by protein profiling with SELDI-TOF MS

Abstract

In the search for breast cancer biomarkers, many high abundant acute phase reactants have been previously detected as potential biomarkers. However, these proteins are unlikely to be specific for breast cancer. It is thought that cancer specific biomarkers can be primarily found among lower abundant proteins. Serum fractionation before protein profiling would facilitate a better detection of lower abundant proteins. In this study we performed serum fractionation by strong anion exchange chromatography preceding protein profiling with surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) with the aim of detecting lower abundant proteins that differentiate between breast cancer cases and controls. The six fractions resulting from the serum fractionation were tested on three different array types (IMAC30, CM10 and Q10) in a pilot study. Fraction 3 on IMAC30 and Fraction 6 on Q10 yielded the most discriminative proteins and were used for protein profiling of serum samples of 73 incident breast cancer cases and 73 controls matched for age and sample storage duration. Mean peak intensities of 16 of the 129 peaks detected in Fraction 3 on IMAC30, and 29 of the 150 peaks detected in Fraction 6 on Q10, were statistically significantly different between cases and controls at a critical p-value of 0.05. After correction for multiple testing with the False Discovery Rate (FDR) method, eight peaks (two on IMAC30, and six on Q10) had less than 10% chance to be false positive. Seven of these peaks could be tentatively identified: apolipoprotein C-II (mass-to-charge ratio (*m*/*z*) 8909), oxidized apolipoprotein C-II (*m*/*z* 8925), apolipoprotein C-III (*m*/*z* 8746), fragment of coagulation factor XIIIa (*m*/*z* 3959), heterodimer of apolipoprotein A-I and apolipoprotein A-II (*m*/*z* 45435), haemoglobin ß-chain (*m*/*z* 15915), and post-translational modified haemoglobin (*m*/*z* 15346). Due to the extensive fractionation of the serum samples before protein profiling, we detected many more proteins here than in previous studies without fractionation. However, the discriminating proteins detected in this study were still high abundant proteins. We did not detect discriminative lower abundant proteins. These results indicate that either lower abundant proteins are less distinctive than high abundant (acute-phase) proteins, or that more rigorous fractionation and selective protein depletion is needed to detect these lower abundant proteins.

Introduction

In the last few years many proteomics studies using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) have been carried out in search of diagnostic blood markers for several types of cancer (1-12). The discovery of such markers could enable the detection of tumors in early stages of the disease, with easy to perform and less-invasive blood test. Consequently, breast cancer would be diagnosed in earlier stages, which gives patients higher chances of survival with possibly less invasive therapies.

However, the potential biomarkers that have been detected with SELDI-TOF MS so far, such as apolipoprotein C-I, complement component 3a (C3a), fibrinogen, haptoglobin and inter-alpa trypsin inhibitor heavy chain 4 (ITIH4) are mainly high abundant blood proteins involved in coagulation and acute phase responses (13). These responses are not likely to be specific for cancer, let alone for one type of cancer. This limits the usefulness of these markers as cancer type specific markers.

The fact that primarily acute phase reactants are found is most likely due to the complexity of the serum proteome. The serum proteome contains a large number of proteins spanning a wide dynamic range of concentrations (14). Only a few, high abundant proteins comprise about 99% of the total amount of proteins (15). Acute phase reactants are examples of such high abundant proteins, and for this reason they are easily detected. Cancer specific proteins that are exclusively expressed by one type of malignant cells are expected to be much less abundant, with normal blood concentrations of low nanograms per milliliter or less (13,14). SELDI-TOF MS suffers from the fact that these proteins are largely 'masked' by high abundant proteins. The concentration range of known blood proteins is greater than 10^{10}, while SELDI-TOF only has the capacity to detect proteins in a concentration range of 10^2 (14).

Serum fractionation has been proposed as a promising method to measure low abundant proteins (16-19). In this study we performed fractionation by anion exchange chromatography; based on differences in isoelectric point (pI) of the proteins we divided the serum proteome into six protein fractions. High abundant proteins are segregated into a limited number of fractions, which reduces the signal

suppression effects on proteins of lower abundance in the other fractions. This increases the number of peaks detected, and facilitates detection of low abundant proteins (14). We did not perform depletion of the most abundant proteins since this may cause elimination of lower abundant proteins that are bound to the depleted proteins (20). In this study we compared the protein profile in fractionated serum samples of incident breast cancer patients with that of healthy controls. The aim of this study was to detect proteins, other than high abundant acute phase reactants, that differentiate between breast cancer patients and controls.

Material and Methods

Study population

To investigate serum protein profiles, we performed a case-control study. Serum samples of both the cases and the controls were obtained from a serum bank at the Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands. Samples were collected from March 2003 until July 2005, from women who were just diagnosed with primary breast cancer, and from healthy female relatives or friends of the patients. Serum samples of the cases were collected before surgery was performed, or any other kind of treatment was started. All samples were collected after receiving the individuals' informed consent, under approval of the Institutional Review Board control.

Blood collection, processing, and storage of the serum samples were performed under strictly defined conditions, which were the same for cases and controls. All serum samples were collected with the use of BD Vacutainer SST plastic serum tubes with clot activator and gel (Becton-Dickinson, Franklin Lakes, NJ, USA). After collection, blood samples were allowed to clot for 30 minutes at room temperature and were subsequently centrifuged for 15 minutes at 3000 rpm at room temperature. After centrifugation, samples were aliquoted and stored at -30°C.

Menopausal status at diagnosis was obtained through examination of the cases' medical records. Tumor stage, tumor size, estrogen receptor (ER) status, progesterone receptor (PR) status, HER2/neu expression, and p53 expression were determined by pathological examination of the removed tumor. Lymph node involvement and the presence of metastasis were also examined.

From a group of 157 women with primary diagnosed breast cancer and 131 healthy controls we matched cases and controls for age and serum sample storage duration. Finally, 73 cases and 73 controls could be matched with a maximum age difference of three years and a maximum difference in sample storage duration of three months.

Pilot study for selection of experimental conditions

In this study, we carried out serum fractionation before protein profiling with SELDI-TOF MS. To obtain the most informative testing conditions, different combinations of fraction and array type were tested in a pilot study with nine randomly selected case-control pairs. Serum fractionation was performed with strong anion exchange Q ceramic resin (Bio-Rad Labs, Hercules, CA, USA) according to the manufacturers' protocol (see **Appendix 1**), which resulted in six fractions.

Next, these six fractions were applied to three different array types with an appropriate binding buffer to test these combinations. Firstly, we tested the Immobilized Metal Affinity Capture (IMAC30) array (Bio-Rad Labs) charged with copper sulfate (Merck, Darmstadt, Germany) with a binding buffer containing 0.01 M phosphate buffered saline pH 7.4 (Sigma, St. ouis, MO, USA) with 0.5 M sodium chloride (Merck). Furthermore, we tested the weak cation exchange (CM10) array (Bio-Rad Labs) with a binding buffer containing 100 mM sodium acetate pH 4 (Sigma). Lastly, we tested the strong anion exchange (Q10) array (Bio-Rad Labs) with a binding buffer containing 20 mM Tris-HCl pH 9 (Sigma).

Ultimately, the IMAC30 array with Fraction 3 and the Q10 array with Fraction 6 were the two conditions which yielded the most discriminative proteins relative to the total number of detected peaks, mainly in the mass range 2 to 10 kDa.

Profiling of fractionated serum samples of cases and controls

Selected conditions were subsequently used for the analysis of the total sample set. The samples were fractionated and applied to the ProteinChip arrays in three batches, on three consecutive days, with a Biomek pipetting robot (Beckman Coulter, Brea, CA, USA). For application of the serum samples to the arrays, we

used the same protocol as in the pilot study (see **Appendix 2**). Samples of matched cases and controls were analyzed in the same batch. In addition to the case and control samples, in every batch, two aliquots of two quality control (QC) samples were analyzed. Aliquots of these two QC samples were used for all three batches. All samples were randomly applied to the well plates. On the fourth day we performed SELDI-TOF MS on all arrays with the PCS 4000 ProteinChip Reader (Bio-Rad Labs). See **Appendix 2** for the settings of the ProteinChip Reader for protein profiling, for method of processing the spectra, and for settings for peak detection. Spectra in which normalization revealed too low or too high total ion current (TIC) were excluded from further analysis.

To estimate the reproducibility of the analysis we calculated the within batch variation and the between batch variation in the QC samples. To determine the within batch variation, we first averaged the peak intensities measured in the duplicates of each QC sample for every peak, per batch, and we calculated the standard deviation (SD). Then, we calculated the median coefficient of variance (CV) (the SD as percentage of average) within every batch, of all peaks detected on one type of array. To determine the between batch variation, we averaged the averaged peak intensities of each QC sample in the three batches for every peak, and we calculated the SD. Subsequently, we calculated the median CV between batches of all peaks detected on one type of array.

Data analysis

Peak information was subsequently exported as CSV-files and imported into SPSS 15.0 for statistical analysis. Data analysis was performed separately for the IMAC30 peaks and the Q10 peaks. The sera were fractionated and applied to the arrays on three consecutive days, a parameter likely to influence spectral data (21-23). Therefore, before merging peak intensity data of the three batches, peak intensities were transformed into Z-values within each batch (histograms showed normally distributed peak intensities within the batches). In this way peak intensities were expressed as the number of standard deviations above or below the mean intensity of that peak across all samples in a batch. The Z-transformed data of the three batches were subsequently merged in one file. To test whether the mean Z-

transformed peak intensities in the breast cancer cases were statistically significantly different from those in the matched healthy controls, a paired samples T test was performed. P-values <0.05 were considered statistically significant.

Correction for multiple testing was performed on all detected IMAC30 and Q10 peaks together, using the False Discovery Rate (FDR) method suggested by Benjamini and Hochberg (24). The FDR controls the expected proportion of falsely rejected hypotheses. For this method, individual p-values of all tested peaks were sorted from smallest to largest. Subsequently, 10% was chosen as an acceptable proportion of false positive results (q-value=0.10). Then, for every tested peak the FDR threshold was calculated, which is the rank of the p-value divided by the total number of tested peaks, multiplied by the q-value. Tests with a p-value smaller than the FDR threshold had less than 10% chance to be false positive (24).

We also performed a conditional multivariate logistic regression analysis in which we simultaneously included the peaks that had less than 10% chance to be a false positive finding. We performed backward selection (p-value <0.20) to determine which peaks statistically significantly contributed to the discrimination of cases and controls. We subsequently determined the area under the curve (AUC) of the Receiver Operating Characteristic (ROC) curve based on the predicted probabilities resulting from the model, with 95% confidence interval (CI). We executed this analysis to determine which peaks were independently related to breast cancer, and to find a combination of peaks that could optimally distinguish breast cancer cases from healthy controls.

Identification of the most discriminative peaks

Based on their mass-to-charge ratio (*m/z)*, the sample type (serum) and fraction in which they were found, the ProteinChip surface used, as well as data from previously performed serum profiling studies, we tentatively identified the proteins with a chance of less than 10% to be a false-positive finding.

Results

Study population

Table 1 gives an overview of the characteristics of the breast cancer cases, the matched controls and their serum samples. The median age of both the cases and the controls was 55 years at the time of blood collection. The menopausal status at diagnosis of two women was not reported in their medical record. Information on menopausal status was not available for the controls. The median storage duration of the samples of both the cases and the controls was 52 months. The median duration between diagnosis and sample collection was 15 days, and all samples of the cases were collected before the start of treatment.

Table 1 - Characteristics of the breast cancer cases, the healthy controls and their serum samples

	Breast cancer cases (*n*=73)	Healthy controls (*n*=73)
Age at diagnosis (years)		
Median (IQR)	55 (46 - 60.5)	55 (44.5 - 60)
Menopausal status, *n* (%)		
Premenopausal	28 (39.4)	
Postmenopausal	43 (60.6)	
Missing	2	
Sample storage duration (months)		
Median (IQR)	52 (42 - 59)	52 (42 - 59)
Time from diagnosis to blood sampling (days)		
Median (IQR)	15 (6 - 22)	

IQR: inter-quartile range

Tumor characteristics of the breast cancer cases are listed in **Table 2**. Most of the patients were diagnosed with Stage I (23%) or Stage IIA (44%) breast cancer, and 6% of the patients were diagnosed with a carcinoma in situ. More than half of the patients with an invasive tumor had lymph node involvement, but none of the patients was affected with metastases.

Table 2 - Tumor characteristics of the breast cancer cases

All breast cancer cases	*n*=73
TNM stage, *n* (%)	
0	4 (5.5)
I	17 (23.3)
IIA	32 (43.8)
IIB	9 (12.3)
IIIA and IIIC	11 (15.1)
Breast cancer cases with an invasive tumor	***n*=69**
Tumor size, *n* (%)	
>0.1-0.5 cm	4 (5.8)
>0.5-1 cm	6 (8.7)
>1-2 cm	29 (42.0)
>2 cm	30 (43.5)
Lymph node involvement, *n* (%)	
No	30 (43.5)
Yes	39 (56.5)
ER status, *n* (%)	
Negative	16 (23.2)
Positive	53 (76.8)
PR status, *n* (%)	
Negative	30 (43.5)
Positive	39 (56.5)
HER2/neu expression, *n* (%)	
Negative	55 (79.7)
Positive	14 (20.3)
P53 expression, *n* (%)	
Negative	23 (33.3)
Positive	46 (66.7)

ER: estrogen receptor; PR: progesterone receptor

Peak detection

After normalization, 22 of the 146 spectra (from 73 cases and 73 controls) that resulted from the analysis using the IMAC arrays showed divergent total ion current. These outliers included 9 spectra of controls and 13 spectra of cases. These subjects belonged to 17 matched case-control pairs, which were excluded from the paired analyses. Of the 146 spectra that resulted from the analysis using the Q10 arrays, 20 spectra showed divergent total ion current. These outliers included 8 spectra of controls and 12 spectra of cases. These subjects belonged to 16 matched case-control pairs, which had to be excluded from the paired analyses.

In the spectra resulting from the analysis of Fraction 3 profiled on the IMAC arrays, 129 peaks were detected: 84 peaks in the 2 to 12 kDa mass range and 45 in the 12 to 300 kDa mass range. In the spectra that were obtained from Fraction 6 profiled on the Q10 arrays, 150 peaks were detected: 83 peaks in the 2 to 12 kDa mass range and 67 in the 12 to 300 kDa mass range.

The within batch reproducibility, expressed as the median CV of all peaks detected on the IMAC arrays, was 15%, 24% and 15% for Batch 1, Batch 2 and Batch 3, respectively. Of all peaks detected on the Q10 arrays, the within batch reproducibility was 11%, 14% and 15% for Batch 1, Batch 2 and Batch 3, respectively. The median CV expressing the between batch reproducibility was 32% for the peaks detected on the IMAC arrays and 18% for the peaks detected on the Q10 arrays.

Relations between peak intensities and breast cancer

Based on the paired samples T Test, the intensities of 16 of the 129 peaks detected on the IMAC30 array were found to be significantly different between cases and controls (p-value <0.05). Five of these peaks were detected in the 2 to 12 kDa mass range. Fourteen of the 16 peaks were lower in the cases than the controls. After correction for multiple testing using the FDR, only two of the 16 peaks (*m/z* 15915 and *m/z* 15346) had less than 10% chance to be a false positive finding. The p-value of these peaks was lower than the FDR threshold. The other 14 peaks had a higher chance to be a false positive finding. The *m/z* of the 16 peaks, their mean Z-transformed intensities, the results of the T test, and the FDR thresholds are listed in **Table 3**.

Table 4 shows that the intensities of 29 of the 150 peaks detected on the Q10 array were significantly different between cases and controls. Fifteen of these peaks were detected in the 2 to 12 kDa mass range. The intensity of 16 of the 29 peaks was lower in cases than controls. After FDR correction, six of the 29 peaks appeared to have less than 10% chance to be a false positive finding (*m/z* 8926, *m/z* 8909, *m/z* 4162, *m/z* 8746, *m/z* 45435 and *m/z* 3959).

When we performed a paired samples T test in which the sets that were used for the pilot study were excluded, the same peaks were in the top of the ranking.

Table 3 - Mean Z-transformed intensities of the discriminative peaks detected on IMAC arrays in cases and controls in order of significance.

IMAC	Breast cancer cases (*n*=56)	Healthy controls (*n*=56)				Tentative identity		
M/z	*Mean intensity* (SD)*	*Mean intensity* (SD)*	*Intensity in cases vs. controls*	*p-value*[#]	*FDR threshold*	*Correlated*[†]	*Protein*	*Molecular weight (Da)*
15,915	-0.31 (0.69)	0.33 (1.21)	Lower	.0013[§]	.0022	A	Haemoglobin ß-chain	15,867
15,346	-0.28 (0.70)	0.30 (1.22)	Lower	.0035[§]	.0036	A	Post-translational modified haemoglobin	
15,143	-0.26 (0.60)	0.30 (1.27)	Lower	.0043	.0039	A	Haemoglobin α-chain	15,126
6923	-0.24 (0.98)	0.26 (0.79)	Lower	.0044	.0043			
94,790	-0.27 (0.84)	0.27 (1.07)	Lower	.0051	.0047	B	Albumin/Apo A-I heterodimer	94,500
28,269	-0.27 (0.86)	0.25 (1.07)	Lower	.0081	.0057	B	Apo A-1 + ..	
29,114	-0.25 (0.68)	0.28 (1.22)	Lower	.0082	.0061			
55,991	-0.25 (0.89)	0.24 (1.06)	Lower	.0173	.0082			
28,100	-0.24 (0.85)	0.23 (1.10)	Lower	.0178	.0086	B	Apo A-1	28,080
14,061	-0.24 (0.82)	0.21 (1.10)	Lower	.0212	.0104	B	Apo A-1 2+	
3095	0.20 (1.05)	-0.21 (0.89)	Higher	.0259	.0115	C	Albumine fragment + oxide atom	3099
5856	-0.21 (1.05)	0.21 (0.86)	Lower	.0290	.0129			
47,482	-0.24 (0.81)	0.15 (1.13)	Lower	.0292	.0133	B	Albumin/Apo A-I heterodimer 2+	
3079	0.17 (1.09)	-0.20 (0.88)	Higher	.0383	.0140	C	Albumine fragment	3083
14,163	-0.21 (0.85)	0.20 (1.07)	Lower	.0390	.0143	B	Apo A-1 + .. 2+	
6414	-0.19 (0.87)	0.14 (1.04)	Lower	.0416	.0151			

M/z : mass-to-charge ratio; SD: standard deviation; Apo: apoliopoprotein; 2+: double charged ion; * Z-transformed peak intensities; [#] Paired samples T test; [§] Less than 10% chance to be a false positive finding after FDR correction (p-value lower than FDR threshold based on 90% certainty (q=0.10)); [†] Peaks of which transformed intensities were correlated (A: R^2 >0.887; B: R^2 >0.729 (except for *m/z* 47482: R^2 >0.652; C: R^2 =0.886)

Table 4 - Mean Z-transformed intensities of the discriminative peaks detected on Q10 arrays in cases and controls in order of significance.

Q10	Breast cancer cases (*n*=57)	Healthy controls (*n*=57)					Tentative identity	
M/z	*Mean intensity* (SD)*	*Mean intensity* (SD)*	*Intensity in cases vs. controls*	*p-value*[#]	*FDR threshold*	*Correlated*[†]	*Protein*	*Molecular weight (Da)*
8926	-0.29 (0.83)	0.28 (1.10)	Lower	.0003[§]	.0004	A	Apo C-II + oxide atom	8930
8909	-0.30 (0.62)	0.30 (1.22)	Lower	.0005[§]	.0007	A	Apo C-II	8914
4162	0.31 (0.96)	-0.26 (0.88)	Higher	.0007[§]	.0011			
8746	0.28 (1.07)	-0.29 (0.90)	Higher	.0008[§]	.0014		Apo C-III	8765
45,435	-0.29 (0.95)	0.31 (0.98)	Lower	.0010[§]	.0018		Apo A-I/Apo A-II heterodimer	45,470
3959	0.30 (1.18)	-0.24 (0.72)	Higher	.0015[§]	.0025		Factor XIIIa fragment	3951
28,094	-0.27 (1.06)	0.24 (0.91)	Lower	.0029	.0029	B	Apo A-I	28,080
14,058	-0.25 (1.05)	0.23 (0.87)	Lower	.0033	.0032	B	Apo A-I 2+	
8819	0.17 (1.04)	-0.25 (0.76)	Higher	.0055	.0050		Apo A-II	8810
14,158	-0.23 (1.08)	0.21 (0.87)	Lower	.0078	.0054	B	Apo A-I +.. 2+	
7608	0.22 (1.05)	-0.22 (0.77)	Higher	.0110	.0065		Apo L-I	7616
8191	-0.22 (0.64)	0.22 (1.24)	Lower	.0112	.0068	A	Apo C-II truncated	8204
56,202	-0.23 (1.02)	0.20 (0.95)	Lower	.0116	.0072	B	Apo A-I dimer	56,160
28,303	-0.24 (1.06)	0.21 (0.93)	Lower	.0119	.0075	B	Apo A-I +..	
14,261	-0.22 (1.06)	0.22 (0.92)	Lower	.0138	.0079	B		
79,213	0.21 (1.06)	-0.18 (0.96)	Higher	.0184	.0090		Serotransferin	79,000
84,751	-0.20 (0.97)	0.18 (0.99)	Lower	.0186	.0093			
9430	-0.22 (0.94)	0.18 (1.05)	Lower	.0186	.0097		Apo C-III glycosylated	9420
124,174	0.24 (0.91)	-0.17 (0.98)	Higher	.0194	.0100			
2008	0.21 (1.03)	-0.24 (0.97)	Higher	.0237	.0108			
13,092	-0.22 (0.94)	0.17 (0.96)	Lower	.0250	.0111			
33,494	0.20 (1.12)	-0.15 (0.87)	Higher	.0268	.0118			
116,138	0.21 (0.94)	-0.21 (1.01)	Higher	.0278	.0122			

Table 4 - Continued

Q10	Breast cancer cases (*n*=57)	Healthy controls (*n*=57)					Tentative identity	
M/z	*Mean intensity* (SD)*	*Mean intensity* (SD)*	*Intensity in cases vs. controls*	*p-value*[#]	*FDR threshold*	*Correlated*[†]	*Protein*	*Molecular weight (Da)*
5371	0.21 (1.14)	-0.16 (0.85)	Higher	.0286	.0125			
7812	0.16 (0.95)	-0.18 (0.86)	Higher	.0301	.0136			
8209	-0.18 (0.87)	0.17 (1.12)	Lower	.0394	.0147	A	Apo C-II truncated + oxide atom	8220
6428	-0.18 (1.05)	0.15 (0.95)	Lower	.0475	.0154	C	Apo C-I truncated	6432
23,741	0.13 (1.04)	-0.22 (0.94)	Higher	.0488	.0158			
6622	-0.19 (1.05)	0.13 (0.96)	Lower	.0494	.0161	C	Apo C-I	6630

M/z : mass-to-charge ratio; SD: standard deviation; Apo: apolipoprotein; 2+: Double charged ion; * Z-transformed peak intensities; [#] Paired samples T test; [§] Less than 10% chance to be a false positive finding after FDR correction (p-value lower than FDR threshold based on 90% certainty (q=0.10)); [†] Peaks of which transformed intensities were correlated (A: R^2 >0.706; B: R^2 >0.702; C: R^2 >0.884)

Conditional multivariate logistic regression analysis

The multivariate analysis, including all peaks that had less than 10% chance to be false positive result, revealed that *m/z* 3959, *m/z* 4162, *m/z* 8909 and *m/z* 15915 significantly contributed to the distinction between breast cancer cases and healthy controls. A ROC curve of the predicted probabilities for breast cancer, based on the intensities of these peaks, resulted in an AUC of 0.77 (95%CI: 0.69-0.86).

Proposed identities of the peaks

Based on their *m/z*, the sample type and fraction in which they were found, the ProteinChip surface used, as well as data from previously performed serum profiling studies (N. Harris, unpublished data, and (25-30)), we tentatively identified the peaks with <10% chance to be a false positive finding. The peak with *m/z* 15915 that was detected in Fraction 3 on the IMAC30 array, was identified as haemoglobin ß-chain. Peaks with similar mass were previously structurally identified as haemoglobin ß-chain by SDS-PAGE, followed by in-gel trypsin digestion and analysis using tandem MS (e.g. Q-TOF), and/or by an immunoassay (25-29). The molecular weight (MW) of this protein is 15867 Da and it has a pI of 6.81. This pI fits with the assumption that this peak, detected in Fraction 3, is haemoglobin ß-chain, because due to the nature of the method of fractionation, Fraction 3 can only contain proteins with a pI >5 and <7. The Z-transformed intensities of *m/z* 15915 were highly correlated with the Z-transformed intensities of the peak with *m/z* 15346 (R^2=0.887), which was also found in Fraction 3 on IMAC. This peak was therefore identified as a post-translational modified form of haemoglobin. The tentative identities of these two peaks and the other identified peaks on the IMAC array are listed in **Table 3**.

The peaks with *m/z* 8909 and *m/z* 8925, which were both detected in Fraction 6 on the Q10 array, were identified as apolipoprotein C-II and its oxidized form. The MW of apolipoprotein C-II is 8914 Da and its pI is 4.66. The difference in *m/z* of the two peaks is the exact mass of an oxide-atom (16 Da). The peak intensities of these peaks were also highly correlated (R^2=0.875), which is to be expected if one is an oxidized form of the other. Another peak that was detected in the same fraction, *m/z* 8746, was identified as apolipoprotein C-III (MW: 8765 Da),

which has a pI of 4.72. The peak with *m/z* 3959, also found in this fraction, was identified as a fragment of coagulation factor XIIIa (30). This fragment has a MW of 3951 Da and a pI of 4.03. A peak with *m/z* 3950 was previously structurally identified as a fragment of coagulation factor XIIIa using nanoelectrospray ionization quadrupole time-of-flight mass spectrometry (nESI-qTOF-MS) (30). The peak with *m/z* 45435 was identified as a heterodimer of apolipoprotein A-I and apolipoprotein A-II. This dimer has a MW of 45470 Da. The tentative identities of these peaks and the other identified peaks on the Q10 array are listed in **Table 4**. The fraction in which the peaks detected on the Q10 array were found is not informative for the identification. This is because Fraction 6 results from the elution with an organic buffer, which elutes all remaining proteins. By this, also strongly bound or high abundant proteins with pI above 3, which should have been eluted in previous fractions, can end up in Fraction 6. We have no indications for the identity of the last discriminating peak that was detected in Fraction 6 on Q10; *m/z* 4162.

Discussion

In this study we detected eight peaks that were statistically significantly related to the presence of breast cancer with less than 10% chance of being a false positive finding. The tentative identities of seven of these peaks are: apolipoprotein C-II, an oxidized form of apolipoprotein C-II, apolipoprotein C-III, a fragment of coagulation factor XIIIa, a heterodimer of apolipoprotein A-I and apolipoprotein A-II, haemoglobin ß-chain, and post-translational modified haemoglobin. A peak with *m/z* 4162 could not be identified. Apolipoprotein C-II, the fragment of coagulation factor XIIIa, haemoglobin ß-chain, and *m/z* 4162 appeared to contribute significantly to the discrimination of cases and controls.

Apolipoprotein C-II and apolipoprotein C-III were not previously described in relation to the presence of a primary breast cancer tumor. In a study by Jiang et al. (31) coagulation factor XIII itself was found to be lower in breast tumor tissue compared to normal breast tissue (31). Contrary, a fragment of coagulation factor XIIIa with *m/z* 2602 was higher in serum of breast cancer patients compared to controls in another study (32). In the current study, another fragment of coagulation

factor XIIIa was higher in serum samples of breast cancer patients. Differences in regulation may be related to the form of the protein; elevated fragment concentrations are not necessarily the consequence of higher precursors as argued in a study by Villanueva et al. (32). Additionally, differences may be due to differences in type of sample that was investigated; tissue versus serum. In a previous study by Engwegen et al. (11) a specific protein was found to be lower in serum samples of colorectal cancer patients compared to serum samples of controls, while the same protein was higher in colorectal cancer tissue compared to healthy colon tissue (hyperplastic polyps) of the same subjects (11). The heterodimer of apolipoprotein A-I and apolipoprotein A-II was not previously reported in relation to the presence of a primary breast tumor. However, apolipoprotein A-I was found to be lower in post-operative serum samples of high-risk breast cancer patients who developed metastatic relapse despite adjuvant therapy, compared to high-risk breast cancer patients who became long-term metastatic-free survivors (33). Apolipoprotein A-I itself (MW: 28080 Da) was also detected in the current study, both in Fraction 6 on Q10 (m/z 28094) and in Faction 3 on IMAC (m/z 28100). Intensities of this protein were also found to be lower in breast cancer patients compared to controls, however, after correction for multiple testing this result had a more than 10% chance to be a false positive finding. Apolipoprotein A-II has not been reported in relation to the presence of breast cancer before. A peak with a mass likely representing haemoglobin ß-chain (15940 Da), was found to be higher in nipple aspirate fluid (NAF) of breast cancer patients compared to NAF of controls (34). This study comprised only 20 breast cancer patients and 13 controls, but the discriminative value of this protein was very high (expressed in 16 cases and only 1 control) (34). In the current study, however, haemoglobin ß-chain was lower in breast cancer patients. Again, difference in type of body fluid that was investigated, as well as differences in the subject characteristics between the studies could have caused this difference in expression.

The discriminative proteins found in this study are involved in lipid metabolism (apolipoprotein C-II, A-I, and A-II), blood coagulation (coagulation factor XIIIa) and oxygen transport (haemoglobin ß-chain), all processes that do not seem to be cancer specific. Moreover, these proteins are high abundant. Even though it is

found by Villanueva et al. (32) that fragments of high abundant, acute phase reactants can be cancer specific, the substrates itself are unlikely to be good cancer biomarkers. The hypothesized underlying process, namely the fragmentation by exoproteases released by the tumor, is probably best reflected by the concentration of the end products, instead of by the concentration of the substrates. Unfortunately, the SELDI-TOF MS technique is not sensitive enough to be able to detect these low mass fragments.

Despite the extensive fractionation of serum samples in this study we did not detect the expected low abundant discriminative proteins. Serum fractionation by strong anion exchange chromatography has previously been performed in only a few studies, mainly to increase the number of detectable peaks (25,35-37). In three studies the number of detected peaks was found to be increased by serum fractionation (25,35,37), in one study investigating plasma it was not (36). Solassol et al. (37) found that prostate-specific antigen (PSA), which is a low abundant protein, only could be detected in fractionated serum. However, they tested this only with two-dimensional gel electrophoresis and not with SELDI-TOF MS (37).

One explanation for the fact that we did not detect the expected low abundant discriminating proteins could be that the low abundant proteins are not as distinctive as thought, and that they were therefore not found. Another explanation could be that despite the extensive fractionation, we were not able to detect the low abundant proteins after all. Possibly, the relatively high storage temperature of our samples (-30°C) has led to degradation of the serum proteins during the four to six year storage period (38). Especially the concentrations of the low abundant proteins would then have been decreased to levels undetectable for SELDI-TOF MS. A third reason could be that despite extensive fractionation, too many abundant proteins were left that suppressed the signal of low abundant proteins. The fact that we detected a three-fold increased number of peaks in our spectra compared to those in a previous 'unfractionated' SELDI-TOF MS study by our group (4), suggests that we were able to eliminate the most abundant proteins from the investigated fractions, which resulted in enriched spectra. However, it may not have been sufficient to detect the least abundant, possibly highly discriminative proteins. More thorough removal of the highest abundant proteins without excluding all other

proteins with pI equal to that of these high abundant proteins may be needed. Immunodepletion of the top six most abundant proteins (albumin, IgG, IgA, transferrin, haptoglobin and α-1-antitrypsin) could already remove 83% of the total protein content (16), but this may at the same time cause elimination of discriminative low abundant proteins that are bound to the highly abundant ones (20). Other methods of serum fractionation that separate the most abundant proteins from the low abundant proteins are therefore needed to reduce the high dynamic range of serum protein concentrations and to enable detection of low abundant, possibly discriminative proteins. The cancer type specificity of the discriminative proteins detected in the current study should be investigated in future studies to determine their usefulness in the diagnosis of breast cancer in clinical practice.

Acknowledgements

This study was financially supported by a grant from the board of the University Medical Center Utrecht and Julius Center for Health Sciences and Primary Care ('Strategische Impuls') and by the Netherlands Laboratory for Anticancer Drug Formulation (NLADF), Amsterdam, The Netherlands. We would like to acknowledge Leo Kruijt from the Animal Science Group Lelystad for his hospitality at his laboratory and for the possibility to make use of the PCS 4000 ProteinChip Reader.

References

1. Belluco C, Petricoin EF, Mammano E, Facchiano F, Ross-Rucker S, Nitti D et al. Serum Proteomic Analysis Identifies a Highly Sensitive and Specific Discriminatory Pattern in Stage 1 Breast Cancer. ***Ann. Surg. Oncol.*** *2007;* 14(9): 2470-2476.
2. Fung ET, Yip TT, Lomas L, Wang Z, Yip C, Meng XY et al. Classification of cancer types by measuring variants of host response proteins using SELDI serum assays. ***Int. J. Cancer.*** *2005;* 115(5): 783-789.
3. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. ***Clin. Chem.*** *2002;* 48(8): 1296-1304.
4. van Winden AW, Gast MC, Beijnen JH, Rutgers EJ, Grobbee DE, Peeters PH et al. Validation of previously identified serum biomarkers for breast cancer with SELDI-TOF MS: a case control study. ***BMC. Med. Genomics*** *2009;* 2: 4.
5. Li J, Orlandi R, White CN, Rosenzweig J, Zhao J, Seregni E et al. Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. ***Clin. Chem.*** *2005;* 51(12): 2229-2235.
6. Mathelin C, Cromer A, Wendling C, Tomasetto C, Rio MC. Serum biomarkers for detection of breast cancers: a prospective study. ***Breast Cancer Res. Treat.*** *2006;* 96(1): 83-90.
7. Song J, Patel M, Rosenzweig CN, Chan-Li Y, Sokoll LJ, Fung ET et al. Quantification of fragments of human serum inter-alpha-trypsin inhibitor heavy chain 4 by a surface-enhanced laser desorption/ionization-based immunoassay. ***Clin. Chem.*** *2006;* 52(6): 1045-1053.
8. Hu Y, Zhang S, Yu J, Liu J, Zheng S. SELDI-TOF-MS: the proteomics and bioinformatics approaches in the diagnosis of breast cancer. ***Breast.*** *2005;* 14(4): 250-255.
9. Vlahou A, Laronga C, Wilson L, Gregory B, Fournier K, McGaughey D et al. A novel approach toward development of a rapid blood test for breast cancer. ***Clin. Breast Cancer.*** *2003;* 4(3): 203-209.
10. Engwegen JY, Helgason HH, Cats A, Harris N, Bonfrer JM, Schellens JH et al. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. ***World J. Gastroenterol.*** *2006;* 12(10): 1536-1544.
11. Engwegen JY, Depla AC, Smits ME, Cats A, Tuynman H, van Heukelem HA et al. Detection of Colorectal Cancer by Serum and Tissue Protein Profiling: A Prospective Study in a Population at Risk. ***Biomark. Insights.*** *2008;* 3: 375-385.
12. Engwegen JY, Mehra N, Haanen JB, Schellens JH, Voest EE, Beijnen JH. Identification of two new serum protein profiles for renal cell carcinoma. ***Oncol. Rep.*** *2009;* 22(2): 401-408.
13. Gast MC, Schellens JH, Beijnen JH. Clinical proteomics in breast cancer: a review. ***Breast Cancer Res. Treat.*** *2009;* 116(1): 17-29.
14. Hoffman SA, Joo WA, Echan LA, Speicher DW. Higher dimensional (Hi-D) separation strategies dramatically improve the potential for cancer biomarker detection in serum and plasma. ***J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*** *2007;* 849(1-2): 43-52.

15. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. ***Mol. Cell Proteomics***. *2002;* 1(11): 845-867.
16. Faca V, Pitteri SJ, Newcomb L, Glukhova V, Phanstiel D, Krasnoselsky A et al. Contribution of protein fractionation to depth of analysis of the serum and plasma proteomes. ***J. Proteome. Res***. *2007;* 6(9): 3558-3565.
17. Vermeulen R, Lan Q, Zhang L, Gunn L, McCarthy D, Woodbury RL et al. Decreased levels of CXC-chemokines in serum of benzene-exposed workers identified by array-based proteomics. ***Proc. Natl. Acad. Sci. U. S. A*** *2005;* 102(47): 17041-17046.
18. Restuccia U, Boschetti E, Fasoli E, Fortis F, Guerrier L, Bachi A et al. pI-based fractionation of serum proteomes versus anion exchange after enhancement of low-abundance proteins by means of peptide libraries. ***J. Proteomics***. *2009;* 72(6): 1061-1070.
19. Sahab ZJ, Iczkowski KA, Sang QX. Anion exchange fractionation of serum proteins versus albumin elimination. ***Anal. Biochem***. *2007;* 368(1): 24-32.
20. Yocum AK, Yu K, Oe T, Blair IA. Effect of immunoaffinity depletion of human serum during proteomic investigations. ***J. Proteome. Res***. *2005;* 4(5): 1722-1731.
21. Karsan A, Eigl BJ, Flibotte S, Gelmon K, Switzer P, Hassell P et al. Analytical and preanalytical biases in serum proteomic pattern analysis for breast cancer diagnosis. ***Clin. Chem***. *2005;* 51(8): 1525-1528.
22. Pelikan R, Bigbee WL, Malehorn D, Lyons-Weiler J, Hauskrecht M. Intersession reproducibility of mass spectrometry profiles and its effect on accuracy of multivariate classification models. ***Bioinformatics***. *2007;* 23(22): 3065-3072.
23. Hu J, Coombes KR, Morris JS, Baggerly KA. The importance of experimental design in proteomic mass spectrometry experiments: some cautionary tales. ***Brief. Funct. Genomic. Proteomic***. *2005;* 3(4): 322-331.
24. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. ***J. R. Statistic. Soc. B*** *1995;* 57(1): 289-300.
25. Timms JF, Arslan-Low E, Gentry-Maharaj A, Luo Z, T'Jampens D, Podust VN et al. Preanalytic influence of sample handling on SELDI-TOF serum protein profiles. ***Clin. Chem***. *2007;* 53(4): 645-656.
26. Trak-Smayra V, Dargere D, Noun R, Albuquerque M, Yaghi C, Gannage-Yared MH et al. Serum proteomic profiling of obese patients: correlation with liver pathology and evolution after bariatric surgery. ***Gut*** *2009;* 58(6): 825-832.
27. Kozak KR, Su F, Whitelegge JP, Faull K, Reddy S, Farias-Eisner R. Characterization of serum biomarkers for detection of early stage ovarian cancer. ***Proteomics***. *2005;* 5(17): 4589-4596.
28. Sundsten T, Zethelius B, Berne C, Bergsten P. Plasma proteome changes in subjects with Type 2 diabetes mellitus with a low or high early insulin response. ***Clin. Sci. (Lond)*** *2008;* 114(7): 499-507.
29. Woong-Shick A, Sung-Pil P, Su-Mi B, Joon-Mo L, Sung-Eun N, Gye-Hyun N et al. Identification of hemoglobin-alpha and -beta subunits as potential serum biomarkers for the diagnosis and prognosis of ovarian cancer. ***Cancer Sci***. *2005;* 96(3): 197-201.

30. Tammen H, Mohring T, Kellmann M, Pich A, Kreipe HH, Hess R. Mass spectrometric phenotyping of Val34Leu polymorphism of blood coagulation factor XIII by differential peptide display. ***Clin. Chem.*** *2004;* 50(3): 545-551.
31. Jiang WG, Ablin R, Douglas-Jones A, Mansel RE. Expression of transglutaminases in human breast cancer and their possible clinical significance. ***Oncol. Rep.*** *2003;* 10(6): 2039-2044.
32. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. ***J. Clin. Invest.*** *2006;* 116(1): 271-284.
33. Goncalves A, Esterni B, Bertucci F, Sauvan R, Chabannon C, Cubizolles M et al. Postoperative serum proteomic profiles may predict metastatic relapse in high-risk primary breast cancer patients receiving adjuvant chemotherapy. ***Oncogene.*** *2006;* 25(7): 981-989.
34. Sauter ER, Zhu W, Fan XJ, Wassell RP, Chervoneva I, Du Bois GC. Proteomic analysis of nipple aspirate fluid to detect biologic markers of breast cancer. ***Br. J. Cancer*** *2002;* 86(9): 1440-1443.
35. Rollin D, Whistler T, Vernon SD. Laboratory methods to improve SELDI peak detection and quantitation. ***Proteome. Sci.*** *2007;* 5: 9.
36. Harezlak J, Wang M, Christiani D, Lin X. Quantitative quality-assessment techniques to compare fractionation and depletion methods in SELDI-TOF mass spectrometry experiments. ***Bioinformatics.*** *2007;* 23(18): 2441-2448.
37. Solassol J, Marin P, Demettre E, Rouanet P, Bockaert J, Maudelonde T et al. Proteomic detection of prostate-specific antigen using a serum fractionation procedure: potential implication for new low-abundance cancer biomarkers detection. ***Anal. Biochem.*** *2005;* 338(1): 26-31.
38. Gast MC, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ et al. Influence of sample storage duration on serum protein profiles assessed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS). ***Clin. Chem. Lab Med.*** *2009;* 47(6): 694-705.

Appendix 1

Serum fractionation

Twenty µl serum was denatured in 30 µl 9 M urea / 2% CHAPS in 50 mM Tris-HCl pH 9 (Bio-Rad Labs). After this, samples were applied to the wells of a 96-well ProteinChip Q filtration plate containing Q ceramic HyperD F resin (Bio-Rad Labs), and the plate was incubated for 30 minutes on a MicroMix5 platform shaker (DPC Cirrus Inc., Los Angeles, Ca, USA) to allow binding of the proteins to the beads. Following incubation, the flow through was collected using a vacuum manifold (Millipore, Billerica, MA, USA). Bound proteins were subsequently eluted with a stepwise pH gradient, using wash buffers ranging from pH 9 to pH 3, followed by an organic buffer for the elution of remaining proteins (Bio-Rad Labs). Between additions of the buffers, the filtration plate was placed on the vacuum manifold to collect the fractions. This resulted in six fractions per sample; Fraction 1 (flow-through plus pH 9), Fraction 2 (pH 7), Fraction 3 (pH 5), Fraction 4 (pH 4), Fraction 5 (pH 3) and Fraction 6 (organic buffer).

Appendix 2

Application of the collected fractions to the arrays

Throughout the assay, arrays were assembled in a 96-well bioprocessor (Bio-Rad Labs). IMAC30 arrays were charged with 50 µl 100 mM copper sulfate (Merck) for 10 minutes after which they were neutralized with 100 µl 100 mM sodium acetate buffer pH 4 (Sigma) for 10 minutes and rinsed quickly with 100 µl of MilliQ water (Millipore). After that, the IMAC30 and Q10 arrays were equilibrated twice with 100 µl of their binding buffer for 5 minutes. Next, 85 µl binding buffer was applied to each well, and 15 µl of Fraction 3 and Fraction 6, of every sample, were randomly added to the IMAC30 array and the Q10 array, respectively. Arrays were subsequently incubated for 30 minutes, followed by three washes with 100 µl binding buffer for 5 minutes. During all these steps, the bioprocessor was shaken on the MicroMix 5 platform shaker (DPC Cirrus Inc.) at setting 20/7. Finally, arrays were quickly rinsed with 100 µl MilliQ water (Millipore). After air-drying, 1 µl of a 50% solution of sinapinic acid (SPA; Bio-Rad Labs) in 50% acetonitrile (ACN; Lab-scan Ltd., Dublin, Ireland) and 0.5% trifluoroacetic acid (TFA; Merck) was applied to every spot twice.

Protein profiling and processing of the spectra

Using the PCS 4000 ProteinChip Reader, protein profiles were collected between 0 and 300 kDa, with 530 laser shots with 3500 nJ intensity, at focus mass 7,500 Da, and matrix attenuation till 1,000 Da. To optimally calibrate the m/z's over the whole mass range, we externally calibrated the instrument once with the All-in-one standard peptide mixture, and once with the All-in-one standard protein mixture (Bio-Rad Labs). This resulted in two calibration-equations. These two equations were applied to two copies of each spectrum. Spectra were processed with the ProteinChip Software package version 3.07 (Bio-Rad Labs), separately for spectra obtained with the two different experimental conditions and calibrated with the two different equations. Following baseline subtraction, noise was estimated from 2,000 Da, and spectra were normalized to the average total ion current (TIC) of all spectra from 2,000 Da. Spectra with divergent TIC (normalization factor (NF)

deviating more than two standard deviations from the mean NF) were eliminated from the analysis.

Subsequently, we compared two spectra of one subject on which the two calibration-equations were applied. In this comparison, we determined the mass range in which both spectra reported the same *m/z* for a peak. We observed a minimal difference in *m/z*'s in the mass range 12 to 16 kDa. Therefore, we chose to detect peaks from 2 to 12 kDa in the spectra on which the calibration-equation based on the peptide standard was applied. Peaks from 12 to 300 kDa were detected in the spectra on which the calibration-equation based on the protein standard was applied.

Peak detection

The Expression Difference Mapping (EDM) software application (Bio-Rad Labs) was applied for peak detection. We auto-detected the peaks with a signal-to-noise ratio (S/N) greater than 4, which were present in at least 10 percent of the spectra. In the second pass, peaks were detected with a S/N greater than 2, within a 0.3% mass window of the already detected peaks. In the spectra with no peak in a detected peak cluster, a mark was placed at the average *m/z* of that peak cluster. Additionally, we manually detected clearly visible peaks (S/N>3) that were present in less than 10% of the spectra. We also manually detected 'shoulder-peaks'; peaks that were very close to other peaks, which were not automatically detected as separate peaks.

Chapter 4

Searching for early breast cancer biomarkers by serum protein profiling of pre-diagnostic serum; a nested case-control study

Abstract

Serum protein profiles have been investigated frequently to discover early biomarkers for breast cancer. So far, these studies used biological samples collected *at* or *after* diagnosis. This may limit their value in the search for cancer biomarkers because of the often advanced tumor stage and consequently a risk of reverse causality. Here we present for the first time pre-diagnostic serum protein profiles in relation to breast cancer, using the Prospect-EPIC (European Prospective Investigation into Cancer and nutrition) cohort. In a nested case-control design we compared 68 women diagnosed with breast cancer within three years after enrollment with 68 matched controls for differences in serum protein profiles. All samples were analyzed with SELDI-TOF MS (surface enhanced laser desorption/ionization time-of-flight mass spectrometry). In a subset of 20 case-control pairs, the serum proteome was identified and relatively quantified using isobaric Tags for Relative and Absolute Quantification (iTRAQ) and online two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry (2D-nanoLC-MS/MS). Two SELDI-TOF MS peaks identified as doubly charged apolipoprotein C-I and C3a des-arginine anaphylatoxin ($C3a_{desArg}$) were higher in pre-diagnostic breast cancer serum ($p=0.02$ and $p=0.06$, respectively). With 2D-nanoLC-MS/MS, afamin, apolipoprotein E and isoform 1 of inter-alpha trypsin inhibitor heavy chain H4 (ITIH4) were found to be higher in pre-diagnostic breast cancer serum ($p<0.05$), while alpha-2-macroglobulin and ceruloplasmin were lower ($p<0.05$). $C3a_{desArg}$ and ITIH4 have previously been related to the presence of symptomatic and/or mammographically detectable breast cancer. Here we show for the first time that serum protein profiles are already altered up to three years before breast cancer detection.

Introduction

Early diagnosis of breast cancer by mammography is one of the most important factors contributing to the successful treatment of breast cancer. Further improvement of early diagnosis might be possible with the use of blood-based biomarkers. Such markers could indicate the presence of a breast tumour already in an early stage, preferably even before the lesion is visual on a mammogram. This would be particularly relevant for young women for whom mammographic screening is less effective due to lower sensitivity (25 to 59%) (1). Although the addition of magnetic resonance imaging (MRI) to mammography could improve sensitivity (1), a blood test would be less expensive and easier to perform on a large scale.

Many studies have been executed in an attempt to find such early breast cancer biomarkers, for example using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) (2-9). Several proteins in the blood were indeed found to be related to the presence of breast cancer (2-9). However, only few of these proteins were reported to be discriminative for breast cancer in more than one study, and even then, some proteins found to be higher in patients in one study, were found to be lower in another study (2-9). These discrepancies may be caused by differences between cases and controls in collection, processing and storage of their blood samples, both within and between studies (10-16). On the other hand, it cannot be excluded that findings were simply due to chance.

Until now, all studies used biological samples collected *at* or *after* diagnosis of breast cancer and thus findings may reflect consequences rather than predictors of malignancy. Thus, it remains unclear whether these proteins are able to identify women with a breast lesion which is not yet visible on a mammogram and does not induce clinical symptoms yet. In the present study we performed protein profiling of breast cancer samples for the first time in a nested case-control study. For this we used the Prospect-EPIC (European Prospective Investigation into Cancer and nutrition) cohort (17), where at study enrollment blood samples of approximately 17,000 healthy women were collected and stored. For the current study we selected those women who were diagnosed with breast cancer within 3 years after enrollment in the cohort. Pre-diagnostic protein profiles of their serum samples,

taken at enrollment, were compared to those of matched controls who remained healthy.

Our first aim was to assess whether previously reported proteins are also discriminative in serum samples taken up to three years *before* breast cancer diagnosis. We also set out to discover new discriminating proteins. To this end, we used SELDI-TOF MS that has the possibility to measure multiple proteins simultaneously in a high-throughput fashion. Next, in a subset of the case-control pairs, we analyzed the serum protein profiles with isobaric Tags for Relative and Absolute Quantification (iTRAQ)-labeling and two-dimensional online nano-liquid chromatography coupled with tandem mass spectrometry (2D-nanoLC-MS/MS), by which the detected proteins are relatively quantified and immediately identified. SELDI-TOF MS and 2D-nanoLC-MS/MS cover different mass ranges and therefore are able to detect different proteins.

In summary, we set out to find new proteins as well as to test previously detected proteins in patients still free of symptomatic breast cancer.

Material and Methods

Study population

We performed a case-control study nested within the Prospect-EPIC cohort. Prospect-EPIC is one of the two Dutch cohorts participating in the European Prospective Investigation into Cancer and nutrition, which includes ten European countries. From 1993 to 1997, 17,357 women from Utrecht and vicinity, aged 50-69 years, enrolled in this cohort through the national population-based breast cancer screening program (17). Women filled out an extensive food frequency questionnaire and a general questionnaire. The latter contained questions on demographic characteristics, medical history, lifestyle characteristics, and risk factors for cancer and other chronic diseases (17,18).

Prospect-EPIC participants also donated a blood sample. Blood collection, processing and storage was performed following a strict protocol. After collection, blood samples were stored in a climate controlled refrigerator at 5ºC overnight. The next day blood samples were centrifuged at 1500 g for 20 minutes. After centrifuging, the serum was put in 0.5 ml straws. These straws were stored in a

-86ºC freezer until they were transported to liquid nitrogen tanks (-196ºC), where they have been stored since then.

Participants were followed for vital and health status. Through the municipal registries information on dates of death and migration was obtained. Causes of death were obtained from the Central Bureau for Statistics (CBS). Through yearly linkage with the regional and national cancer registries information about cancer incidence and stage of disease at diagnosis (tumor behavior, tumor size, lymph node involvement and metastasis) was obtained (17). Until December 31st 2006, 687 women were diagnosed with breast cancer in the Prospect-EPIC cohort.

For the current study we selected women who were diagnosed with breast cancer within three years after enrollment into the cohort, and who were postmenopausal at enrollment (no menstrual periods in last 12 months). Women were excluded if they had had cancer before, were suffering from diabetes, were current smokers or were currently using oral contraceptives or menopausal hormone therapy (HT). This was done to obtain a homogeneous group with respect to hormone levels, smoking status, and metabolic status because these factors may influence serum protein profiles. Sixty-eight women were eventually included as a case. Controls were participants of the same cohort. We matched each case with one postmenopausal control that remained free of breast cancer up to the time the case was diagnosed. Additional matching factors were age at enrollment (± 1 year) and date of enrollment (± ½ year). For controls the same exclusion criteria were applied as for cases.

SELDI-TOF MS analysis

We performed serum protein profiling on immobilized metal affinity capture (IMAC30) ProteinChip arrays (Bio-Rad Labs, Hercules, Ca, USA) activated with nickel as described in our previous study (9). The total sample set was analyzed in duplicate, in three separate batches, within two weeks time. Duplicates were analyzed within the same batch, but on different arrays, to correct for inter-array variability. Cases and controls were evenly, and randomly, distributed over the three batches. Samples in one batch were prepared and applied to the arrays, followed by detection of the proteins bound to the arrays with SELDI-TOF MS, on the

same day. SELDI-TOF MS was performed using the PBS-IIC ProteinChip Reader (Bio-Rad Labs). See **Appendix 1** for settings of the ProteinChip Reader.

Since analyzing samples in different batches, on different days, introduces inter-batch variation (16,19,20), spectra were processed per batch. For this, we used the ProteinChip Software package, version 3.1 (Bio-Rad Labs). Spectra in which normalization revealed too low or too high total ion current were excluded from further analysis. The cases and controls matched with these subjects were also excluded from the paired analyses. Subsequently, the Biomarker Wizard (BMW) software application (Bio-Rad Labs) was used to detect peaks. This was performed in each batch separately. See **Appendix 1** for way of processing the spectra and for the settings for peak detection.

SELDI-TOF MS data analysis

Peak information from all acquired spectra was exported from the ProteinChip Software to SPSS 15.0 for statistical analysis. First, we estimated the reproducibility of the duplicates, by calculating the median coefficient of variance (CV) for each detected peak, in cases and controls together. The averaged intensities of the peaks with the same mass in the duplicate spectra of a subject were used for further analysis. To be able to merge peak intensity data of the three batches, averaged peak intensities were first Z-log-transformed per batch (21).

Paired samples T tests were used to test if the mean Z-log-transformed peak intensities in the pre-diagnostic breast cancer serum samples were statistically significantly different from those in the controls samples. We also investigated whether any significant relation could be explained by any of the subject characteristics other than breast cancer status. To this end, bivariate conditional logistic regression analyses were performed including the peak intensity (continuous) and one of the following characteristics: Body Mass Index (BMI), former use of oral contraceptives, former use of HT, number of children, smoking habits, alcohol consumption, blood sample's time in refrigerator between blood collection and centrifugation, and sample's time in -86ºC freezer until storage at liquid nitrogen. The adjusted odds ratios (OR) resulting from the analyses were compared with the crude breast cancer OR in relation to peak intensity.

Sample preparation for 2D-nanoLC-MS/MS

We restricted the 2D-nanoLC-MS/MS analysis to 20 case-control sets, because of costs and time restrictions. The cases included in this sub-analysis were diagnosed with breast cancer within the first 14 months after enrollment in the study.

The serum samples were depleted of the high abundant proteins albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin, using the Multiple Affinity Removal Spin Cartridge (Hu-6HC, Agilent Technologies, Santa Clara, CA, USA) as described in the manufacturer's protocol. Thereafter the samples were desalted using Microcon Centrifugal Filter units (Millipore, Billerica, MA. USA). The total protein content of the depleted sera was determined using a protein assay kit (BCA™, Pierce, Thermo Scientific, Rockfort, IL, USA). The proteins (50 µg per sample) were reduced using tris(2-carboxyethyl)phosphine, alkylated using iodoacetamide and then trypsin digested (Roche Diagnostics Gmbh, Mannhein, Germany) overnight and evaporated to dryness using a SpeedVac. Peptides were labeled with 4-plex iTRAQ reagents (iTRAQ reagent kit-plasma, Applied Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer.

Two case-control pairs were labeled with different isobaric tags in each iTRAQ-labeling set. The first case was labeled with tag114 and the matching control with tag115, the next case was labeled with tag116 and the matching control with tag117. The 4 labeled samples were finally pooled into a new sample tube. A total of 10 iTRAQ-labeled sample sets consisting of two case-control pairs were generated.

2D-nanoLC-MS/MS analysis

The 10 iTRAQ-labeled samples sets were analyzed using quadrupole-time-of-flight mass spectrometer (QSTAR pulsar; Applied Biosystems), equipped with a nanoelectrospray source (Proxeon, Odense, Denmark), and connected to a 2D-nanoLC system equipped with a capillary and nano pump (1100 series; Agilent Technologies). See **Appendix 2** for details about the used columns and mobile phases. The LC system was coupled on-line to a fused-silica PicoTip (50 µm i.d. × 360 µm o.d. × 8 µm tip; New Objective, Woburn, MA, USA). Details about acquisition and calibration are also described in **Appendix 2**.

2D-nanoLC-MS/MS data analysis

Protein identifications and quantifications were performed using Protein Pilot 1.0 (Applied Biosystems) in which the paragon search algorithm was applied. Proteins were searched against the IPI human protein database (IPI human v3.40) downloaded from www.ebi.ac.uk (22). See **Appendix 3** for details on search parameters and data processing.

In some runs, some peptides were unusable for quantification due to an artificial low signal of the signature ions or because the peptide sequence was shared by other proteins. In those cases the peptides were excluded from quantification. No iTRAQ ratio was calculated if there was not one usable peptide left. If only one peptide was usable for quantification of a protein then no error factor (EF) was calculated. A case-control pair was excluded when no ratio and/or EF could be calculated for this pair. Only proteins that could be measured in at least 14 of the 20 case-control pairs were selected for further analysis.

The ratios and the EFs for a protein, in the different pairs, were used to model a random effect model. We used the random effect model since we assumed heterogeneity between the ratios of the different pairs that is partly based on variation by coincidence, but also on true variation between the pairs. The random effect model resulted in a weighted mean ratio with a 95% confidence interval (95%CI) for every protein.

Results

Study population

Characteristics of the total study population are presented in **Table 1**. About half of both cases and controls used oral contraceptives in the past, but the cases used them for a longer period of time than the controls (median: 10 years and 4.5 years, respectively). Cases were somewhat more often nulliparous (15%) than controls (7%). Among women with children, controls had more children than the cases; 3 and 2 (median), respectively. About half of both cases and controls had smoked in the past, for about 8 and 4 pack-years (median), respectively. The energy adjusted intake of alcohol was somewhat higher for controls than for cases; 2.5 and 2.0 g/day respectively (median). Other characteristics were distributed equally in cases and

Table 1 - Study population characteristics

	Cases (*n*=68)	Controls (*n*=68)
Age at enrollment (years)		
Mean (SD)	60.2 (5.6)	60.3 (5.7)
BMI		
Mean (SD)	26.6 (3.1)	26.3 (3.6)
Missing	1	-
Use of oral contraceptives, *n* (%)		
No, but used to in the past	36 (52.9)	40 (58.8)
No, never	32 (47.1)	28 (41.2)
Duration of oral contraceptives use* (years)		
Median (IQR)	10.0 (4.3-15.8)	4.5 (2.0-10.0)
Use of HT, *n* (%)		
No, but used to in the past	7 (10.3)	6 (8.8)
No, never	61 (89.7)	62 (91.2)
Duration of HT use* (years)		
Median (IQR)	1.0 (1.0-8.0)	2.0 (1.0-10.0)
Ovariectomy, *n* (%)		
Both ovaries removed	5 (7.4)	3 (4.5)
Missing	-	1
Parity, *n* (%)		
Nulliparous	10 (14.7)	5 (7.4)
Number of children[†]		
Median (IQR)	2.0 (2.0-3.0)	3.0 (2.0-3.0)
Smoking, *n* (%)		
No, but used to in the past	31 (45.6)	34 (50.0)
No, never	37 (54.4)	34 (50.0)
Pack-years smoking until stop date[‡]		
Median (IQR)	7.9 (1.9-16.4)	4.1 (1.4-10.2)
Missing	1	3
Alcohol intake (g/day)[§]		
Median (IQR)	2.0 (0.2-7.2)	2.5 (0.2-8.4)
Use of medicines, minerals or vitamins[#], *n* (%)		
Yes	46 (67.6)	44 (64.7)
No	22 (32.4)	24 (35.3)
Time since last meal and/or drink** (minutes)		
Median (IQR)	108 (87-137)	116 (88-137)

SD: standard deviation; BMI: body mass index; IQR: inter-quartile range; HT: menopausal hormone therapy; * Among former oral contraceptives/HT users; [†] Among women with children; [‡] Among former smokers; [§] Energy-adjusted alcohol intake at enrollment; [#] In last week before blood collection; ** At moment of blood collection

controls. Characteristics of the serum samples and the sample collection are listed in **Table 2**. There was no difference between cases and controls regarding sample collection and storage. Characteristics of the subjects in the subset analyzed by 2D-nanoLC-MS/MS and of their serum samples are shown in **Appendix 4** and **5**.

Table 2 - Characteristics of the serum samples

	Cases (*n*=68)	Controls (*n*=68)
Serum sample storage duration* (years)		
Mean (SD)	11.2 (1.1)	11.2 (1.1)
Hours in refrigerator†		
Median (IQR)	22 (21-23)	22 (20-23)
Days at -86°C‡		
Median (IQR)	8 (6-11)	7 (5-11)

SD: standard deviation; IQR: inter-quartile range; * Until experiment; † Between collection and centrifugation; ‡ Between centrifugation and storage at liquid nitrogen

Breast cancer was diagnosed after a median time of 21.3 months (IQR: 0.7-26.6) after enrollment. More than 80% of the cases had an invasive tumor. More than half of the invasive tumors were diagnosed in Stage I and a quarter of the invasive tumors were diagnosed in Stage IIA. Only one tumor was diagnosed in Stage IIIA. The invasive tumors were more or less equally distributed over the three size categories (>0.1-1 cm, 1-2 cm and >2 cm). In almost 30% of the invasive tumors, lymph nodes were involved. None of the cases was diagnosed with distant metastasis. We reported the pathologically determined tumor size and lymph node involvement unless this was unknown; in that case we reported the clinically determined stage. Cases in the subset analyzed by 2D-nanoLC-MS/MS were diagnosed 0.9 months (median) (inter-quartile range (IQR): 0.6-7.5) after enrolment. Two of the 20 cases were diagnosed with carcinoma in situ. Two thirds of the invasive tumors were diagnosed in Stage I and almost a quarter in Stage IIA, the remaining tumors were diagnosed in Stage IIB. Half of the invasive tumors were sized <1 cm, and in only three invasive tumors lymph nodes were involved.

Peaks detected with SELDI-TOF MS

After normalization, 25 of the 272 spectra (68 cases and 68 controls in duplicate) had to be eliminated from the analysis. These outliers included 12 spectra of cases and 13 spectra of controls. Of one case and two controls both spectra (duplicates) had to be eliminated. With the BMW software application, in total 47 different peaks were auto-detected in the three batches. Twenty-two of these peaks were present with an S/N >2 in at least 50% of the spectra in each batch. The median CV's of these peaks varied between 12% and 35%.

The intensity of a peak with mass-to-charge ratio (*m/z*) 3323 was statistically significantly higher in pre-diagnostic breast cancer serum samples than in serum samples of controls (p=0.02). The intensity of a peak with *m/z* 8938 was borderline statistically significantly higher in cases than in controls (p=0.06) (**Figure 1**). No statistically significant relations were found between the intensities of the other detected peaks and the early presence of breast cancer. The 22 detected peaks ordered by their *m/z*, together with their mean Z-log-transformed peak intensities in cases and controls, and the results of the paired T test are listed in **Table 3**.

Bivariate conditional logistic regression analysis revealed that the relations between *m/z* 3323 and breast cancer, and *m/z* 8938 and breast cancer, were independent of age, BMI, oral contraceptives use, HT use, number of children, smoking habits, alcohol intake, duration of blood sample in refrigerator between collection and centrifugation, or serum sample storage duration at -86ºC before storage at liquid nitrogen (data not shown).

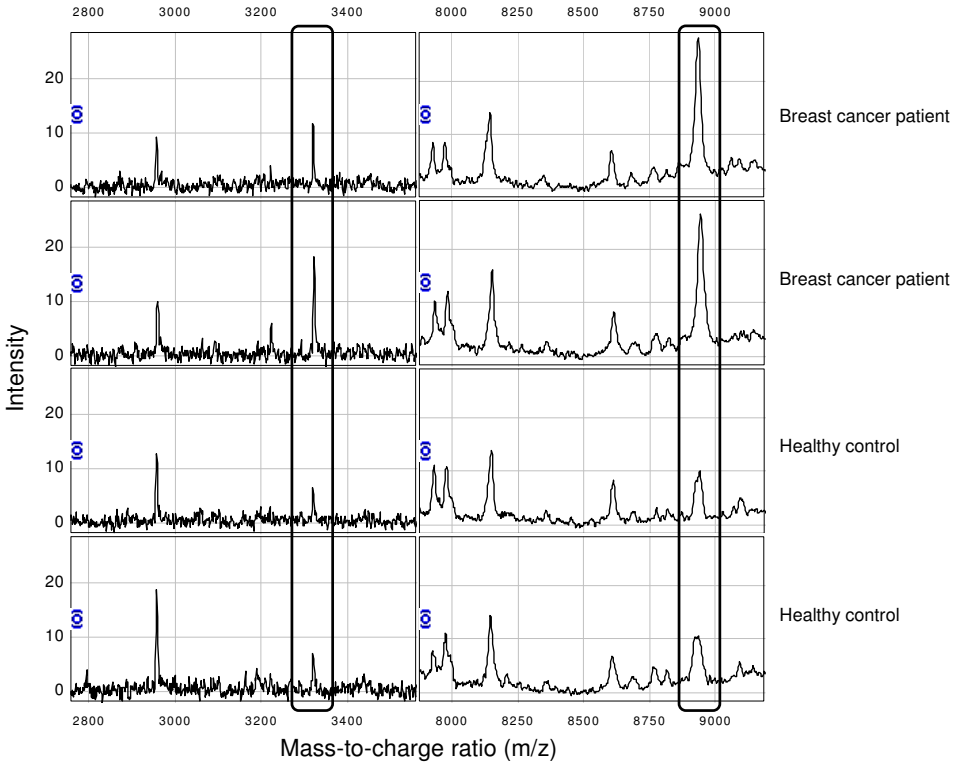

Figure 1 - Difference in protein expression of *m/z* 3323 and *m/z* 8938, detected with SELDI-TOF MS, between breast cancer cases and healthy controls

Table 3 - The Z-log-transformed intensities of the peaks detected with SELDI-TOF MS, ordered by their *m/z*.

	Cases (*n*=65)	Controls (*n*=65)		Paired T test
M/z	*Mean Z-log-transformed intensity (SD)*	*Mean Z-log-transformed intensity (SD)*	*Intensity in cases vs. controls*	*p-value*
2958	0.06 (0.95)	-0.03 (1.02)	-	.61
3323	0.21 (0.98)	-0.19 (0.97)	Higher	.02
3888	0.12 (1.00)	-0.13 (0.99)	-	.18
4649	0.03 (0.95)	-0.02 (1.00)	-	.80
4824	-0.09 (0.85)	0.13 (1.11)	-	.21
5343	0.09 (0.87)	-0.05 (1.08)	-	.43
5911	0.00 (0.76)	0.03 (1.16)	-	.86
6117	0.03 (0.90)	-0.01 (1.06)	-	.81
6439	0.09 (0.99)	-0.11 (1.01)	-	.27
6637	0.11 (0.97)	-0.10 (1.03)	-	.23
6842	0.08 (1.01)	-0.07 (1.00)	-	.43
6948	-0.12 (1.01)	0.14 (0.93)	-	.15
7476	-0.04 (0.94)	0.05 (1.06)	-	.62
7772	0.06 (0.95)	-0.08 (1.05)	-	.45
7978	0.11 (0.97)	-0.12 (1.02)	-	.21
8148	0.11 (0.97)	-0.12 (1.02)	-	.21
8609	-0.04 (1.02)	0.07 (0.97)	-	.55
8938	0.19 (1.02)	-0.14 (0.94)	Higher	.06
9294	0.03 (0.89)	0.00 (1.03)	-	.88
9427	0.15 (1.09)	-0.17 (0.88)	-	.08
9501	0.06 (0.87)	-0.05 (1.06)	-	.54
13892	-0.08 (0.97)	0.12 (0.95)	-	.26

M/z: mass-to-charge ratio; SD: standard deviation; - : No statistically significant difference in intensity

Identities of the SELDI-TOF MS peaks

Based on results of a previous study performed by our group (23), the peak with *m/z* 3323 was identified as doubly charged apolipoprotein C-I. We previously identified a 6.6 kDa peak as apolipoprotein C-I (molecular weight (MW): 6631 Da) by biomarker purification, in-gel tryptic digestion and peptide mapping. Its identity was confirmed with an immunoassay. In the same study, a highly correlated 3.3 kDa peak was found to be the result of double charged apolipoprotein C-I ions (23). As expected, besides *m/z* 3323, we also detected the peak representing apolipoprotein C-I itself in the current study (*m/z* 6637), although its relationship with early stage breast cancer

was not statistically significant (p=0.23). The Z-log-transformed intensities of *m/z* 6637 and *m/z* 3323 detected in the current study were also correlated (Pearson R^2=0.558 (p<0.001) in the controls), as expected between a protein and its doubly charged ion.

The peak with *m/z* 8938 was identified as C3a des-arginine anaphylatoxin ($C3a_{desArg}$) (MW: 8939 Da), based on a previous study by our group (24). In that study a peak with *m/z* 8937 was identified as $C3a_{desArg}$ by protein purification and in-gel tryptic digestion, followed by peptide mapping. The identity of the peak was confirmed by sequencing the tryptic digest peptides by quadrupole-time-of-flight MS and by an immunoassay on ProteinA beads (24).

Proteins detected with 2D-nanoLC-MS/MS

In total, 110 different proteins were detected in the samples of the 20 cases-control pairs with 2D-nanoLC-MS/MS. For only 32 of the detected proteins, ratios and EF's could be calculated for at least 14 of the 20 case-control pairs (**Table 4**). Afamin, apolipoprotein E and an isoform of inter-alpha trypsin inhibitor heavy chain H4 (ITIH4) were statistically significantly higher (p<0.05) in cases than in controls (weighted mean ratio: 1.10 (95%CI: 1.02-1.18), 1.13 (95%CI: 1.01-1.26) and 1.08 (95%CI: 1.03-1.14), respectively). Alpha-2-macroglobulin and ceruloplasmin were statistically significantly lower (p<0.05) in cases than in controls (weighted mean ratio: 0.94 (95%CI: 0.88-1.00) and 0.94 (95%CI: 0.89-0.99), respectively).

Table 4 - Proteins detected with 2D-nanoLC-MS/MS in 14 pairs or more

		Pairs*	Weighted ratio†		Random fixed effects model
Protein Name	*Function*	*n*	*Mean*	*95%CI*	*p-value*
Vitronectin		16	1.06	0.99-1.13	.07
Transthyretin		15	0.98	0.88-1.09	.71
Alpha-1B-glycoprotein		20	1.03	0.99-1.07	.17
Alpha-2-macroglobulin	**Proteinase inhibitor**	**20**	**0.94**	**0.88-1.00**	**.04**
Afamin	**Serum transport protein**	**17**	**1.10**	**1.02-1.18**	**.02**
AMBP protein		18	1.04	0.97-1.12	.26
Apolipoprotein A-I		20	1.04	0.97-1.12	.25
Apolipoprotein A-II		20	0.98	0.91-1.06	.61
Apolipoprotein A-IV		20	1.05	0.95-1.18	.32
Apolipoprotein B-100		20	1.06	0.99-1.12	.08
Complement C3 (Fragment)		20	1.02	0.98-1.06	.34
Isoform 1 of Complement factor H		18	1.02	0.97-1.07	.39
Ceruloplasmin	**Acute phase reactant**	**20**	**0.94**	**0.89-0.99**	**.03**
Hemopexin		20	0.96	0.90-1.02	.17
Histidine-rich glycoprotein		20	0.96	0.89-1.04	.30
Inter-alpha trypsin inhibitor heavy chain H1		16	1.00	0.94-1.07	.89
Alpha-1-acid glycoprotein 2		16	1.00	0.93-1.08	.91
Inter-alpha (Globulin) inhibitor H2		18	0.96	0.89-1.04	.33
Orosomucoid 1		20	1.06	0.98-1.14	.16
Alpha-1-antichymotrypsin		19	1.00	0.92-1.08	.94
B-factor, properdin		18	0.99	0.94-1.05	.76
Plasminogen		18	0.98	0.90-1.07	.69
Alpha-2-HS-glycoprotein		18	1.00	0.94-1.07	.88
Beta-2-glycoprotein 1		18	0.99	0.92-1.05	.66
C4B1		16	0.99	0.93-1.05	.74
Prothrombin (Fragment)		16	0.98	0.88-1.10	.76
Apolipoprotein E	**Lipid metabolism**	**16**	**1.13**	**1.01-1.26**	**.04**
Apolipoprotein C-I		14	1.02	0.94-1.12	.57
13 kDa protein		14	1.03	0.92-1.16	.55
Isoform LMW of Kininogen-1		18	1.06	0.98-1.14	.13
Isoform 1 of inter-alpha trypsin inhibitor heavy chain H4	**Acute phase reactant**	**16**	**1.08**	**1.03-1.14**	**<.01**
Vitamin D-binding protein		20	1.03	0.99-1.07	.17

95%CI: 95% confidence interval; * Number of pairs in which a ratio could be determined and a EF could be calculated; † Ratio case/control

Discussion

We found several proteins that showed different intensities in pre-diagnostic serum samples of breast cancer cases not yet showing clinical symptoms compared to samples of healthy controls. Two proteins detected with SELDI-TOF MS, one with *m/z* 3323 identified as a double charged ion of apolipoprotein C-I, and another with *m/z* 8938 identified as $C3a_{desArg}$, were found to be related to pre-diagnostic breast cancer. Of the proteins detected with 2D-nanoLC-MS/MS, afamin, apolipoprotein E and an isoform of ITIH4 were slightly, but significantly higher and alpha-2-macroglobulin and ceruloplasmin slightly, but significantly lower in pre-diagnostic breast cancer samples compared to control samples.

A protein detected with SELDI-TOF MS showed the largest difference between cases and controls and was identified as the double charged ion of apolipoprotein C-I (*m/z* 3323). Apolipoprotein C-I itself, detected both with SELDI-TOF MS (*m/z* 6637) and 2D-nanoLC-MS/MS, showed results in the same direction, i.e. higher in cases, but not statistically significantly. In a study by Engwegen et al. (23), examining serum samples taken after diagnosis, the doubly charged ion of apolipoprotein C-I was lower in breast cancer cases, but not statistically significantly. Apolipoprotein C-I itself (6631 Da), was statistically significantly lower in breast cancer cases in that study (23). It is striking that the same protein was found to be related with breast cancer in both studies, but in different directions. This may be due to differences in sample collection, processing and storage, but also to the differences in stage of disease of the two study populations. We included samples collected up to three years before diagnosis, while in the study by Engwegen et al. (23) samples were collected after diagnosis. Apolipoprotein C-I may be differently expressed in pre-diagnostic stages of breast cancer compared to stages visible on a mammogram and/or leading to clinical symptoms. It is also possible that the result is a chance finding.

$C3a_{desArg}$ (*m/z* 8938), that we found to be higher in pre-diagnostic breast cancer samples, has been found to be related to breast cancer in several previous SELDI-TOF MS studies (2-5,7,25). In the majority of these studies the protein was higher in patients compared to controls (3-5,7), but in two studies it was lower (2,9). ITIH4 was higher in our pre-diagnostic breast cancer samples than in the

control samples. This is a protein of which fragments have been frequently described in relation to symptomatic and/or mammographically detectable breast cancer (4,5,7,9,26-28). In these studies levels of a 4.3 kDa ITIH4 fragment were found either to be significantly higher (7,26), or significantly lower (4,5,9) in breast cancer. Levels of other fragments of ITIH4, which were investigated by Villanueva et al. (27), Song et al. (26), and our own group (28), were usually found to be higher in breast cancer or were not related at all (26-28).

To our knowledge, afamin, apolipoprotein E, alpha-2-macroglobulin and ceruloplasmin have not been found before to differ between breast cancer serum samples and control serum samples in studies using SELDI-TOF MS or other profiling methods. In the 1980s however, the acute phase proteins alpha-2-macroglobulin and ceruloplasmin were already studied in relation to breast cancer using immunoassay methods (29,30). Serum levels of alpha-2-macroglobulin did not differ between breast cancer patients and women with benign breast disease (29). In our study, alpha-2-macroglobulin and ceruloplasmin were both lower in pre-diagnostic breast cancer samples compared to the control samples.

The most important strength of our study is that we were able to investigate proteomic profiles in serum of patients with asymptomatic breast cancer (diagnosed after a median time of 21.3 months (IQR: 0.7-26.6) after enrollment). Our study population therefore is more appropriate for finding early breast cancer biomarkers than all previous studies where mostly symptomatic cases were included. The case-control design nested in a cohort of apparently healthy screening participants also ensures that all serum samples were collected, processed and stored uniformly under strictly defined conditions, at a time when none of the participants were diagnosed with breast cancer yet. These factors have shown to be important in protein profiling studies (10-16). In this way systematic errors due to differences in these factors between cases and controls were prevented in our study.

To our knowledge, protein profiling has not been performed before in breast cancer serum samples that were stored for more than 10 years at -196°C. Despite this long storage duration, and potential related breakdown of proteins, we were still able to show differences in serum protein profiles between asymptomatic breast cancer cases and healthy controls. This is promising for other cohorts that

may have samples stored for several years, since this study design is ideal to study early biomarkers in an unbiased way.

By measuring the protein profiles both with SELDI-TOF MS and 2D-nanoLC-MS/MS we benefited of the advantages of two complementary methods. SELDI-TOF MS has the advantage to simultaneously measure parts of the serum proteome in a high-throughput fashion with relative simple sample preparation, high analytical sensitivity and high speed of data acquisition (31,32). Although with 2D-nanoLC-MS/MS fewer samples can be measured simultaneously, this method has the advantage that it can identify the detected proteins immediately. Moreover, the protein detection by these two methods is complementary. With SELDI-TOF MS mainly measuring proteins in the 2 to 10 kDa mass range, many break-down products can be detected. Additionally, by measuring exact mass-to-charge ratios with SELDI-TOF MS, it is also possible to detect post-translational modified forms of proteins; for example proteins with additional amino acids or truncated forms. With 2D-nanoLC-MS/MS in combination with iTRAQ-labeling a higher selectivity is reached because of analysis of tryptic peptides with protein identification based on sequence information. This allows proteins with higher mass to be identified which cannot be detected with high sensitivity by SELDI-TOF MS.

In summary, we detected several serum proteins that differed in concentration between women with asymptomatic breast cancer and matched healthy controls. This study also shows for the first time the possibility to measure protein profiles in serum samples stored for more than 10 years in liquid nitrogen, both with SELDI-TOF MS and with 2D-nanoLC-MS/MS. This may open the way to find early (breast) cancer biomarkers in blood samples from large cohorts, where samples were collected several years before the clinical manifestation of the disease. Remarkably, high abundant, acute phase proteins, which we expected only to be detectable in symptomatic cancer cases, were also found to be significantly higher *before* diagnosis. However, it is still not clear whether these proteins are breast cancer specific. Given that the currently identified proteins are high abundant, they are unlikely to be breast cancer specific, at least on their own. It is likely that specific tumor markers are less abundant, as it is typical for known circulating tumor markers to have low concentrations (33). Using techniques that

give insight into 'the deeper/low abundant proteome', e.g. by fractionation of the samples or depletion of a higher number of the most abundant proteins, which was already partially done in the 2Dnano-LC-MS/MS analysis, may help to find these low abundant and probably more specific tumor markers.

Acknowledgements

The Prospect-EPIC study was funded by "Europe Against Cancer" Program of the European Commission (SANCO); the Dutch Ministry of Health; the Dutch Cancer Society; ZonMw the Netherlands Organization for Health Research and Development; World Cancer Research Fund (WCRF). We thank Integral Cancer Registration IKMN and the Integral Cancer Registration IKO for follow-up data on cancer. This study was financially supported by a grant from the board of the University Medical Center Utrecht and Julius Center for Health Sciences and Primary Care ('Strategische Impuls') and by the Netherlands Laboratory for Anticancer Drug Formulation (NLADF), Amsterdam, The Netherlands. Part of the proteomics work funded by the ECNIS Network of Excellence (Environmental Cancer Risk, Nutrition and Individual Susceptibility), operating within the European Union 6th Framework Program, Priority 5: "Food Quality and Safety" (FOOD-CT-2005-513943). Dr. Lützen Portengen is greatly acknowledged for his statistical assistance in the 2D-nanoLC-MS/MS data analysis.

References

1. Lord SJ, Lei W, Craft P, Cawson JN, Morris I, Walleser S et al. A systematic review of the effectiveness of magnetic resonance imaging (MRI) as an addition to mammography and ultrasound in screening young women at high risk of breast cancer. ***Eur. J. Cancer*** *2007;* 43(13): 1905-1917.
2. Hu Y, Zhang S, Yu J, Liu J, Zheng S. SELDI-TOF-MS: the proteomics and bioinformatics approaches in the diagnosis of breast cancer. ***Breast.*** *2005;* 14(4): 250-255.
3. Belluco C, Petricoin EF, Mammano E, Facchiano F, Ross-Rucker S, Nitti D et al. Serum proteomic analysis identifies a highly sensitive and specific discriminatory pattern in stage 1 breast cancer. ***Ann. Surg. Oncol.*** *2007;* 14(9): 2470-2476.
4. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. ***Clin. Chem.*** *2002;* 48(8): 1296-1304.
5. Mathelin C, Cromer A, Wendling C, Tomasetto C, Rio MC. Serum biomarkers for detection of breast cancers: a prospective study. ***Breast Cancer Res. Treat.*** *2006;* 96(1): 83-90.
6. Vlahou A, Laronga C, Wilson L, Gregory B, Fournier K, McGaughey D et al. A novel approach toward development of a rapid blood test for breast cancer. ***Clin. Breast Cancer.*** *2003;* 4(3): 203-209.
7. Li J, Orlandi R, White CN, Rosenzweig J, Zhao J, Seregni E et al. Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. ***Clin. Chem.*** *2005;* 51(12): 2229-2235.
8. Gast MC, Bonfrer JM, van Dulken EJ, de Kock L, Rutgers EJ, Schellens JH et al. SELDI-TOF MS serum protein profiles in breast cancer: assessment of robustness and validity. ***Cancer Biomark.*** *2006;* 2(6): 235-248.
9. van Winden AW, Gast MC, Beijnen JH, Rutgers EJ, Grobbee DE, Peeters PH et al. Validation of previously identified serum biomarkers for breast cancer with SELDI-TOF MS: a case control study. ***BMC. Med. Genomics*** *2009;* 2: 4.
10. Hsieh SY, Chen RK, Pan YH, Lee HL. Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. ***Proteomics.*** *2006;* 6(10): 3189-3198.
11. Timms JF, Arslan-Low E, Gentry-Maharaj A, Luo Z, T'Jampens D, Podust VN et al. Preanalytic influence of sample handling on SELDI-TOF serum protein profiles. ***Clin. Chem.*** *2007;* 53(4): 645-656.
12. Villanueva J, Philip J, Chaparro CA, Li Y, Toledo-Crow R, DeNoyer L et al. Correcting common errors in identifying cancer-specific serum peptide signatures. ***J. Proteome. Res.*** *2005;* 4(4): 1060-1072.
13. West-Nielsen M, Hogdall EV, Marchiori E, Hogdall CK, Schou C, Heegaard NH. Sample handling for mass spectrometric proteomic investigations of human sera. ***Anal. Chem.*** *2005;* 77(16): 5114-5123.

14. Banks RE, Stanley AJ, Cairns DA, Barrett JH, Clarke P, Thompson D et al. Influences of blood sample processing on low-molecular-weight proteome identified by surface-enhanced laser desorption/ionization mass spectrometry. ***Clin. Chem.*** *2005;* 51(9): 1637-1649.
15. Engwegen JY, Alberts M, Knol JC, Jimenez CR, Depla AC, Tuynman H et al. Influence of variations in sample handling on SELDI-TOF MS serum protein profiles for colorectal cancer. ***Proteomics Clin. Appl.*** *2008;* 2: 936-945.
16. Karsan A, Eigl BJ, Flibotte S, Gelmon K, Switzer P, Hassell P et al. Analytical and preanalytical biases in serum proteomic pattern analysis for breast cancer diagnosis. ***Clin. Chem.*** *2005;* 51(8): 1525-1528.
17. Boker LK, van Noord PA, van der Schouw YT, Koot NV, Bueno de Mesquita HB, Riboli E et al. Prospect-EPIC Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. ***Eur. J. Epidemiol.*** *2001;* 17(11): 1047-1053.
18. Pols MA, Peeters PH, Ocke MC, Slimani N, Bueno-de-Mesquita HB, Collette HJ. Estimation of reproducibility and relative validity of the questions included in the EPIC Physical Activity Questionnaire. ***Int. J. Epidemiol.*** *1997;* 26 Suppl 1: S181-S189.
19. Hu J, Coombes KR, Morris JS, Baggerly KA. The importance of experimental design in proteomic mass spectrometry experiments: some cautionary tales. ***Brief. Funct. Genomic. Proteomic.*** *2005;* 3(4): 322-331.
20. Pelikan R, Bigbee WL, Malehorn D, Lyons-Weiler J, Hauskrecht M. Intersession reproducibility of mass spectrometry profiles and its effect on accuracy of multivariate classification models. ***Bioinformatics.*** *2007;* 23(22): 3065-3072.
21. Gast MC, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ et al. Serum protein profiling for diagnosis of breast cancer using SELDI-TOF MS. ***Oncol. Rep.*** *2009;* 22(1): 205-213.
22. Kersey PJ, Duarte J, Williams A, Karavidopoulou Y, Birney E, Apweiler R. The International Protein Index: an integrated database for proteomics experiments. ***Proteomics.*** *2004;* 4(7): 1985-1988.
23. Engwegen JY, Helgason HH, Cats A, Harris N, Bonfrer JM, Schellens JH et al. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. ***World J. Gastroenterol.*** *2006;* 12(10): 1536-1544.
24. Gast MC, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ et al. Influence of sample storage duration on serum protein profiles assessed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS). ***Clin. Chem. Lab Med.*** *2009;* 47(6): 694-705.
25. Goncalves A, Esterni B, Bertucci F, Sauvan R, Chabannon C, Cubizolles M et al. Postoperative serum proteomic profiles may predict metastatic relapse in high-risk primary breast cancer patients receiving adjuvant chemotherapy. ***Oncogene.*** *2006;* 25(7): 981-989.

26. Song J, Patel M, Rosenzweig CN, Chan-Li Y, Sokoll LJ, Fung ET et al. Quantification of fragments of human serum inter-alpha-trypsin inhibitor heavy chain 4 by a surface-enhanced laser desorption/ionization-based immunoassay. ***Clin. Chem.*** *2006;* 52(6): 1045-1053.
27. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. ***J. Clin. Invest.*** *2006;* 116(1): 271-284.
28. van Winden AW, van den Broek I, Gast MC, Engwegen JY, Sparidans RW, Dulken EJ et al. Serum degradome markers for the detection of breast cancer. *Submitted for publication.*
29. Kreienberg R, Koehler P, Kasemeyer R, Melchert F. Clinical utility of different tumor markers in breast cancer and gynecological malignancies. ***Cancer Detect. Prev.*** *1983;* 6(1-2): 221-225.
30. Lamoureux G, Mandeville R, Poisson R, Legault-Poisson S, Jolicoeur R. Biologic markers and breast cancer: a multiparametric study--1. Increased serum protein levels. ***Cancer*** *1982;* 49(3): 502-512.
31. Hutchens TW, Yip TT. New desorption strategies for the mass spectrometric analysis of macromolecules. ***Rapid Commun. Mass Spectrom.*** *1993;* 7: 576-580.
32. Merchant M, Weinberger SR. Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. ***Electrophoresis*** *2000;* 21(6): 1164-1177.
33. Lee SM, Hwang KS, Yoon HJ, Yoon DS, Kim SK, Lee YS et al. Sensitivity enhancement of a dynamic mode microcantilever by stress inducer and mass inducer to detect PSA at low picogram levels. ***Lab Chip.*** *2009;* 9(18): 2683-2690.

Appendix 1

SELDI-TOF MS data collection

With the PBS-IIC ProteinChip Reader (Bio-Rad Labs), 96 shots were fired on every spot, with a laser intensity of 153, and a detector sensitivity of 6. This was done after two warming shots per position, with a laser intensity of 155, which were not included in the final spectrum. Masses up to 160,000 Da were detected with an optimization range from 1,500 Da to 12,000 Da and with a focus at 8,000 Da. Mass-to-charge ratios (*m*/*z*'s) were calibrated externally with the All-in-one standard peptide mixture (Bio-Rad Labs).

Spectra were first baseline subtracted, noise was estimated in the range from 2,000 to 160,000 Da, and spectra were normalized to the total ion current in the same range. Spectra with a normalization factor >2.00 or <0.50 were excluded from further analysis.

Peaks with a signal-to-noise ratio (S/N) greater than 5, and present in at least 20% of the spectra, were auto-detected in the first pass. In the second pass, peaks were detected with an S/N greater than 2, within a 0.3% mass window of the already detected peaks. Since not all peaks were auto-detected in each batch, all peaks auto-detected in at least one batch were subsequently manually detected in all batches. Thereafter, the BMW was applied on these user-detected peaks. Finally, the peaks with an S/N >2 in at least 50% of the spectra per batch were selected for further analysis.

Appendix 2

2D-nanoLC-MS/MS analysis

In the first dimension peptides were separated on a strong cation exchange (SCX) column (35 × 0.3 mm ZORBAX SCX Bioseries II, Agilent technologies) with mobile phase A (water with 3% acetonitrile and 0.1% formic acid) and B (0.5 M ammonium formate, 3% acetonitrile pH set to 3.5 using formic acid) using the capillary pump. The flow was set to 10 µl/min. The sample was eluted in 9 steps:;1% B, 1-6% B, 6-6.5% B, 6.5-10% B, 10-12.5% B, 12.5-15% B, 15-22% B, 22-30% B and 30-80% B (thus yielding a total of 9 fractions). The eluted peptides were trapped on a C18 trap column (5 µm 5×0.3 mm, ZORBAX; Agilent Technologies), the valve was then switched so that the nanoflow path was redirected through the trap-column and then onto a C18 analysis column (3.5 µm 150 mm × 100 µm, ZORBAX; Agilent Technologies). The mobile phases of the nanopump were A: water and B: acetonitrile both acidified with 0.1% formic acid. In each run a gradient from 5-40% B for 85 min, from 40-70% B in 5 min and then back to 5% B was applied in a flow rate of 400 nl/min.

Information dependent acquisitions (IDA) were performed with a total cycle time of 9 s including a TOF MS survey scan (1 s, *m/z* 300-1100), followed by acquiring of MS/MS spectra (2 s, *m/z* 100-2000) of the 4 most intense ions with charge state 2 to 5. Former target ions were excluded for 50 s and IDA were collected for 120 min for each fraction. The instrument was calibrated with CsI (*m/z* 132.9054) and the pentapeptide iPDI (*m/z* 829.5398).

Appendix 3

2D-nanoLC-MS/MS data analysis

Search parameters were as follows: cystein modification, iodoacetamide; digestion, trypsin; search effort, thorough ID; instrument, QSTAR ESI; detected protein threshold, >1,3 (only proteins identified with at least 95% confidence). To compare expression levels in cases vs. controls tag114-labeled samples were normalized to the tag115-labeled samples and tag116 to tag117, respectively. Also, the ratios were corrected for unequal mixing of proteins when preparing the samples by dividing the ratios with the median average protein ratio calculated for each pair of an iTRAQ set (bias correction).

Appendix 4

Characteristics of the subset

	Cases (*n*=20)	Controls (*n*=20)
Age at enrollment (years)		
Mean (SD)	59.6 (6.0)	59.7 (6.1)
BMI		
Mean (SD)	26.8 (3.5)	26.5 (3.6)
Missing	1	-
Use of oral contraceptives, *n* (%)		
No, but used to in the past	11 (55.0)	10 (50.0)
No, never	9 (45.0)	10 (50.0)
Duration of oral contraceptives use* (years)		
Median (IQR)	10 (4-12)	4.5 (2.5-10.5)
Use of HT, *n* (%)		
No, but used to in the past	-	-
No, never	20 (100)	20 (100)
Duration of HT use* (years)		
Median (IQR)	-	-
Ovariectomy, *n* (%)		
Both ovaries removed	1 (5.0)	-
Missing	-	-
Parity, *n* (%)		
Nulliparous	2 (10.0)	-
Number of children[†]		
Median (IQR)	2.0 (2.0-3.0)	3.0 (2.0-3.8)
Smoking, *n* (%)		
No, but used to in the past	10 (50.0)	10 (50.0)
No, never	10 (50.0)	10 (50.0)
Pack-years smoking until stop date[‡]		
Median (IQR)	3.1 (1.6-7.9)	7.2 (1.1-16.4)
Missing	1	1
Alcohol intake[§] (g/day)		
Median (IQR)	5.1 (0.8-17.0)	3.4 (0.1-6.4)
Use of medicines, minerals or vitamins[#], *n* (%)		
Yes	16 (80.0)	11 (55.0)
No	4 (20.0)	9 (45.0)
Time since last meal and/or drink** (minutes)		
Median (IQR)	122 (97-160)	134 (87-145)

SD: standard deviation; BMI: body mass index; IQR: inter-quartile range; HT: menopausal hormone therapy; * Among former oral contraceptives/HT users; [†] Among women with children; [‡] Among former smokers; [§] Energy-adjusted alcohol intake at enrollment; [#] In last week before blood collection; ** At moment of blood collection

Appendix 5

Characteristics of the serum samples in the subset

	Cases (*n*=20)	Controls (*n*=20)
Serum sample storage duration* (years)		
Mean (SD)	11.5 (1.1)	11.5 (1.1)
Hours in refrigerator†		
Median (IQR)	21 (19-22)	22 (18-22)
Days at -86°C‡		
Median (IQR)	9 (5-21)	9 (5-21)

SD: standard deviation; IQR: inter-quartile range; * Until experiment; † Between collection and centrifugation; ‡ Between centrifugation and storage at liquid nitrogen

Part 2

Candidate-based approach

Chapter 5

Serum degradome markers
for the detection of breast cancer

Abstract

Many proteins have been proposed as potential biomarkers for breast cancer. Yet, validation of their discriminative value using quantitative methods has scarcely been performed. In this study we investigated the discriminative value of six peptides that were previously proposed to be generated by breast cancer specific exoproteases: bradykinin, des-Arg^{9}-bradykinin, Hyp^{3}-bradykinin, and fragments of fibrinogen α-chain (Fib-α $_{[605-629]}$), complement component 4a (C4a $_{[1337-1350]}$) and inter-alpha trypsin inhibitor heavy chain 4 (ITIH4 $_{[666-687]}$). Their absolute serum concentrations were measured with a validated liquid chromatography-tandem mass spectrometric assay (LC-MS/MS), and compared between 62 newly diagnosed breast cancer patients and 62 controls matched for age and sample storage duration. Both ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin showed statistically significantly higher median concentrations in breast cancer samples than in matched control samples. In a combined analysis, ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin independently contributed to the discrimination between cases and controls. Additionally, we analyzed of the same patients serum samples collected after surgical removal of the tumor, in which median ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin concentrations were significantly decreased, and not statistically significantly different from concentrations in the controls anymore. In this study we confirmed that the exoprotease breakdown peptides, ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin, differed between breast cancer cases and controls, supporting the potential of degradome markers for the diagnosis of breast cancer.

Introduction

Many studies have been performed to find breast cancer biomarkers in blood that can be used for early detection of the disease (1-5). The only breast cancer blood biomarkers identified until now are cancer antigen 15.3 (CA15.3), cancer antigen 27.29 (CA27.29) and carcinoembryonic antigen (CEA). Although these proteins are useful in combination with imaging and physical examination for monitoring treatment in breast cancer patients with metastatic disease, they lack sensitivity to detect primary breast cancer in an early stage (6). In the last decade further attempts have been made in the search for breast cancer blood biomarkers using mass spectrometric (MS) techniques like surface enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF MS) and matrix assisted LDI-TOF MS (MALDI-TOF MS) (7-16). These platforms allow simultaneous measurement of a large part of the proteome, in e.g. serum or plasma, in a high-throughput fashion. However, until now, this technique mainly detected high abundant acute phase proteins that could discriminate between breast cancer cases and healthy controls. Unfortunately, these proteins are likely to lack cancer type specificity, and could be related to other inflammatory processes as well (17).

Villanueva et al. (18) recently discovered that not the abundant proteins themselves, but the pattern in which the abundant proteins are degraded, the degradome, harbors cancer specificity, as well as cancer type specificity. They found 61 signature peptides that were able to make a distinction between bladder, breast and prostate cancer patients, and controls without cancer. After tandem mass spectrometry (MS/MS) based sequence identification, these signature peptides appeared to be breakdown products of abundant blood proteins. Interestingly, different breakdown products from the same proteins were found to be related to different types of tumors. It was suggested that cancer cells contribute unique proteases that result in specific degradation patterns. The patterns appeared not only to be cancer specific, but also cancer *type* specific. They may therefore have clinical utility as surrogate markers for the detection and classification of cancer (18).

Peptides that showed the greatest differences between breast cancer patients and controls ($p<1*10^{-11}$), and which were also higher or lower compared to controls exclusively in breast cancer patients and not in other cancers, were bradykinin (*m/z* 1060.57), and fragments of apolipoprotein A-IV (apo A-IV$_{[256-278]}$; *m/z* 2508.16), fibrinogen α-chain (Fib-α $_{[605-629]}$; *m/z* 2659.03), complement component 4a (C4a $_{[1337-1350]}$; *m/z* 1626.85), and inter-alpha trypsin inhibitor heavy chain 4 (ITIH4 $_{[666-687]}$; *m/z* 2358.09) (18). These peptides from the paper by Villanueva et al. (18) are listed in **Table 1** together with the reported p-values for differences between breast cancer cases and controls, and the ratios of intensities between them.

Table 1 - Description of most discriminative peptides for breast cancer as observed by Villanueva et al. (18)

Peptide*	Average theoretical mass (Da)	Amino acid sequence	p-value#	Ratio of medians†
Apo A-IV $_{[256-278]}$	2508.8	ISASAEELRQRLAPLAEDVRGNL	$5.56*10^{-13}$	7.96
ITIH$_4$ $_{[666-687]}$	2358.6	SSRQLGLPGPPDVPDHAAYHPF	$4.07*10^{-12}$	531
C4a $_{[1337-1350]}$	1626.8	NGFKSHALQLNNRQ	$6.69*10^{-12}$	2.78
Fib-α $_{[605-629]}$	2659.8	DEAGSEADHEGTHSTKRGHAKSRPV	$7.39*10^{-12}$	2.45
Bradykinin	1060.2	RPPGFSPFR	$1.03*10^{-11}$	1.9
Des-Arg^9-bradykinin	904.1	RPPGFSPF	$7.39*10^{-10}$	1.62
Hyp^3-bradykinin	1076.2	RPP^{o}GFSPFR	-	-

* Average theoretical mass (Da) and amino acid sequences of precursor proteins derived from NCBI Protein database (25); # Mann-Whitney U test breast cancer cases versus controls; † Ratio of breast cancer patients / controls

In our laboratory we recently developed and validated an assay using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify potential breast cancer specific degradome markers in human serum (19). The validated assay included the peptides: Fib-α $_{[605-629]}$, C4a $_{[1337-1350]}$, ITIH4 $_{[666-687]}$, and bradykinin, and fulfilled the FDA guidelines on bioanalytical method validation (20). A pro^3-hydroxylated form of bradykinin, Hyp^3-bradykinin, and the des-Arg^9-bradykinin metabolite were added to the panel of peptides as in the study by Villanueva et al. (18) they showed the same relation with breast cancer as bradykinin, and were easily detectable in human serum samples. Characteristics of these peptides are

also shown in **Table 1**. Due to different chromatographic behavior and the lack of a proper internal standard, Apo A-IV $_{[256-278]}$ could not be accurately and precisely quantified in the same assay.

In the current study we used this assay to measure the concentrations of the six potential degradome markers in samples of 62 incident breast cancer cases and 62 matched controls, to evaluate the discriminative value of these quantitatively measured peptides alone, and in combination. This is the first study measuring these peptides quantitatively in clinical samples. For the control group we used samples of women with an indication for colonoscopy. They experienced altered bowel habits/movements or abdominal discomfort, or they were screened because of a family history of adenomatous polyps or colorectal cancer, but they all appeared to be free of adenomatous polyps or colorectal cancer. Samples of these subjects were collected in the same time frame and in the exact same way as the samples of the breast cancer cases. From the breast cancer cases, a second serum sample was collected two weeks or more after the surgical removal of the tumor. Peptide concentrations in these samples were compared with the peptide concentrations in the pre-surgery samples of the breast cancer cases, as well as with the concentrations in the samples of the controls to determine whether the concentrations changed after removal of the tumor, and whether this change was in the direction of the concentrations in the controls.

Material and Methods

Study population

In this study we analyzed serum samples of women who were above 18 years old presenting with an indication for breast cancer surgery at the Department of Surgery of the Slotervaart Hospital, Amsterdam, between April 2005 and December 2007. Of the 84 women who agreed to participate, seven participants were excluded because they had had prior malignancies. Another 15 breast cancer patients had to be excluded, because no pre-surgery sample was available, either because collection failed or because not enough material was collected. This resulted in a total of 62 breast cancer patients of whom serum samples could be analyzed in this study. Post-

surgery samples were also collected from these patients. These samples were collected at least 2 weeks after surgery, but prior to any administration of adjuvant therapy. Post-surgery samples were available of 57 breast cancer cases. Of the other subjects, collection failed or took place following initiation of adjuvant therapy, or not enough material was collected.

The control group consisted of women in the same age range, presenting in the same period with an indication for colonoscopy at the Department of Gastroenterology and Hepatology of two Dutch hospitals (the Slotervaart Hospital and the Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital). All were free of adenomatous polyps or colorectal cancer. Women with a history of malignancies (excluding curatively treated basal cell carcinoma of the skin and cervix carcinoma in situ) were excluded. Blood samples of the participants were collected before colonoscopy. We matched each breast cancer case with one of these controls with respect to age (± 6 years) and sample storage duration (± 8 months).

All samples were collected after approval by the local medical ethics committees of both hospitals, and after receiving individuals' written informed consent. Serum collection was uniformly performed following a strict protocol. Blood samples were collected in 9.5 ml BD Vacutainer® SST™ tubes (Beckton-Dickinson, Breda, The Netherlands) and allowed to clot for exactly 30 minutes at room temperature (RT), after which they were centrifuged at 1500 g for 15 minutes at RT. Sera were then immediately aliquotted and stored at -80°C.

Menopausal status at diagnosis was obtained through examination of the cases' medical records. Tumor stage, tumor size, differentiation, estrogen receptor (ER) status, progesterone receptor (PR) status, and HER2/neu expression were determined by pathological examination of the removed tumor. Lymph node involvement and the presence of metastasis were also examined.

LC-MS/MS analysis

The LC-MS/MS analysis was performed using an Accela high-speed chromatographic system coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization (H-ESI) probe operating in the

positive ion mode (both from Thermo Scientific, San Jose, CA, USA). Multiple reaction monitoring (MRM) was used for detection.

Samples were randomized over four batches, by which it was taken into account that the samples of a breast cancer case and its matched control, as well as the pre- and post-surgery sample of one subject, were analyzed in the same batch. Within each batch, eight calibration standards (nine for the bradykinins) were prepared by serial dilution of one standard sample containing all six peptides and measured to enable quantification of the peptides. These standards were in the range: 120-3000 ng/ml for Fib-α $_{[605-629]}$, 1-25 ng/ml for C4a $_{[1337-1350]}$, 0.4-10 ng/ml for ITIH4 $_{[666-687]}$, 10-500 ng/ml for bradykinin, 2-100 ng/ml for des-Arg^{9}-bradykinin and 4-200 ng/ml for Hyp^{3}-bradykinin. In addition, three quality control (QC) samples with low (LQC), mid (MQC), or high (HQC) concentration of all peptides of interest were measured in triplicate to assure analytical performance. These samples were randomly analyzed between the other samples.

Detailed information on the LC-MS/MS conditions and the sample preparation are described in the study by Van den Broek et al. (19). Accuracies and precisions of the method complied with the FDA guidelines for bioanalytical method validation (19).

Data analysis

The quality of the measurements was accepted when deviations from the expected concentrations and variations were below 15%. Measurements that were under the detection limit (missing), under the lowest limit of quantification (LLOQ) or above the upper limit of quantification (ULOQ) could not be quantified. Missing values and values under the LLOQ were therefore imputed by the LLOQ of that peptide, and values above the ULOQ by the ULOQ of that peptide.

Peptide concentrations were imported into SPSS 15.0 for statistical analysis. To test if the peptide concentrations in the breast cancer cases were statistically significantly different from those in the matched controls, we performed the Wilcoxon signed rank test. Subsequently, we used this test to investigate whether there were differences in peptide concentrations between the samples collected from the breast cancer cases, before and after surgery, and between the post-

surgery samples of the breast cancer cases and the samples of the matched controls. For all tests, p-values < 0.05 were considered statistically significant. Finally, we investigated the ability of the peptides to discriminate between the breast cancer cases and the controls using multivariate logistic regression including all peptides that univariately appeared to be related to the presence of breast cancer. Subsequently, we determined the area under the curve (AUC) of the Receiver Operating Characteristic (ROC) curve based on the predicted probabilities resulting from this model, with 95% confidence interval.

Results

Description of study population

The subject and sample characteristics of the different groups are listed in **Table 2**. The breast cancer patients were of similar age as the controls (median age: 56 years). No information about menopausal status was available for the controls. The median sample storage duration for both the cases and the controls was 35 months.

Table 2 - Subject and sample characteristics of the breast cancer cases and the controls

	Breast cancer cases (*n*=62)	Matched controls (*n*=62)
Age at diagnosis (years)		
Median (IQR)	55.6 (47.4-69.5)	56.1 (49.0-69.7)
Menopausal status, *n* (%)		
Premenopausal	15 (26.3)	-
Perimenopausal	5 (8.8)	-
Postmenopausal	37 (64.9)	-
Missing	5	62
Sample storage duration (months)		
Median (IQR)	35 (28-41)	35 (28-40)

IQR: inter-quartile range

The characteristics of the tumors of the cases are shown in **Table 3**. The majority of the cases were diagnosed with Stage I (42%) or Stage IIA (29%) breast cancer. Three cases were diagnosed with carcinoma in situ. About two thirds of the invasive tumors were not spread to the lymph nodes. All cases were free of metastasis.

Table 3 - Characteristics of the tumor of the breast cancer cases

All breast cancer cases	*n*=62
TNM stage, *n* (%)	
0	3 (4.8)
I	26 (41.9)
IIA	18 (29.0)
IIB	9 (14.5)
IIIA and IIIC	6 (9.6)
Of the cases with an invasive tumor	***n*=59**
Tumor size, *n* (%)	
>0.1-0.5 cm	3 (5.1)
>0.5-1 cm	11 (18.6)
>1-2 cm	21 (35.6)
>2 cm	24 (40.7)
Lymph node involvement, *n* (%)	
No	38 (64.4)
Yes	21 (35.6)
Differentiation, *n* (%)	
High	13 (22.4)
Intermediate	21 (36.2)
Low	24 (41.4)
Missing	1
ER status, *n* (%)	
Negative	19 (33.3)
Positive	38 (66.7)
Missing	2
PR status, *n* (%)	
Negative	28 (49.1)
Positive	29 (50.9)
Missing	2
HER2/neu expression, *n* (%)	
Negative	42 (75.0)
Positive	14 (25.0)
Missing	3

ER: estrogen receptor; PR: progesterone receptor

Controls were referred for colonoscopy because they experienced altered bowel habits/movements (26%), abdominal discomfort (19%) or rectal blood loss (15%), or they were screened because of a family history of adenomatous polyps or colorectal cancer (15%), or a combination of these reasons (25%). Colonoscopy revealed that they were all free of adenomatous polyps or colorectal cancer.

ITIH4 [666-687] and Des-Arg^9-bradykinin related to breast cancer

The serum concentrations of ITIH4 [666-687] and des-Arg^9-bradykinin appeared to be statistically significantly different between the breast cancer cases and their matched controls ($p<0.001$ and $p=0.031$, respectively). The median concentration of ITIH4 [666-687] was higher in the cases than in the controls (0.58 ng/ml (inter-quartile range (IQR): 0.40-1.30) and 0.40 ng/ml (IQR: 0.40-0.57), respectively). The median concentration of des-Arg^9-bradykinin was also higher in the cases compared to the controls (98.4 ng/ml (IQR: 72.7-139.3) and 80.8 ng/ml (IQR: 60.0-109.1), respectively) We could not detect a significant difference in concentration between breast cancer cases and controls for Fib-α [605-629], C4a [1337-1350], bradykinin and Hyp^3-bradykinin (**Table 4**).

Table 4 - Differences in peptide concentrations between matched breast cancer cases and controls

	Breast cancer cases (*n*=62)	Controls (*n*=62)		Wilcoxon signed ranks test
Peptide	*Median concentration* (IQR)*	*Median concentration* (IQR)*	*Cases vs. controls*	*p-value*
Fib-α [605-629]	174.8 (128.6-249.4)	185.5 (143.2-225.0)	-	.375
C4a [1337-1350]	2.92 (1.48-5.20)	2.45 (1.45-4.24)	-	.136
ITIH$_4$ [666-687]	0.58 (0.40-1.30)	0.40 (0.40-0.57)	Higher	<.001
Bradykinin	71.4 (35.8-115.7)	63.1 (41.9-93.1)	-	.418
Des-Arg^9-bradykinin	98.4 (72.7-139.3)	80.8 (60.0-109.1)	Higher	.031
Hyp^3-bradykinin	18.2 (7.0-35.0)	21.5 (9.0-46.3)	-	.270

IQR: inter-quartile range; * in ng/ml

Comparison of the peptide concentrations of ITIH4 [666-687] and des-Arg^9-bradykinin in the pre-surgery sample and the post-surgery sample of each breast cancer case, revealed statistically significant differences for both peptides ($p=0.002$ and $p=0.002$, respectively). Median concentrations of ITIH4 [666-687] and des-Arg^9-

bradykinin were both decreased in the post-surgery samples compared to the pre-surgery samples (ITIH4 $_{[666-687]}$: 0.44 (IQR: 0.40-0.78) and 0.58 (IQR: 0.40-1.34), des-Arg^9-bradykinin: 77.3 (IQR: 53.6-126.7) and 98.0 (IQR: 68.4-138.5), respectively). When post-surgery concentrations of these peptides were compared to the concentrations in the matched controls, it appeared that there was no statistically significant difference between breast cancer cases and controls (anymore) (p=0.299 and p=0.790, respectively). Median concentrations in the three groups, and p-values of the Wilcoxon signed ranks tests are shown in **Table 5**.

Table 5 - Differences in peptide concentrations between the pre-surgery and post-surgery breast cancer samples and between post-surgery breast cancer samples and matched controls

	Pre-surgery cases (*n*=57)	Post-surgery cases (*n*=57)	Controls (*n*=57)	Difference pre vs. post	Difference post vs. controls
Peptide	*Median concentration* (IQR)*	*Median concentration* (IQR)*	*Median concentration* (IQR)*	*p-value#*	*p-value#*
ITIH$_{4\ [666-687]}$	0.58 (0.40-1.34)	0.44 (0.40-0.78)	0.40 (0.40-0.58)	.002	.299
Des-Arg^9-bradykinin	98.0 (68.4-138.5)	77.3 (53.6-126.7)	83.4 (60.5-109.2)	.002	.790

IQR: inter-quartile range; * in ng/ml ; # Wilcoxon signed ranks test

ITIH4 $_{[666-687]}$ and Des-Arg^9-bradykinin independently discriminate between cases and controls

Multivariate logistic regression analysis including the discriminative peptides ITIH4 $_{[666-687]}$ and des-Arg^9-bradykinin revealed that they independently contributed to the discrimination between cases and controls (p=0.026 and p=0.089, respectively). The AUC of the ROC curve based on the probabilities resulting from the model was 0.66 (95%CI: 0.56-0.76). In comparison, the AUC of the ROC curve based on the concentrations of ITIH4 $_{[666-687]}$ alone was 0.65 (95%CI: 0.56-0.75). The AUC for des-Arg^9-bradykinin alone was 0.62 (95%CI: 0.52-0.72).

Discussion

Both ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin appeared to be independently related with the presence of breast cancer in this study. The serum concentrations of these two peptides were higher in the breast cancer cases than in the matched controls. After removal of the tumor, serum concentrations of these two protein fragments were at the same level as in the samples of the controls, confirming their relations with the presence of a breast tumor. Both peptides were previously discovered by Villanueva et al. (18), and supposed to be generated ex vivo, after degradation of endogenous serum proteins, by cancer-specific proteases that were released by the cancer cells. In this study we now confirm the discriminative value of these peptides found by Villanueva et al. (18) in samples of 62 breast cancer cases and 62 matched controls. The higher concentrations of these peptides in breast cancer cases compared to controls were in accordance with the results of Villanueva et al. (18).

We measured the concentrations of these peptides with a validated, quantitative LC-MS/MS assay. In previous studies, SELDI-TOF MS (9,11,13,14,21) or MALDI-TOF MS (18) was used for the measurement of differences in peptide and protein concentrations between cases and controls. However, with both methods only relative quantities can be measured. With the assay used in this study, which was designed and validated specifically for the six peptides investigated in this study, absolute quantities could be highly accurately and precisely measured with the use of calibration standards of the synthetic peptides in bovine plasma samples and three structural or isotope-labeled internal standards (19).

The other peptides that were investigated in this study; Fib-α $_{[605-629]}$, C4a $_{[1337-1350]}$, bradykinin and Hyp^{3}-bradykinin were not significantly related to the presence of breast cancer. One explanation may be that the total time the samples were held at RT, at which exoproteases are active, was too short for some of the proteins to be sufficiently degraded. Our samples were allowed to clot for 30 minutes, followed by centrifugation for 30 minutes, both at RT, and they were only thawed once. The samples investigated in the study by Villanueva et al. (18) were allowed to clot for 1 hour at RT and they were centrifuged for 10 minutes, while they were thawed twice (18). The speed at which proteins are degraded by exoproteases is believed to be substrate specific (22), and therefore, it is possible

that the cancer-specific exoprotease fragmentation of the precursor-proteins of above mentioned peptides was not discriminative yet. For future research, optimization of the time at RT for the different peptides is suggested, possibly resulting in peptide concentrations that are more discriminative.

It should also be noted that, although our sample size is larger than that of Villanueva et al. (18) (21 breast cancer patients and 33 controls), the power of our study may still be limited for detecting small differences between cases and controls, because a number of samples showed concentrations below the LLOQ (12, 22, 5 and 14 of the 181 samples for Fib-α $_{[605-629]}$, C4a $_{[1337-1350]}$, bradykinin and Hyp^{3}-bradykinin, respectively), which we all imputed by the LLOQ (see **Appendix**).

In this study we investigated women with an indication for a colonoscopy as the control group. They have previously been included as part of a control group in a study of Engwegen et al. (23) on protein biomarkers for early detection of colorectal cancer. We chose this control group because the samples were collected and processed following the identical, strict and uniform protocol as was used for the collection of the breast cancer serum samples, and also in the same period and time frame. Conditions for sample collection and storage are known to have a great impact on spectral data (24). By choosing this control group we were able to prevent any sampling handling differences from influencing our results. One could argue that healthy controls would have been preferable to obtain maximal contrast between cases and controls. However, if this would lead to the detection of other peptides that differed between cases and controls, these peptides might well be markers of circumstantial processes such as inflammatory processes, which are unlikely to be cancer specific. These markers may have been present in both colonoscopy controls and breast cancer cases in the current study.

Although ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin appear to be specific degradome markers for breast cancer, the AUC of the ROC curve, which was 0.66, shows that the combination of these peptides is still insufficient for diagnostic purposes. Additional factors, like other blood markers, and maybe also known risk factors could be added to improve the diagnostic value. The decrease of these peptide concentrations to normal levels after removal of the tumor, also suggests their potential value in prediction of the need for adjuvant therapy. However, this

possibility has to be further investigated first. In future studies it would also be interesting to investigate the relation of these peptides with breast cancer subtypes and with tumor stage, for which the power in current study was insufficient.

In conclusion, in this study we confirmed that the exoprotease breakdown peptides, ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin, differed between breast cancer cases and controls. Our findings support the hypothesis of Villanueva et al. (18) that degradome markers have potential for the diagnosis of breast cancer.

Acknowledgements

This study was financially supported by a grant from the board of the University Medical Center Utrecht and Julius Center for Health Sciences and Primary Care ('Strategische Impuls') and by the Netherlands Laboratory for Anticancer Drug Formulation (NLADF), Amsterdam, The Netherlands. We would like to thank the breast cancer patients and the women with an indication for colonoscopy for participation in the study and for donating a blood sample. We acknowledge the nurses from the Clinical Chemical laboratories for collecting the blood samples.

References

1. Barros AC, Fry W, Jr., Nazario AC, Santos MO, Sato MK. Experience with CA 15.3 as a tumor marker in breast cancer. ***Eur. J. Surg. Oncol.*** *1994;* 20(2): 130-133.
2. Eskelinen M, Kataja V, Hamalainen E, Kosma VM, Penttila I, Alhava E. Serum tumour markers CEA, AFP, CA 15-3, TPS and Neu in diagnosis of breast cancer. ***Anticancer Res.*** *1997;* 17(2B): 1231-1234.
3. Fabre-Lafay S, Monville F, Garrido-Urbani S, Berruyer-Pouyet C, Ginestier C, Reymond N et al. Nectin-4 is a new histological and serological tumor associated marker for breast cancer. ***BMC. Cancer*** *2007;* 7: 73.
4. Rodriguez de PL, Arnaiz F, Estenoz J, Ortuno B, Lanzos E. Study of serum tumor markers CEA, CA 15.3 and CA 27.29 as diagnostic parameters in patients with breast carcinoma. ***Int. J. Biol. Markers*** *1995;* 10(1): 24-29.
5. Gast MC, Schellens JH, Beijnen JH. Clinical proteomics in breast cancer: a review. ***Breast Cancer Res. Treat.*** *2009;* 116(1): 17-29.
6. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. ***J. Clin. Oncol.*** *2007;* 25(33): 5287-5312.
7. Belluco C, Petricoin EF, Mammano E, Facchiano F, Ross-Rucker S, Nitti D et al. Serum Proteomic Analysis Identifies a Highly Sensitive and Specific Discriminatory Pattern in Stage 1 Breast Cancer. ***Ann. Surg. Oncol.*** *2007;* 14(9): 2470-2476.
8. Gast MC, Bonfrer JM, van Dulken EJ, de Kock L, Rutgers EJ, Schellens JH et al. SELDI-TOF MS serum protein profiles in breast cancer: assessment of robustness and validity. ***Cancer Biomark.*** *2006;* 2(6): 235-248.
9. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. ***Clin. Chem.*** *2002;* 48(8): 1296-1304.
10. Li J, Orlandi R, White CN, Rosenzweig J, Zhao J, Seregni E et al. Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. ***Clin. Chem.*** *2005;* 51(12): 2229-2235.
11. Mathelin C, Cromer A, Wendling C, Tomasetto C, Rio MC. Serum biomarkers for detection of breast cancers: a prospective study. ***Breast Cancer Res. Treat.*** *2006;* 96(1): 83-90.
12. van Winden AW, Gast MC, Beijnen JH, Rutgers EJ, Grobbee DE, Peeters PH et al. Validation of previously identified serum biomarkers for breast cancer with SELDI-TOF MS: a case control study. ***BMC. Med. Genomics*** *2009;* 2: 4.
13. Hu Y, Zhang S, Yu J, Liu J, Zheng S. SELDI-TOF-MS: the proteomics and bioinformatics approaches in the diagnosis of breast cancer. ***Breast.*** *2005;* 14(4): 250-255.
14. Vlahou A, Laronga C, Wilson L, Gregory B, Fournier K, McGaughey D et al. A novel approach toward development of a rapid blood test for breast cancer. ***Clin. Breast Cancer.*** *2003;* 4(3): 203-209.

15. van der Werff MP, Mertens B, de Noo ME, Bladergroen MR, Dalebout HC, Tollenaar RA et al. Case-control breast cancer study of MALDI-TOF proteomic mass spectrometry data on serum samples. ***Stat. Appl. Genet. Mol. Biol.*** *2008;* 7(2): Article2.
16. Engwegen JY, Gast MC, Schellens JH, Beijnen JH. Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. ***Trends Pharmacol. Sci.*** *2006;* 27(5): 251-259.
17. Fung ET, Yip TT, Lomas L, Wang Z, Yip C, Meng XY et al. Classification of cancer types by measuring variants of host response proteins using SELDI serum assays. ***Int. J. Cancer.*** *2005;* 115(5): 783-789.
18. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. ***J. Clin. Invest.*** *2006;* 116(1): 271-284.
19. van den Broek I, Sparidans R.W., Schellens J.H.M., Beijnen JH. Quantitative assay for six potential breast cancer biomarker peptides in human serum by liquid chromatography coupled to tandem mass spectrometry. ***J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*** *2010;* 878(5-6): 590-602.
20. U.S.Food and Drug Administration: Centre for Drug Evaluation and Research. Guidance for Industry: Bioanalytical Method Validation. Available at www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf *Accessed October 2009.*
21. Belluco C, Petricoin EF, Mammano E, Facchiano F, Ross-Rucker S, Nitti D et al. Serum Proteomic Analysis Identifies a Highly Sensitive and Specific Discriminatory Pattern in Stage 1 Breast Cancer. ***Ann. Surg. Oncol.*** *2007;* 14(9): 2470-2476.
22. Jimenez CR, Piersma S, Pham T. High-throughput and targeted in-depth mass spectrometry-based approaches for biofluid profiling and biomarker discovery. ***Biomarkers in Medicine*** *2007;* 1(4): 541-565.
23. Engwegen JY, Depla AC, Smits ME, Cats A, Tuynman H, van Heukelem HA et al. Detection of Colorectal Cancer by Serum and Tissue Protein Profiling: A Prospective Study in a Population at Risk. ***Biomark. Insights.*** *2008;* 3: 375-385.
24. Gast MC, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ et al. Influence of sample storage duration on serum protein profiles assessed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS). ***Clin. Chem. Lab Med.*** *2009;* 47(6): 694-705.
25. National Center for Biotechnology Information Web Site. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val= NP_000473 (Apo A-IV), NP_002209 (ITIH4), NP_009224 (C4a), NP_000499 (Fibrinogen alpha-chain). *October 2009.*

Appendix

Concentration ranges

The quality of the measurements was acceptable since deviations from the expected concentrations and variations were below 15%. However, measurement of the 181 samples revealed that found concentration levels of the peptides were not always within the validated concentration ranges. Therefore, one additional validation-run was performed to extend the concentration range for accurate and precise quantification of the peptides up to 100 ng/ml for Fib-α $_{[605-629]}$, 4 ng/ml for bradykinin, 2 ng/ml for Hyp^3-bradykinin and 200 ng/ml for Des-Arg^9-bradykinin. Nevertheless, the concentration of Fib-α $_{[605-629]}$ was below 100 ng/ml in 12 samples (four pre-surgery and four post-surgery breast cancer samples, and four control samples). Twenty-two samples had a C4a $_{[1337-1350]}$ concentration below the LLOQ (1.0 ng/ml) (eight pre-surgery and four post-surgery breast cancer samples, and 10 control samples). In 79 samples, ITIH4 $_{[666-687]}$ was not detected or the concentration was below the LLOQ (0.4 ng/ml) (20 pre-surgery and 26 post-surgery breast cancer samples, and 33 control samples). In five samples bradykinin could not be detected, or the concentration was below 4 ng/ml (four pre-surgery and one post-surgery breast cancer samples). In 14 samples hyp^3-bradykinin could not be detected, or the concentration was below 2 ng/ml (six pre-surgery and five post-surgery breast cancer samples, and three control samples). Missing values and values below the LLOQ were imputed by the LLOQ for that peptide. For peptides for which the concentration range for accurate and precise quantification could be extended, values below the minimum concentrations were imputed by the extended minimum concentration. The concentration of des-Arg^9-bradykinin was above 200 ng/ml in 13 samples (nine pre-surgery and three post-surgery samples, and one control sample). Values above this concentration were imputed by 200 ng/ml.

Chapter 6

Bead-based multiplexed immunoassay to identify early breast cancer biomarkers in pre-diagnostic serum

Abstract

In this study we investigated whether a set of ten potential breast cancer serum biomarkers and cancer antigens can predict the presence of early stage breast cancer. The ten markers studied were: osteopontin (OPN), haptoglobin, cancer antigen 15-3 (CA15-3), carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), prolactin, cancer antigen 19-9 (CA19-9), α-fetoprotein (AFP), leptin and migration inhibitory factor (MIF). Our study population consisted of participants of the Prospect-EPIC (European Prospective Investigation into Cancer and nutrition) cohort. In a nested case-control design we examined to what extent the biomarker panel could discriminate between 68 women diagnosed with breast cancer up to three years after enrollment and 68 matched healthy controls. Using a quantitative bead-based multiplexed assay we determined protein concentrations in pre-diagnostic serum samples collected at enrollment into the cohort. Discriminatory power of the panel was assessed with Principal Component Analysis (PCA) and Random Forest (RF) analysis. PCA and RF revealed that based on all ten proteins, cases could not be separated from controls. Adding subject characteristics, such as BMI, age at menarche, age at menopause, former use of oral contraceptives, former use of hormone therapy, number of children, smoking habits, alcohol consumption and level of education to the protein data in the RF analysis did not result in classification accuracy scores that could correctly classify the samples (sensitivity: 51%, specificity: 51%). Restriction of the analysis to the cases diagnosed very shortly after enrollment and their matched controls did not change the results. Our findings indicate that the panel of selected tumor markers cannot be used for diagnosis of early breast cancer.

Introduction

The identification of blood biomarkers for early detection of breast cancer is an important target of research. Mammography, the current routine method for early detection lacks sensitivity to detect invasive breast lesions in dense breast tissue (1-4). Besides being less-invasive and easy to perform, a blood test would omit the imaging-related problem of high breast density (4). Thereby, it would also offer potential for younger women who are now excluded from most breast cancer screening programs, mainly because the prevalence of dense breast tissue is very high in this group.

The discovery of blood-based breast cancer biomarkers has long been a major objective in breast cancer research. Three serum proteins; cancer antigen 15-3 (CA15-3), carcinoembryonic antigen (CEA) and HER2/neu, have so far been approved by the FDA to monitor chemotherapy in patients with advanced breast cancer (5,6). However, these markers have been proven to be ineffective as a stand-alone assay in early detection of breast cancer or in distinguishing between benign and malignant tumors due to low diagnostic sensitivity and specificity (7-13). Another tumor marker, cancer antigen 125 (CA125), is primarily used together with transvaginal ultrasound for early detection of ovarian cancer in women with hereditary syndromes (5) but has also been suggested as tumor marker for breast cancer (14).

Within the past decade, a growing number of cancers as encountered in human patients are modeled in mice (15,16). Animal studies may help in finding appropriate and valid biomarkers. Animal experiments have a shorter time span than human studies and methods of specimen ascertainment are easier to standardize. Using transgenic mouse models that spontaneously develop mammary tumors mimicking human breast cancer we previously reported two markers that completely distinguished mammary-tumor bearing mice from non-tumor mice. These markers were osteopontin (OPN) and haptoglobin (17).

In the current study we evaluated the value of tumor markers for early detection of breast cancer in human females. We composed a panel of biomarkers to study their combined predictive value, as single tumor markers have been found

to show limited sensitivity and specificity for early detection (5-14). Furthermore, it is likely that, due to the heterogeneity of breast cancer in humans (18), a panel of several biomarkers is needed to be able to detect the different subtypes.

In addition to the tumor markers haptoglobin and osteopontin identified in our mouse models, our panel contained CA15-3, CEA and CA-125, serum markers for which a relation with breast cancer is found in previous studies, but which by themselves were not discriminative enough (5-14). We also included prolactin which has previously been found to be a risk marker for breast cancer in postmenopausal women, particularly for estrogen receptor positive (ER+) and progesterone receptor positive (PR+) cancers (19). Furthermore we included cancer antigens that are risk markers for multiple cancer types such as CA19-9 for pancreatic, gastric and colon cancer (6,20) and α-fetoprotein (AFP) for ovary, testis and liver cancer (6). Finally, we selected two markers previously found to be higher in mammary tumor tissues; leptin and migration inhibitory factor (MIF) (21,22). The concentrations of these proteins, listed in **Table 1**, were assessed using bead-based multiplexed immunoassays. This is a novel proteomics technique enabling analysis of many potential markers simultaneously and processing large sets of sera relatively rapidly (23,24).

Table 1 - List of biomarkers tested

Biomarker	Rational	Reference
OPN	Higher in humanized Mouse models for breast cancer	(17)
Haptoglobin	Higher in humanized Mouse models for breast cancer	(17)
CA15-3	Monitoring marker breast cancer*	(5,6)
CEA	Monitoring marker breast cancer*	(5,6)
CA-125	Monitoring marker breast and ovarian cancer*	(14)
Prolactin	Risk marker for breast cancer*	(19)
CA19-9	Monitoring marker pancreatic and gastrointestinal cancer*	(6,20)
AFP	Staging marker testis, ovary and liver cancer*	(6)
Leptin	Higher expression in mammary tumors	(21,22)
MIF	Higher expression in mammary tumors	(21,22)

OPN: osteopontin; CA15-3: cancer antigen 15-3; CEA: carcinoembryonic antigen; CA-125: cancer antigen 125; CA19-9: cancer antigen 19-9; AFP: α-fetoprotein; MIF: migration inhibitory factor; * Higher in advanced disease

We investigated the discriminative value of this serum tumor marker panel in a nested case-control study using the Prospect-EPIC (European Prospective Investigation into Cancer and nutrition) cohort (25). We studied women who were diagnosed with breast cancer up to three years after enrollment in the cohort. The concentrations of the tumor markers were measured in the pre-diagnostic serum samples of these women and compared to those of matched controls who remained healthy. This design creates the unique opportunity to evaluate the discriminative power of this panel of tumor markers for detection of early, a-symptomatic stages of breast cancer.

Material and Methods

Study population

We performed a case-control study nested within the Prospect-EPIC cohort. Prospect-EPIC is one of the two Dutch cohorts participating in the European Prospective Investigation into Cancer and nutrition, a large multicenter cohort study, including participants from ten European countries. From 1993 to 1997, 17,357 women from Utrecht and vicinity, then aged between 50 and 69 years, enrolled in this cohort through the breast cancer screening program. Together with their invitation for the breast cancer screening, they were invited to participate in this cohort (25). Women filled out an extensive food frequency questionnaire and a general questionnaire. The latter contained questions on demographic characteristics, medical history, lifestyle characteristics and risk factors for cancer and other chronic diseases (25,26).

At enrollment, Prospect-EPIC participants also donated a blood sample. After collection, blood samples were stored in a climate controlled refrigerator at 5ºC overnight. The next day blood samples were centrifuged at 1500 g for 20 minutes. After centrifuging, the serum was put in 0.5 ml straws. These straws were stored in a -86ºC freezer until they were transported to liquid nitrogen tanks (-196ºC), where they have been stored ever since.

Participants were followed for vital and health status. Through the municipal registries information on death and migration was obtained. Causes of

death were obtained from the Central Bureau for Statistics (CBS). Through yearly linkage with the regional and national cancer registries information about cancer incidence and stage of disease at diagnosis (tumor behavior, tumor size, lymph node involvement and metastasis) was obtained (25). Until December 31st 2006, 687 women in the Prospect-EPIC cohort were diagnosed with breast cancer.

For the current study we selected women who were diagnosed with breast cancer within three years after enrollment into the cohort, and who were postmenopausal then (no menstrual periods in last 12 months). Women were excluded if they had had cancer before, were suffering from diabetes, were current smokers or were currently using oral contraceptives or menopausal hormone therapy (HT). This was done to obtain a homogeneous group with respect to hormone levels, smoking status and metabolic status because these factors may influence serum protein concentrations. Sixty-eight women were eventually included as a case. Controls were participants of the same cohort. We matched each case with one postmenopausal control that remained free of breast cancer up to the time the case was diagnosed. Additional matching factors were age at enrollment (± 1 year) and date of enrollment (± ½ year). For controls the same exclusion criteria were applied as for cases.

Multiplex Analysis

The candidate breast cancer markers were analyzed with the use of three different kits. For OPN, leptin and MIF we used the Beadlyte® Cancer Biomarker Panel kit (Millipore, St Charles, MO, USA) and for CA15-3, CEA, CA19-9 and AFP the WideScreen™ kit for Human Cancer Panel 1 (Novagen, Darmstadt, Germany) was used. CA-125 and prolactin were included in both kits. For haptoglobin we used the WideScreen™ kit for Human CVD Panel 5 (Novagen, Darmstadt, Germany). Samples were analyzed in two plates. After retrieval of the serum samples from the liquid nitrogen, samples had been thawed and aliquoted, and aliquots had been refrozen at -80C. These aliquots were later used for this analysis, meaning that all samples have been thawed twice.

The sample analysis was performed according to manufacturers' instructions. In short, assays were carried out in 96-wells filter plates. Standard

curve samples were prepared using serial dilution steps in standard diluent. Standard curve samples, diluted quality control samples, diluted serum samples and blanks were added to the plates randomly and blinded to status of the sample. The standard curve samples and the two quality control samples were applied to each plate in duplicate. Fluorescently labeled, antibody-conjugated beads were applied to each well and incubated in the dark overnight at 4°C for the Beadlyte® plate, and 1 hour at room temperature (RT) for the WideScreen™ plates. Next, biotin-conjugated detection antibodies were added to the wells and incubated at RT in the dark (1.5 hour for Beadlyte® and 1 hour for WideScreen™). Subsequently Streptavidin-Phycoerythrin was applied to each well and incubated for 30 minutes at RT in the dark. After each incubation, the plates were vacuumed and washed twice. Sheath fluid was applied to the wells of the Beadlyte® plate, and a supplied buffer to the wells of the WideScreen™ plates. Finally, plates were shaken for 1-5 minutes in the dark and fluorescence intensity of the beads was analyzed on the Bio-plex instrument.

The quality of the assay was evaluated by the measurements of the quality control samples. The reproducibility of the measurements within a plate and between plates was expressed by the coefficient of variance (CV) of the measurements. The reproducibility was considered good if the CVs were smaller than 15%.

Values of samples below the standard curve were replaced with the lowest limit of quantification for that protein. In nine subjects (four cases and five controls) the measurements of one or more proteins were missing due to air bubbles or leaking wells. These missing concentrations were imputed using the *k*-nearest neighbor (KNN) procedure (27,28). By this procedure the missing value is imputed using nearest neighbor averaging. For each missing value, a value was imputed by averaging data from three subjects that were most similar with respect to the concentration of the other proteins and for which that protein was not missing (27).

CA-125 and prolactin were measured both in the Beadlyte® and the Widescreen™ kit. For CA-125 the Beadlyte® data was excluded, because in more than 50% of the samples the concentration was below the detection range. For

prolactin the Widescreen™ kit data were excluded for further analysis because they contained four missing values due to technical issues.

Data analysis

Since the data were not normally distributed we applied a log-transformation to the protein concentrations. To determine whether the mean log-transformed concentration of any of the candidate breast tumor markers was statistically significantly different between pre-diagnostic serum samples of breast cancer patients and serum samples of healthy controls, we performed a paired samples T test using SPSS 15.0. P-values <0.05 were considered statistically significant. Geometric mean concentration and 95% confidence intervals (95%CI) were calculated for each protein in cases and controls. For analysis of combinations of proteins principle component analysis (PCA) and Random Forests (RF) were applied. PCA was performed to study clustering of pre-diagnostic breast cancer patients and healthy controls based on the concentrations of all 10 proteins combined (29). For PCA analysis log transformed values of all proteins were imported into GenemathXT version 2.12 (Applied Math, Sint-Martens-Latem, Belgium). With RF analysis using all components, we investigated whether any combination of the proteins could discriminate between the cases and controls (30). RF is an algorithm for classification that uses an ensemble of classification trees (30,31). It has recently been used in several classification studies based on proteomic or genomic data and is capable of identifying the optimal combinations of proteins that can still achieve good predictive performance (32,33). For Mtry the default value was chosen since similar prediction errors were obtained for different Mtry values. RF provides an importance factor for each protein that allows relative ranking of all proteins. To perform the RF analyses we used the scaled mean decrease in classification accuracy. Proteins were ranked according to their importance. To obtain stable estimates of the Im, large numbers of trees in the forest are needed (31,34). Also, to capture as many important interactions as possible, large number of trees are required. RF does not overfit; therefore we performed the analyses with a large number of trees (40000). We used al proteins in the dataset in the analysis, and Im was used as measure to rank the proteins. We compared the classification accuracy

based on all proteins and on the optimal subset of proteins. The optimal subset of proteins is the marker combination with the best predictive performance. The dataset was randomly split in a training set and a test set, where each set consisted of half the cases with their matched controls, this process was repeated 1000 times and the average classification accuracy score was calculated. We also added information on subject characteristics, such as BMI, age at menarche, age at menopause, former use of oral contraceptives, former use of hormone therapy, number of children, smoking habits, alcohol consumption, and level of education to the protein data in the RF analysis to investigate whether they could add diagnostic value to the panel of proteins. RF analysis was performed in R program packages Random Forests (28,31).

Furthermore we investigated whether the relations between the proteins and breast cancer were stronger in subjects who were closest to diagnosis at the time of blood collection. To this end we selected those cases who were diagnosed with breast cancer as the result of a positive screening mammogram at the time of enrollment, and we compared their protein concentrations with those of the matched controls with a paired sample T test. With PCA we tested whether the cases and controls in this subset could be separated based on the concentrations of all proteins. RF was performed as described above.

Results

Study population

Characteristics of the study population are presented in **Table 2**. About half of both cases and controls used oral contraceptives in the past, but the cases used them for a longer time period than the controls (median duration 10 and 4.5 years, respectively). Cases were somewhat more often nulliparous (15%) than controls (7%). Among women with children, controls had more children than the cases; 3 and 2 (median), respectively. About half of both cases and controls had smoked in the past, for about 8 and 4 years (median), respectively. The energy adjusted intake of alcohol was somewhat higher for controls than for cases; 2.5 and 2.0 g/day, respectively (median). Other characteristics were distributed equally in cases and

Table 2 - Study population characteristics

	Cases (*n*=68)	Controls (*n*=68)
Age at enrollment (years)		
Mean (SD)	60.2 (5.6)	60.3 (5.7)
BMI		
Mean (SD)	26.6 (3.1)	26.3 (3.6)
Missing	1	-
Use of oral contraceptives, *n* (%)		
No, but used to in the past	36 (52.9)	40 (58.8)
No, never	32 (47.1)	28 (41.2)
Duration of oral contraceptives use* (years)		
Median (IQR)	10.0 (4.0-16.0)	4.5 (2.0-10.0)
Use of HT, *n* (%)		
No, but used to in the past	7 (10.3)	6 (8.8)
No, never	61 (89.7)	62 (91.2)
Duration of HT use* (years)		
Median (IQR)	1.0 (1.0-8.0)	2.0 (1.0-10.0)
Ovariectomy, *n* (%)		
Both ovaries removed	5 (7.4)	3 (4.4)
Missing	-	1
Parity, *n* (%)		
Nulliparous	10 (14.7)	5 (7.4)
Number of children#		
Median (IQR)	2 (2-3)	3 (2-3)
Smoking, *n* (%)		
No, but used to in the past	31 (45.6)	34 (50.0)
No, never	37 (54.4)	34 (50.0)
Pack-years smoking until stop date†		
Median (IQR)	7.9 (1.9-16.4)	4.1 (1.4-10.2)
Missing	1	3
Alcohol intake‡ (g/day)		
Median (IQR)	2.0 (0.2-7.2)	2.5 (0.2-8.4)
Use of medicines, minerals or vitamins last week, *n* (%)		
Yes	46 (67.6)	44 (64.7)
No	22 (32.4)	24 (35.3)
Time since last meal and/or drink** (minutes)		
Median (IQR)	108 (87-137)	116 (88-137)

SD: standard deviation; BMI: body mass index; IQR: inter-quartile range; HT: menopausal hormone therapy; * Among former oral contraceptives/HT users; # Among women with children; † Among former smokers; ‡ Energy-adjusted alcohol intake at enrollment; ** At moment of blood collection

controls. Characteristics of the serum samples are listed in **Table 3**. There was no difference between the cases and the controls regarding the sample collection and storage.

Table 3 - Characteristics of the serum samples

	Cases (*n*=68)	Controls (*n* =68)
Serum sample storage duration* (years)		
Mean (SD)	13.6 (1.1)	13.5 (1.1)
Hours in refrigerator#		
Median (IQR)	22 (21-23)	22 (20-23)
Days at -86°C†		
Median (IQR)	8 (6-11)	7 (5-11)

SD: standard deviation; IQR: inter-quartile range; * Until experiment; # Between collection and centrifugation; † Between centrifugation and storage at liquid nitrogen

The cases were diagnosed with breast cancer after a median time of 21.3 months (IQR: 0.7-26.6) after enrollment. More than 80% of the cases were affected with an invasive tumor. More than half of the invasive tumors were diagnosed in Stage I and a quarter of the invasive tumors were diagnosed in Stage IIA. Only one tumor was diagnosed in Stage IIIA. The invasive tumors were more or less equally distributed over the three size categories (>0.1-1 cm, 1-2 cm and >2 cm). In 30% of the cases with invasive tumors, lymph nodes were involved. None of the cases was affected with metastases. For seven and nine cases, respectively, the pathologically determined tumor size and lymph node involvement was unknown. For all but two of these cases we were able to report the clinically determined size and lymph node involvement.

Discriminative value of candidate breast cancer markers in pre-diagnostic serum

The quality of the measurements was acceptable since CVs were smaller than 15%. Concentrations of CA15-3 and MIF were slightly, but not significantly lower in the cases compared to the controls (CA15-3 (U/ml): 8.48 (95%CI: 7.08-10.15) versus 10.34 (95%CI: 8.72-12.26) (p=0.11) and MIF (pg/ml): 166.5 (95%CI: 141.9-195.4) versus 199.7 (95%CI: 165.9-240.4) (p=0.13)). Concentrations of prolactin were slightly, but not significantly higher in cases compared to controls (4.79 ng/ml

(95%CI: 4.09-5.60) versus 4.03 ng/ml (95%CI: 3.48-4.66) (p=0.10)). Concentrations of the other individual markers were not found to be different between cases and controls. The geometric mean concentrations and 95% confidence intervals of all proteins in cases and controls, together with the p-values are presented in **Table 4**.

Table 4 - Serum concentrations of all measured proteins in the pre-diagnostic breast cancer- and healthy control samples

	Cases (*n*=68)		Controls (*n*=68)		Paired T test
Biomarker	*Geometric mean concentration*	*95%CI*	*Geometric mean concentration*	*95%CI*	*P-value*
OPN (ng/ml)	1.11	0.86-1.44	1.17	0.83-1.66	.82
Haptoglobin (mg/ml)	1.10	0.86-1.39	1.08	0.87-1.33	.89
CA15-3 (U/ml)	8.48	7.08-10.15	10.34	8.72-12.26	.11
CEA (ng/ml)	0.56	0.48-0.66	0.49	0.40-0.60	.29
CA-125 (U/ml)	2.19	1.64-2.92	1.95	1.45-2.63	.56
Prolactin (ng/ml)	4.79	4.09-5.60	4.03	3.48-4.66	.10
CA19-9 (U/ml)	2.30	1.74-3.02	2.10	1.57-2.79	.66
AFP (ng/ml)	0.50	0.40-0.64	0.46	0.36-0.59	.62
Leptin (ng/ml)	17.34	14.38-20.92	15.67	12.91-19.02	.43
MIF (pg/ml)	166.5	141.9-195.4	199.7	165.9-240.4	.13

95%CI: 95% confidence interval; OPN: osteopontin; CA15-3: cancer antigen 15-3; CEA: carcinoembryonic antigen; CA-125: cancer antigen 125; CA19-9: cancer antigen 19-9; AFP: α-fetoprotein; MIF: migration inhibitory factor

To determine whether cases could be distinguished from controls based on a combination of all 10 protein markers, PCA analysis was performed. **Figure 1A** represents the PCA 3D view in which the cases and controls are plotted based on their scores on the first three principal components. PCA based on concentrations of all proteins could not distinguish between the cases and the controls. The amount of variance explained by the first three components of the total of 9 components was equal to 31.4%, 18.6% and 12.1% respectively. The RF classification accuracy scores of all proteins or the best combination of proteins; OPN and prolactin, showed no evidence that a panel of markers can correctly classify the samples. The average prediction resulted in sensitivity and specificity of only 51%. Adding information on subject characteristics to the protein data in the RF analysis could not improve the classification accuracy scores.

Twenty-four women were diagnosed with breast cancer as a result of a positive screening mammogram at the time of enrollment in the cohort. See for detailed subject and sample characteristics **Appendix 1** and **2**. We hypothesized that in this group of cases the set of biomarkers tested could well be discriminative, but that their discriminative power might be masked when all samples are taken together in the classification analyses. However, restriction of the analyses to the women in this subset did not change the results; cases could again not be discriminated from matched controls (**Figure 1B** and **Appendix 3**).

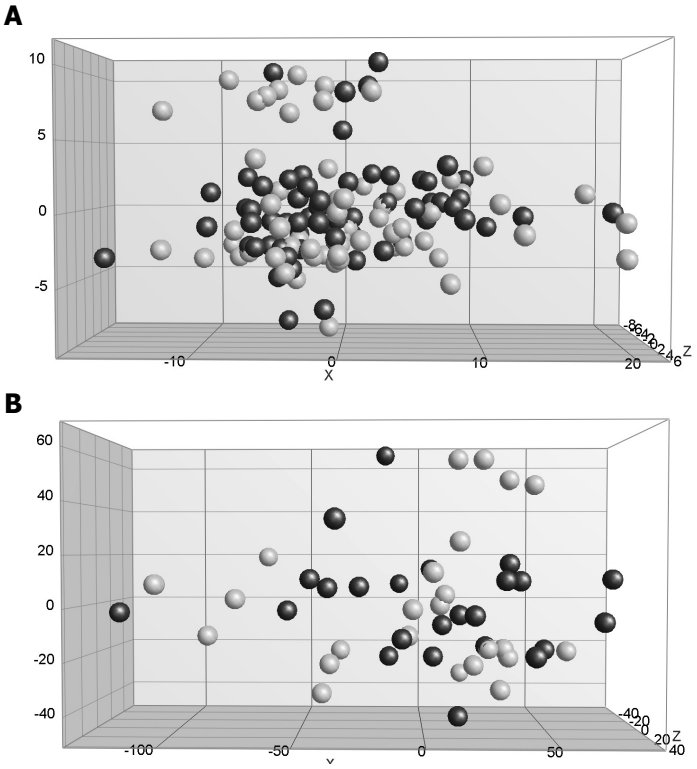

Figure 1 - Principle component analysis (PCA) 3D view for protein profiles from 68 cases (black dots) and 68 controls (gray dots) (A) and for the subset consisting of 24 cases and 24 controls (B). The PCA in both A and B is based on all 10 proteins. The first three components are shown.

Discussion

Using a panel of potential breast cancer markers consisting of OPN, haptoglobin, CA15-5, CEA, CA125, prolactin, CA19-9, AFP, leptin and MIF, we were unable to predict in serum the presence of early stage breast cancer. These proteins could not discriminate between the pre-diagnostic breast cancer samples and the control samples on their own nor in combination with the other proteins, at least not in the samples and experimental set-up used by us.

This is the first study that investigated the predictive value of this combination of proteins. Previous studies have shown that the use of a combination of markers related to breast cancer but with low sensitivity on their own, increases the sensitivity for breast cancer (5-14). Four of the proteins included in our panel, CA15-3, CEA, CA125 and prolactin have been investigated before as markers for breast cancer. In these case-control studies they were found to be slightly higher in women with symptomatic breast cancer (5-14,19). Two other proteins in our panel, OPN and haptoglobin, were identified as very promising breast cancer biomarkers in mouse models for breast cancer (17). MIF and leptin were previously found to be higher in breast cancer tissue (21,22), and CA19-9 and AFP were found before to be related to other cancers (6,20).

An important difference between these previous studies and ours is that we specifically designed our study for detection of breast cancer in very early stages, by studying serum samples that had been collected up to three years prior to the diagnosis of breast cancer. The proteins we selected to study could have predictive value for the presence of more advanced breast cancer, but apparently not up to three years prior to the detection on mammography or to the occurrence of symptoms. One could question whether it is at all possible to detect increased marker levels in the blood at such early stages. In a previous study, however, using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry (2D-nanoLC-MS/MS) for serum protein profiling, we were able to detect differences in the same pre-diagnostic sera of the same cases and controls as studied here (35), but these might be proteins involved in systemic responses rather than tumor derived markers.

However, we also focused specifically on a sub-group of cases with breast cancer detected following a positive screening mammogram at the time of enrollment in the cohort. In line with ovarian cancer, where based on increased CA125 levels 83% of advanced stage ovarian cancers can be detected, but also 50% of patients with stage I disease, our set of 10 markers might well have been discriminative in this specific subset of early breast cancer cases, but it was not.

Another difference with most previous biomarker studies and strength of our study is that our case-control study is nested in a cohort, where blood samples of all participants were collected, processed and stored in the exact same way under strictly defined conditions at a time when none of the participants was diagnosed with breast cancer yet. This ensures that our results are not biased by differences in sampling handling between cases and controls (36). In previous studies sample handling has been shown to have a major impact on protein concentrations (37-43).

Limitations of our study are its limited sample size and the fact that we do not have information on the estrogen receptor, progesterone receptor or HER2/neu status of the breast tumors and therefore cannot separate them by molecular subtype. It cannot be excluded that the heterogeneity among the breast cancers in our study is too large to identify a general predictive (combination) of markers. This might be especially a problem when the focus is on tumor derived markers, as most of our biomarkers were.

In conclusion, our results suggest that the tumor markers selected in the current study, single or in a panel, do not predict the presence of very early staged breast cancer. It cannot be excluded that the proteins may predict better in combinations with other proteins not selected here. It could also be that they predict better for specific molecular subtypes of breast cancer. Future studies should therefore preferentially select a broader target set of potential biomarkers, which could be enabled by new technologies based on antibody arrays that can measure up to 100 proteins in small amounts of serum (44), and include sufficient numbers of the different molecular tumor subtypes so that possibly a distinct predictive combination of biomarkers for each subtype can be identified.

Acknowledgements

The Prospect-EPIC study was funded by “Europe Against Cancer” Program of the European Commission (SANCO); the Dutch Ministry of Health; the Dutch Cancer Society; ZonMw the Netherlands Organization for Health Research and Development; World Cancer Research Fund (WCRF). We thank Integral Cancer Registration IKMN and the Integral Cancer Registration IKO for follow-up data on cancer. This study was financially supported by a grant from the board of the University Medical Center Utrecht and Julius Center for Health Sciences and Primary Care ('Strategische Impuls') and by the Netherlands Laboratory for Anticancer Drug Formulation (NLADF), Amsterdam, The Netherlands.

References

1. Ma L, Fishell E, Wright B, Hanna W, Allan S, Boyd NF. Case-control study of factors associated with failure to detect breast cancer by mammography. ***J. Natl. Cancer Inst.*** *1992;* 84(10): 781-785.
2. Mandelson MT, Oestreicher N, Porter PL, White D, Finder CA, Taplin SH et al. Breast density as a predictor of mammographic detection: comparison of interval- and screen-detected cancers. ***J. Natl. Cancer Inst.*** *2000;* 92(13): 1081-1087.
3. Kolb TM, Lichy J, Newhouse JH. Comparison of the performance of screening mammography, physical examination, and breast US and evaluation of factors that influence them: an analysis of 27,825 patient evaluations. ***Radiology*** *2002;* 225(1): 165-175.
4. Boyd NF, Guo H, Martin LJ, Sun L, Stone J, Fishell E et al. Mammographic density and the risk and detection of breast cancer. ***N. Engl. J. Med.*** *2007;* 356(3): 227-236.
5. Sturgeon CM, Duffy MJ, Stenman UH, Lilja H, Brunner N, Chan DW et al. National Academy of Clinical Biochemistry laboratory medicine practice guidelines for use of tumor markers in testicular, prostate, colorectal, breast, and ovarian cancers. ***Clin. Chem.*** *2008;* 54(12): e11-e79.
6. Ludwig JA, Weinstein JN. Biomarkers in cancer staging, prognosis and treatment selection. ***Nat. Rev. Cancer*** *2005;* 5(11): 845-856.
7. Kulasingam V, Zheng Y, Soosaipillai A, Leon AE, Gion M, Diamandis EP. Activated leukocyte cell adhesion molecule: a novel biomarker for breast cancer. ***Int. J. Cancer*** *2009;* 125(1): 9-14.
8. Arslan N, Serdar M, Deveci S, Ozturk B, Narin Y, Ilgan S et al. Use of CA15-3, CEA and prolactin for the primary diagnosis of breast cancer and correlation with the prognostic factors at the time of initial diagnosis. ***Ann. Nucl. Med.*** *2000;* 14(5): 395-399.
9. Eskelinen M, Kataja V, Hamalainen E, Kosma VM, Penttila I, Alhava E. Serum tumour markers CEA, AFP, CA 15-3, TPS and Neu in diagnosis of breast cancer. ***Anticancer Res.*** *1997;* 17(2B): 1231-1234.
10. Lumachi F, Basso SM, Brandes AA, Pagano D, Ermani M. Relationship between tumor markers CEA and CA 15-3, TNM staging, estrogen receptor rate and MIB-1 index in patients with pT1-2 breast cancer. ***Anticancer Res.*** *2004;* 24(5B): 3221-3224.
11. Molina R, Jo J, Filella X, Zanon G, Pahisa J, Mu nM et al. c-erbB-2 oncoprotein, CEA, and CA 15.3 in patients with breast cancer: prognostic value. ***Breast Cancer Res. Treat.*** *1998;* 51(2): 109-119.
12. Robertson JF, Pearson D, Price MR, Selby C, Pearson J, Blamey RW et al. Prospective assessment of the role of five tumour markers in breast cancer. ***Cancer Immunol. Immunother.*** *1991;* 33(6): 403-410.
13. Hwa HL, Kuo WH, Chang LY, Wang MY, Tung TH, Chang KJ et al. Prediction of breast cancer and lymph node metastatic status with tumour markers using logistic regression models. ***J. Eval. Clin. Pract.*** *2008;* 14(2): 275-280.

14. Norum LF, Erikstein B, Nustad K. Elevated CA125 in breast cancer--A sign of advanced disease. ***Tumour. Biol.*** *2001;* 22(4): 223-228.
15. Wijnhoven SW, Zwart E, Speksnijder EN, Beems RB, Olive KP, Tuveson DA et al. Mice expressing a mammary gland-specific R270H mutation in the p53 tumor suppressor gene mimic human breast cancer development. ***Cancer Res.*** *2005;* 65(18): 8166-8173.
16. Krupke DM, Begley DA, Sundberg JP, Bult CJ, Eppig JT. The Mouse Tumor Biology database. ***Nat. Rev. Cancer*** *2008;* 8(6): 459-465.
17. Rodenburg W, Pennings JL, van Oostrom CT, Roodbergen M, Kuiper RV, Luijten M et al. Identification of breast cancer biomarkers in transgenic mouse models: a proteomics approach. ***Proteomics Clin. Appl.*** *In press.*
18. Perou CM, Sorlie T, Eisen MB, van de RM, Jeffrey SS, Rees CA et al. Molecular portraits of human breast tumours. ***Nature*** *2000;* 406(6797): 747-752.
19. Tworoger SS, Eliassen AH, Sluss P, Hankinson SE. A prospective study of plasma prolactin concentrations and risk of premenopausal and postmenopausal breast cancer. ***J. Clin. Oncol.*** *2007;* 25(12): 1482-1488.
20. Park IJ, Choi GS, Jun SH. Prognostic value of serum tumor antigen CA19-9 after curative resection of colorectal cancer. ***Anticancer Res.*** *2009;* 29(10): 4303-4308.
21. Bando H, Matsumoto G, Bando M, Muta M, Ogawa T, Funata N et al. Expression of macrophage migration inhibitory factor in human breast cancer: association with nodal spread. ***Jpn. J. Cancer Res.*** *2002;* 93(4): 389-396.
22. Garofalo C, Surmacz E. Leptin and cancer. ***J. Cell Physiol*** *2006;* 207(1): 12-22.
23. Schwenk JM, Igel U, Kato BS, Nicholson G, Karpe F, Uhlen M et al. Comparative protein profiling of serum and plasma using an antibody suspension bead array approach. ***Proteomics.*** *2010; 10(3): 532-40.*
24. Schwenk JM, Gry M, Rimini R, Uhlen M, Nilsson P. Antibody suspension bead arrays within serum proteomics. ***J. Proteome. Res.*** *2008;* 7(8): 3168-3179.
25. Boker LK, van Noord PA, van der Schouw YT, Koot NV, Bueno de Mesquita HB, Riboli E et al. Prospect-EPIC Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. ***Eur. J. Epidemiol.*** *2001;* 17(11): 1047-1053.
26. Pols MA, Peeters PH, Ocke MC, Slimani N, Bueno-de-Mesquita HB, Collette HJ. Estimation of reproducibility and relative validity of the questions included in the EPIC Physical Activity Questionnaire. ***Int. J. Epidemiol.*** *1997;* 26 Suppl 1: S181-S189.
27. Troyanskaya O, Cantor M, Sherlock G, Brown P, Hastie T, Tibshirani R et al. Missing value estimation methods for DNA microarrays. ***Bioinformatics.*** *2001;* 17(6): 520-525.
28. R development core team: A language and environment for statistical computing. Available at http://www.R-project.org *Retrieved October 2009.*
29. Jackson JE. A User's Guide to Principal Components.*2003;* Wiley-Interscience, John Wiley & Sons, New York.
30. Breiman L. Random Forests. ***Machine Learning*** *2001;* 45: 5-32.

31. Liaw A, Wiener M. Classification and Regression by Random Forests. ***R News*** *2002;* 2: 18-22.
32. Barrett JH, Cairns DA. Application of the random forest classification method to peaks detected from mass spectrometric proteomic profiles of cancer patients and controls. ***Stat. Appl. Genet. Mol. Biol.*** *2008;* 7(2): Article4.
33. Diaz-Uriarte R, Alvarez de Andrés S. Gene selection and classification of microarray data using random forest. ***BMC. Bioinformatics.*** *2006;* 7: 3.
34. Lunetta KL, Hayward LB, Segal J, Van EP. Screening large-scale association study data: exploiting interactions using random forests. ***BMC. Genet.*** *2004;* 5(1): 32.
35. van Winden AW, Krop EJM, Kåredal MH, Gast MC, Lindh CH, Jeppson MC et al. Searching for early breast cancer biomarkers by serum protein profiling of pre-diagnostic serum. *Submitted for publication.*
36. Ransohoff DF. Bias as a threat to the validity of cancer molecular-marker research. ***Nat. Rev. Cancer*** *2005;* 5(2): 142-149.
37. Banks RE, Stanley AJ, Cairns DA, Barrett JH, Clarke P, Thompson D et al. Influences of blood sample processing on low-molecular-weight proteome identified by surface-enhanced laser desorption/ionization mass spectrometry. ***Clin. Chem.*** *2005;* 51(9): 1637-1649.
38. Engwegen JY, Alberts M, Knol JC, Jimenez CR, Depla AC, Tuynman H et al. Influence of variations in sample handling on SELDI-TOF MS serum protein profiles for colorectal cancer. ***Proteomics Clin. Appl.*** *2008;* 2: 936-945.
39. Hsieh SY, Chen RK, Pan YH, Lee HL. Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. ***Proteomics.*** *2006;* 6(10): 3189-3198.
40. Timms JF, Arslan-Low E, Gentry-Maharaj A, Luo Z, T'Jampens D, Podust VN et al. Preanalytic influence of sample handling on SELDI-TOF serum protein profiles. ***Clin. Chem.*** *2007;* 53(4): 645-656.
41. West-Nielsen M, Hogdall EV, Marchiori E, Hogdall CK, Schou C, Heegaard NH. Sample handling for mass spectrometric proteomic investigations of human sera. ***Anal. Chem.*** *2005;* 77(16): 5114-5123.
42. Karsan A, Eigl BJ, Flibotte S, Gelmon K, Switzer P, Hassell P et al. Analytical and preanalytical biases in serum proteomic pattern analysis for breast cancer diagnosis. ***Clin. Chem.*** *2005;* 51(8): 1525-1528.
43. Villanueva J, Philip J, Chaparro CA, Li Y, Toledo-Crow R, DeNoyer L et al. Correcting common errors in identifying cancer-specific serum peptide signatures. ***J. Proteome. Res.*** *2005;* 4(4): 1060-1072.
44. Carlsson A, Wingren C, Ingvarsson J, Ellmark P, Baldertorp B, Ferno M et al. Serum proteome profiling of metastatic breast cancer using recombinant antibody microarrays. ***Eur. J. Cancer*** *2008;* 44(3): 472-480.

Appendix 1

Characteristics of the subset

	Cases (*n*=24)	Controls (*n*=24)
Age at enrollment (years)		
Mean (SD)	60.9 (5.2)	60.9 (5.3)
BMI		
Mean (SD)	27.7 (3.4)	26.5 (4.2)
Missing	1	-
Use of oral contraceptives, *n* (%)		
No, but used to in the past	12 (50.0)	11 (45.8)
No, never	12 (50.0)	13 (54.2)
Duration of oral contraceptives use* (years)		
Median (IQR)	10 (4-15)	6 (2-10)
Use of HT, *n* (%)		
No, but used to in the past	2 (8.3)	4 (16.7)
No, never	22 (91.7)	20 (83.3)
Duration of HT use* (years)		
Median (IQR)	1.0 (4.5-8.0)	1.5 (1.0-8.0)
Ovariectomy, *n* (%)		
Both ovaries removed	1 (4.2)	-
Parity, *n* (%)		
Nulliparous	1 (4.2)	-
Number of children[#]		
Median (IQR)	2 (2-3)	3 (3-4)
Smoking, *n* (%)		
No, but used to in the past	12 (50.0)	10 (41.7)
No, never	12 (50.0)	14 (58.3)
Pack-years smoking until stop date[†]		
Median (IQR)	3.8 (1.7-9.6)	3.4 (0.9-12.8)
Missing	1	1
Alcohol intake[‡] (g/day)		
Median (IQR)	3.0 (0.4-9.0)	0.5 (0.0-4.6)
Use of medicines, minerals or vitamins last week, *n* (%)		
Yes	17 (70.8)	14 (58.3)
No	7 (29.2)	10 (41.7)
Time since last meal and/or drink** (minutes)		
Median (IQR)	122 (95-160)	131 (90-140)

SD: standard deviation; BMI: body mass index; IQR: inter-quartile range; HT: menopausal hormone therapy; * Among former oral contraceptives/HT users; [#] Among women with children; [†] Among former smokers; [‡] Energy-adjusted alcohol intake at enrollment; ** At moment of blood collection

Appendix 2

Characteristics of the serum samples in the subset

	Cases (*n*=24)	Controls (*n*=24)
Serum sample storage duration* (years)		
Mean (SD)	11.4 (1.3)	11.4 (1.3)
Hours in refrigerator#		
Median (IQR)	22 (20-23)	22 (19-23)
Days at -86°C†		
Median (IQR)	8 (5-21)	7 (5-21)

SD: standard deviation; IQR: inter-quartile range; * Until experiment; # Between collection and centrifugation; † Between centrifugation and storage at liquid nitrogen

Appendix 3

Serum concentrations of all measured proteins in subset of cases diagnosed at 1st screening after inclusion and matched healthy controls

	Cases (*n*=24)		Controls (*n*=24)		Paired T test
Biomarker	*Geometric mean concentration*	*95%CI*	*Geometric mean concentration*	*95%CI*	*p-value*
OPN (ng/ml)	1.21	0.80-1.82	1.18	0.65-2.15	.96
Haptoglobin (mg/ml)	1.34	0.91-1.96	1.10	0.75-1.62	.41
CA15-3 (U/ml)	7.82	5.83-10.49	9.02	6.72-12.11	.45
CEA (ng/ml)	0.57	0.43-0.75	0.44	0.32-0.60	.27
CA-125 (U/ml)	1.99	1.17-3.39	1.33	0.77-2.30	.28
Prolactin (ng/ml)	3.66	3.05-4.38	3.57	2.57-4.97	.90
CA19-9 (U/ml)	1.83	1.16-2.89	2.19	1.50-3.21	.53
AFP (ng/ml)	0.37	0.24-0.57	0.41	0.27-0.63	.69
Leptin (ng/ml)	21.89	17.02 -28.15	18.45	12.91-26.37	.39
MIF (pg/ml)	145.3	101.8-207.5	204.1	146.2-284.9	.15

95%CI: 95% confidence interval; OPN: osteopontin; CA15-3: cancer antigen 15-3; CEA: carcinoembryonic antigen; CA-125: cancer antigen 125; CA19-9: cancer antigen 19-9; AFP: α-fetoprotein; MIF: migration inhibitory factor

General discussion

Chapter 7

Early diagnostic biomarkers for breast cancer:
how far have we come?

Abstract

Many studies have used surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) or matrix assisted laser desorption/ionization (MALDI) -TOF MS to search for blood-based proteins that are related to the presence of breast cancer. We review the biomarkers discovered by these methods and discuss the strengths and weaknesses of these studies. We highlight two proteins that most consistently were related to breast cancer: C3a des-arginine anaphylatoxin ($C3a_{desArg}$) (molecular weight: 8938 Da) and fragments of inter-alpha trypsin inhibitor heavy chain H4 (ITIH4). In addition, we elaborate on three important methodological aspects related to these studies, i.e. protein identification; specificity of the markers; and disease heterogeneity. Finally we propose some points to be addressed in future studies. These include the use of other analytical measurement techniques, need of protein identification, the importance of identical sample handling protocols for cases and controls, and the stratifying of the results according to molecular subtypes and early stages of breast cancer. Ultimately this may lead to the discovery of new and valid breast cancer specific biomarkers.

Introduction

The current routinely used method for early detection of breast cancer is mammography. Breast cancer screening programs were introduced in the late 1980s and 1990s in many Western countries (1). The age range of the programs is generally between 50 and 75 years and mammograms are taken every one to three years (1). In an extensive recent review it was concluded that in countries with long-standing breast cancer screening programs, breast cancer mortality in women having attended at least one screening examination was estimated to be decreased with 16% to 32% (1). However, about a quarter of all breast cancers occur in women aged 49 years or younger. (2) These women are not included in most national screening programs, mainly because young women have higher breast density, which results in lower sensitivity of mammography (3-5). In addition, even among screened women 1 out of 3 breast cancers occur in between two screening examinations (6). Breast cancer biomarkers in blood can be of major aid in the early diagnosis of breast cancer in these women. A blood test would be relatively easy to perform.

In the past years many studies searched for breast cancer biomarkers, mainly in the serum proteome. These proteins could originate from the tumor, but could also be generated by processes that are related to tumorgenesis or a response to the presence of the tumor itself. Improvements in mass spectrometry (MS) based techniques have boosted the use of protein profiling techniques, such as surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS and matrix assisted laser desorption/ionization (MALDI) -TOF MS. These techniques measure a large part of the proteome in one analysis with relatively simple sample preparation, high analytical sensitivity, and in a high-throughput fashion (7,8). These characteristics make these techniques attractive and promising tools for the discovery of new cancer biomarkers. Both techniques are based on the purification of proteins, e.g. from serum or plasma, by arrays with various chromatographic surfaces (SELDI), or with reversed-phase C18 magnetic beads (MALDI). Extracted proteins are subsequently co-crystallized with an energy absorbing matrix and ionized by irradiation, after which an electric field migrates the charged proteins to

the time-of-flight mass analyzer. Proteins are separated based on their mass, which is proportional to their time-of-flight, and their relative amount is measured (8).

This paper reviews the biomarkers from protein profiling studies with potential for the early detection of breast cancer. We summarize protein profiling techniques as a tool to detect cancer biomarkers and discuss recent advances in other proteomic approaches. Finally, we propose how the research may be improved.

Biomarkers from protein profiling studies for breast cancer

Gast et al. recently presented a detailed overview of protein profiling studies for breast cancer (8). In the current review we update that list with studies published after November 2008, using SELDI or MALDI-TOF MS to search for, or validating diagnostic breast cancer biomarkers in serum or plasma (9-22).

Most protein profiling studies using SELDI- or MALDI-TOF MS did not identify the detected proteins but only reported mass-to-charge ratio's (*m*/*z*) of the discriminating peaks (10-19). Results of these studies cannot be compared since different platforms (SELDI- and MALDI-TOF MS) and either serum or plasma were used. Even peaks with similar *m*/*z* detected in serum with SELDI-TOF MS may represent different proteins due to the use of different array surfaces. So these studies therefore only indicate the possibility to discriminate breast cancer cases and controls by serum or plasma protein concentrations, but do not identify new biomarkers. Therefore, validating these results in future studies is hampered. In **Table 1** we show an overview of breast cancer biomarkers detected with SELDI- or MALDI-TOF MS and which were (tentatively) identified. We will highlight two proteins that were detected in several studies as breast cancer biomarkers, i.e. C3a des-arginine anaphylatoxin ($C3a_{desArg}$) (molecular weight: 8938 Da) and fragments of inter-alpha trypsin inhibitor heavy chain H4. The discussion of these two markers should not be seen as an indication that these two proteins are necessarily the most promising breast cancer biomarkers. Merely, these two proteins have rendered the most consistent results to date.

Table 1 - Discriminating proteins detected in breast cancer by SELDI- or MALDI-TOF MS

*M/z**	Platform	Pretreatment	Condition	Expression	Identity	Function	Reference
904, 1061	M	C18 fractionation	-	+	Bradykinin fragments	Inflammation mediator	Villanueva et al. (58)
905-1537	M	C18 fractionation	-	+ and -	Fibrinopeptide A fragments	Blood coagulation	Villanueva et al. (32)
942-1865	M	C18 fractionation	-	-	C3f fragments	Complement activation	Villanueva et al. (32)
1627-1704	M	C18 fractionation	-	+	C4a fragments	Complement activation	Villanueva et al. (32)
2379, 2659	M	C18 fractionation	-	+	Fibrinogen α fragments	Blood coagulation	Villanueva et al. (32)
2661	S	Anion exchange fractionation (F45)[#]	IMAC Cu	-			Shi et al. (32)[†]
2451	M	C18 fractionation	-	+	Transthyretin fragment	Thyroid hormone-binding protein, acute phase reactant	Villanueva et al. (64)
2508	M	C18 fractionation	-	+	Apolipoprotein AIV	Lipid metabolism	Villanueva et al. (32)
2602	M	C18 fractionation	-	+	Factor XIIIa fragment	Blood coagulation	Villanueva et al. (32)
4300	S	-	IMAC Ni	-	Inter-alpha trypsin inhibitor heavy chain H4 (ITIH4) fragments	Acute phase reactant	Li et al. (32)
4300	S	-	IMAC Ni	+			Li et al. (23)
4286	S	-	IMAC Ni	-			Mathelin et al. (25)
4276	S	-	IMAC Ni	-			Van Winden et al. (26)
2271-3272	S	-	Immunoassay	NS			Fung et al. (27)
2271-4293	S	-	Immunoassay	+			Song et al. (33)
998-2358	M	C18 fractionation	-	+			Villanueva et al. (31)
2733-4309	M	-	IMAC Ni	-			Gast et al. (32)
3100	S	-	CM10	+	N-terminal fragment of albumin	Transport protein	Engwegen et al. (21)
3323	S	-	IMAC NI	+	Apolipoprotein CI - double charge	Lipid metabolism	Van Winden et al. (45)

Table 1 - continued

*M/z**	Platform	Pretreatment	Condition	Expression	Identity	Function	Reference
3959	S	Q hyperD fractionation (F6)	Q10	+	Factor XIIIa fragment	Blood coagulation	Van Winden et al. (9)
5900	S	-	CM10	-	Fragment of fibrinogen alphaE chain	Blood coagulation	Engwegen et al. (20)
5909	S	-		+	Platelet factor 4 (PF4)	Modulator of endothelial cell proliferation and angiogenesis	Solassol et al. (45)
6600	S	-	CM10	-	Apolipoprotein CI	Lipid metabolism	Engwegen et al. (22)
8100	S	-	IMAC Ni	+	C3a des-arginine anaphylatoxin C-terminal fragment ($C3a_{desArg\Delta8}$)	Complement activation	Li et al. (45)
8116	S	-	IMAC Ni	+			Li et al. (23)
8126	S	-	IMAC Ni	NS			Mathelin et al. (25)
8129	S	-	IMAC Ni	NS			Van Winden et al. (26)
8746	S	Q hyperD fractionation (F6)	Q10	+	Apolipoprotein CIII	Lipid metabolism	Van Winden et al. (27)
8909	S	Q hyperD fractionation (F6)	Q10	-	Apolipoprotein CII	Lipid metabolism	Van Winden et al. (20)
8925	S	Q hyperD fractionation (F6)	Q10	-	Oxidized apolipoprotein CII	Lipid metabolism	Van Winden et al. (20)
8900	S	-	IMAC Ni	+	C3a des-arginine anaphylatoxin ($C3a_{desArg}$)	Complement activation	Li et al. (20)
8926	S	-	IMAC Ni	+			Li et al. (23)
8919	S	-	IMAC Ni	+			Mathelin et al. (25)
8941	S	-	IMAC Ni	-			Van Winden et al. (26)
8940	S	-	IMAC Ni	-			Gast et al. (27)
8938	S	-	IMAC Ni	+			Van Winden et al. (21)
8926	S	-		+			Solassol et al. (9)

Table 1 - continued

*M/z**	Platform	Pretreatment	Condition	Expression	Identity	Function	Reference
15120	S			+	Haemoglobin α-chain	Oxygen transport	Solassol et al. (22)
15346	S	Q hyperD fractionation (F3)	IMAC Cu	-	Post-translational modified haemoglobin ß-chain		Van Winden et al. (22)
15915	S	Q hyperD fractionation (F3)	IMAC Cu	-	Haemoglobin ß-chain	Oxygen transport	Van Winden et al. (20)
15870	S			+			Solassol et al. (20)
28000	S	-	CM10	-	Apolipoprotein AI	Lipid metabolism	Engwegen et al. (22)
45435	S	Q hyperD fractionation (F6)	Q10	-	Aoplipoprotein AI and AII dimer	-	Van Winden et al. (45)
79235	S			+	Transferrin	Acute phase reactant, iron binding &transport, cell proliferation	Solassol et al. (20)

M/z: mass-to-charge ratio; S: SELDI-TOF MS; M: MALDI-TOF MS; NS: not significantly related; +: higher in cases compared to controls; -: lower in cases compared to controls; Q hyperD fractionation is based on strong anion exchange and resulted in six fractions, Fraction 3 (F3) and Fraction 6 (F6) were used for analysis; * When a range is reported, several fragments with *m/z*'s between these values were detected; [#] Fractionation resulted in four fractions, the third fraction was used for analysis (F45); [†] The only study investigating plasma instead of serum

C3a des-arginine anaphylatoxin

Levels of C3a des-arginine anaphylatoxin ($C3a_{desArg}$) (molecular weight: 8938 Da) were higher in breast cancer cases compared to healthy controls in five studies (9,23-26), while in one of our studies we found lower levels in breast cancer cases (27). Lower levels of $C3a_{desArg}$ among cases were recently also reported in another study (21). One of the studies that reported increased levels of $C3a_{desArg}$ among cases included cases with ductal carcinoma in situ (DCIS) (22). In our study among a-symptomatic cases using pre-diagnostic materials we also found this protein to be higher (9). These higher levels shortly before diagnosis and in non-invasive cancers could indicate that $C3a_{desArg}$ is increased in the very early stages of the disease. The two studies in which $C3a_{desArg}$ was found to be lower among breast cancer cases investigated the same group of breast cancer cases and healthy controls (21,27), which was extended with about 100 cases and 80 controls in the most recent study (21). It is therefore possible that differences in the observed relation between these two studies and the other studies are due to differences related to the study population. $C3a_{desArg}$ was previously found to be lower in metastatic breast cancer patients (25), but this cannot explain the lower levels in the study by Gast et al. (21) or our study (27), since patients in these studies had locally invasive breast cancer without metastases (21,27). So, we did not find clear explanations for these two negative results. In order to further elucidate the potential for $C3a_{desArg}$ as an early detection marker, future studies should use an assay to measure absolute levels of $C3a_{desArg}$. Such a study should also include a large group of patients with various stages of breast cancer to assess the relation with progressing stages of disease. If the marker is higher in early stages but lower in later more progressed disease it would limit the applicability of the marker. A serious shortcoming of the protein, however, is that it is not specific for breast cancer. Increased $C3a_{desArg}$ serum levels have also been reported in hepatocellular and colorectal cancer (28,29), and in chronic lymphoid malignancies (30). In combination with other proteins, however, $C3a_{desArg}$ may still be a valuable marker that contributes to the diagnostic capacity of a panel of markers to detect breast cancer, or it might be valuable in combination with existing or future screening techniques (i.e. magnetic resonance imaging (MRI)).

Fragments of inter-alpha trypsin inhibitor heavy chain H4

Various fragments of inter-alpha trypsin inhibitor heavy chain H4 (ITIH4) have been found to discriminate between breast cancer cases and healthy controls in numerous studies (21,23,25-27,31,32) (**Table 2**). These studies only reported the *m/z* of the peaks representing these fragments and, after identification their amino acid sequence. To be able to compare the various ITIH4 fragments that were reported in the several studies, we calculated the theoretical mass of the different fragments based on the amino acid sequences reported in these studies, using the ExPASy compute pI/MW tool on http://www.expasy.org/tools/pi_tool.html. ITIH4 is a 120 kDa plasma glycoprotein (31,33) that acts as an acute phase protein (31) and is known to be highly sensitive to extensive fragmentation (31,33). ITIH4 is readily cleaved by plasma kallikrein resulting in a 85 kDa fragment and a 35 kDa fragment. The 85 kDa fragment is subsequently cleaved resulting in a 57 kDa fragment and a 28 kDa fragment. The 28 kDa fragment is then rapidly cleaved into unidentified smaller fragments (31).

In a study by Li et al. (23) a 4.3 kDa protein that was later identified as a fragment of ITIH4 (theoretical mass: 4285 Da) (25), was lower in breast cancer, but higher in two subsequent validation studies (25,31). In three other studies, however, proteins with a similar mass were again found to be lower in breast cancer (21,26,27).

Fung et al. used a SELDI-based immunoassay and measured a series of ITIH4 fragments representing sequential N-terminal truncation (33). Of the nine ITIH4 fragments none appeared to discriminate between the 20 breast cancer cases and 41 healthy controls. Song et al. also investigated several ITIH4 fragments (31). Additionally to the fragments measured by Fung et al. (33), they measured the 4285, 3957 and 3158 Da fragments, but they did not measure the 2571 Da fragment (31). The majority of the measured ITIH4 fragments were higher in breast cancer cases (a.o. the 2359 Da fragment) (31). Villanueva et al. also investigated several ITIH4 fragments of which several had not been investigated before, and confirmed the higher intensity of the fragment of 2359 Da. Besides, the 2184, 2115, 1787 and 998 Da fragments were also found to be higher in breast cancer (32). Some of these were also higher in other types of cancer, but a few were exclusively discriminating

Table 2 - ITIH4 fragments found to be discriminating in different studies

Mass*	Structural identity of ITIH4 fragments		References							
Da	*Start-End*	*Amino acid sequence*	(22)	(8)	(23)	(25)	(26)	(27) (AUC)	(33) (p) †	(31)
3158	617-644	R NVHSGSTFFKYYLQGAKIPKPEASFSPR R						NS (.701)	NS (.198)	Lo
2115		R NVHSAGAAGSRMNFRPGVLSS R							Hi	
842	681-687	HAAYHPF							NS (.245)	
1787	671-687	GLPGPPDVPDHAAYHPF							Hi	
2028	669-687	QLGLPGPPDVPDHAAYHPF							NS (.800)	
2272	667-687	SRQLGLPGPPDVPDHAAYHPF					NS	Hi	NS (.098)	
2359	666-687	SSRQLGLPGPPDVPDHAAYHPF					NS	Hi	Hi	
2571	664-687	VLSSRQLGLPGPPDVPDHAAYHPF					NS			
2628	663-687	GVLSSRQLGLPGPPDVPDHAAYHPF					NS	Hi	NS (.887)	
2725	662-687	PGVLSSRQLGLPGPPDVPDHAAYHPF					NS	Hi	NS (4.31E-04)	Lo
2881	661-687	RPGVLSSRQLGLPGPPDVPDHAAYHPF					NS	Hi		
3028	660-687	FRPGVLSSRQLGLPGPPDVPDHAAYHPF					NS	Hi		
3143	659-687	NFRPGVLSSRQLGLPGPPDVPDHAAYHPF					NS	Hi		
3274	658-687	MNFRPGVLSSRQLGLPGPPDVPDHAAYHPF					NS	Hi	NS	Lo
3290	658-687	MNFRPGVLSSRQLGLPGPPDVPDHAAYHPF								Lo
3972	650-687	QAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPF							NS	
4285	650-689	R QAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPFRR L	Lo	Hi	Lo	Lo		Hi		Lo
4301	650-689	QAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPFRR#			Lo	Lo				Lo
3957	654-689	AGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPFRR						NS (.504)		Lo
3973	654-689	AGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPFRR#								Lo
2184	669-688	QLGLPGPPDVPDHAAYHPFR							Hi	
998	681-688	HAAYHPFR							Hi	

The longest peptide is listed with its flanking residues (separated by two spaces); Lo: Lower in cases compared to controls; Hi: Higher in cases compared to controls; NS: not significantly related (Fung et al. (32): NS if p>0.05, Villanueva et al. (21): NS if p>0.0002); * Theoretical mass; # Oxidized fragment (+16 Da); † Ratio peak intensity in the case group versus the control group was >1 for all detected fragments.

in breast cancer, i.e. the fragments of 2359 and 1787 Da (32). A recent study found eight ITIH4 fragments related to breast cancer (21). The 4301 and 4285 Da fragments were lower in breast cancer similar to results of others (23,26,27). Results for some other fragments were less consistent (21).

Villanueva et al. hypothesized that ITIH4 fragments, but also fragments of other proteins (see **Table 1**) are generated by exoprotease activities that confer cancer type-specific differences, and these are superimposed on the proteolytic event of the *ex vivo* coagulation and complement degradation pathways (32). Villanueva et al. developed a test to measure degradation of artificial substrates, the Sequence-Specific Exopeptidase Activity Test (SSEAT) (34). Each fragment that is formed from the degradable, isotopically labeled substrate is quantified by comparison with double-labeled, non-degradable reference peptides (representing truncated sequences) spiked into the samples at the same time as the substrate. In this way measurements can be corrected for adsorptive and processing-related losses. All samples are incubated at RT for the relevant period of time for the substrate under investigation to be optimally degraded (just before decrease of intensity of formed fragments). Using this test, fragmentation patterns of the substrates can be measured independently from factors related to sample collection, storage, and handling as well as possible variability in endogenous peptide precursor levels (34). This test has recently been used to analyze serum samples of metastatic thyroid cancer patients and healthy controls, utilizing three different peptide substrates (complement C3f, fibrinopeptide A and Clus2). Cases and controls could be separated with high sensitivity and specificity when data of the resulting peptide metabolites were used (35). This test should be explored also with ITIH4 as a substrate, and possibly also with other proteins to determine the relevance of exoprotease degradation patterns in diagnosing breast cancer.

Limitations of SELDI- and MALDI-TOF MS studies

Previous studies have extensively described several limitations of SELDI- and MALDI-TOF MS for discovery of cancer biomarkers. These mainly concern the vulnerability of protein profiles for differences in sample collection, sample handling and sample

storage (36-41). Due to the errors introduced by these factors results of SELDI- and MALDI-TOF MS are difficult to reproduce both within and between studies. This issue has been discussed at length in several papers (42,43). Here, we focus on three lesser discussed problems faced in this field of research: protein identification, specificity of detected markers and heterogeneity of breast cancer cases.

Protein identification

Identification of detected proteins is often lacking in SELDI- or MALDI-TOF MS studies. The analyses with these two techniques result in spectra with peaks representing the proteins separated by their mass-to-charge ratio (*m*/*z*). Proteins with similar mass, however, cannot be separated by SELDI- or MALDI-TOF MS, and the result is that peaks will overlap in the spectra. Although the *m*/*z* of peaks detected in different studies may be similar, they still can represent different proteins. Consequently, identification of the discriminating protein is necessary, as well as confirmation of the difference in expression of the identified protein between the two groups. However, identification of detected proteins is not a simple procedure.

A protein can be identified based on the masses of the peptides in which a protein is naturally fragmented, a method called mass fingerprinting. Fragmentation of proteins with a mass below 4,000 Da can usually be performed using MALDI-TOF/TOF MS/MS. First, proteins of interest are extracted from the serum sample using revered-phase C18 magnetic beads. Subsequently, the eluate is mixed with an energy absorbing matrix and spotted on a MALDI target plate. The protein of interest can then be detected and fragmented with MALDI-TOF/TOF MS/MS. Subsequently, the identity of the fragments can be obtained by linking the mass of the fragments to a search engine like MASCOT at http://www.matrixscience.com and the Swiss-Prot database (37,44). Alternatively, Q-TOF MS/MS instead of MALDI-TOF/TOF MS/MS can be used to obtain mass measurements of fragment ions (32). To be able to perform this procedure successfully, the protein of interest should have a high intensity in order to produce enough fragments with intensities high enough to be detectable.

Most proteins detected with SELDI-TOF MS, however, have masses up to 15,000 Da and these can not be fragmented by MALDI-TOF/TOF MS/MS. Proteins with masses higher than about 4,000 Da, therefore, should be digested using trypsin. But, before the sample with the protein of interest can be digested, purification of the protein is required. Different procedures can be used to purify the protein (38,45), and usually several methods are needed. These procedures are based on the separation of proteins in a sample by their isoelectric point (pI) and their size, and further purification by de-salting the fraction of interest (38,45). Between these steps, the proteins in the resulting fractions should be profiled to select the fraction that contains the protein of interest. This fraction can then be further purified. When no other peaks with masses close to the mass of the protein of interest are present in the sample anymore, the fraction can be analyzed with SDS-PAGE. The protein bands of interest then can be excised and collected for digestion. Based on the peptides in the resulting digest, the protein can be identified (38,45). However, the more purification steps are needed, usually the less concentrated the protein will be in the final fraction. Since concentrations of proteins of interest are usually not that high, proteins of interest are often 'lost' during the purification process. This greatly hampers the identification of discriminating proteins detected with SELDI- or MALDI-TOF MS. Identification is however necessary to elucidate molecular mechanisms related to the disease. Moreover, for confirmation by an immunoassay for the protein of interest, but also for replication and validation of the observed relation in other studies the protein has to be identified (46).

Breast cancer specific markers

A second challenge refers to the specificity of new markers. Most of the proteins that have been proposed as candidate biomarkers for breast cancer so far are relatively high abundant proteins. These proteins are mainly involved in acute phase reactions which comprises a cascade of inflammatory signals that can be triggered by the presence of a small tumor, but also in response to inflammation, infection and vascular disease (33). Consequently, the specificity of these proteins for cancer, and in particular breast cancer, is likely to be low. This is probably also the case for $C3a_{desArg}$, which is formed after cleavage of complement C3. Complement C3 is a

high abundant protein that supports the activation of all three pathways of complement activation. Since complement C3 is a positive acute phase reactant, elevated serum levels of $C3a_{desArg}$ are anticipated in breast cancer cases compared to healthy controls (21). Many of these acute phase reactants are easily detected with SELDI- and MALDI-TOF MS because of their high abundance in the serum proteome. Since the dynamic range of the proteins in serum is much larger than the dynamic range that can be detected using SELDI- or MALDI-TOF MS, protein profiles are dominated by high abundant proteins that suppress signals of lower abundant proteins (47). In one of our studies we performed serum fractionation by strong anion exchange chromatography preceding protein profiling with SELDI-TOF MS (20). By eluting the most high abundant proteins in a few fractions, more (lower abundant) proteins should be detectable in the other fractions (24). We indeed detected many proteins that were previously suppressed by the high abundant proteins (20). However, it still did not result in the detection of discriminative low abundant proteins, so probably more rigorous fractionation or selective protein depletion is needed for the detection of lower abundant cancer type specific markers.

On the other hand, Villanueva et al. proposed that differences in concentrations of high abundant proteins and their break-down products are very valuable since they reflect protease activity which is supposed to be cancer type specific (32). Serum peptide signatures of high abundant proteins were found to be significantly higher or lower in specific types of cancer compared to controls. These include: complement C3f, complement C4a, fibrinogen α-chain, fibrinopeptide A (FPA), ITIH4, apolipoprotein A-I, apolipoprotein A-IV, apolipoprotein E, clusterin, bradykinin, high molecular weight (HMW) -kininogen, coagulation factor XIII and transthyretin. These proteins were not just found to be indicators of nonspecific inflammation conditions, such as arthritis or infection, in addition to cancer, but they were specific enough to distinguish different types of cancer from each other and from controls without cancer. Different peptides derived from the same parent protein were found to be higher and lower in one type of cancer, and thus not only the result of higher or lower concentrations of the parent protein (32). This would also explain why different fragments of the same protein, for example ITIH4, can be

found to be related to breast cancer, as shown in **Table 2**. The question remains whether this process of degradation also can explain that opposite relations are found for one specific fragment or protein within one type of cancer in different studies. For example the 4301, 3274 and 2725 Da ITIH4 fragments were found to be higher (25,31) and lower (21,23,26,27) in breast cancer compared to controls in different studies (**Table 2**). It may be that the activity of exoproteases is influenced by stage of disease or sample handling. However, it is unclear whether this can lead to significant relations that are completely opposite. And in that case, the value of such a marker is questionable.

Heterogeneity of breast cancer

An important factor that should be taken into account in biomarker discovery studies is the heterogeneity of breast cancer (34,35). Based on gene expression patterns, six molecular subtypes can be distinguished; luminal A, luminal B, luminal C, normal breast-like, ERBB2+ and basal-like (48,49). The different subtypes distinguish themselves by amongst others the expression of the estrogen receptor (ER) α gene and HER/2neu, and TP53 mutations (48,50). The expression of the different genes in the several subtypes leads to differences in their behavior and are associated with differences in prognosis (50). These differences are most likely due to the different pathways that are activated in the different subtypes. Consequently, it is very likely that a panel of proteins is needed to distinguish patients with these different tumors from controls, and that single biomarkers simply cannot be sensitive enough for all these subtypes. Therefore, it will be important to include information on or perform analyses on specific subtypes in future studies. This may lead to the discovery of proteins that are sensitive for certain subtypes of the disease. In combination, these proteins have the potential to be of major aid in the diagnosis of breast cancer.

Furthermore, it is of major importance to investigate the ability of biomarkers to detect breast cancer at stages that are currently undetectable. Most studies so far included serum samples of patients with symptomatic breast cancer. Biomarkers for these stages may not be sensitive for the early stages of the disease. A blood-based biomarker test for diagnosis of breast cancer would only have added

value when it is already able to detect breast cancer in stages that are currently undetectable by mammography. In one of our studies we used serum samples that were collected up to 3 years before the diagnosis of breast cancer (50). For this nested case-control study we included participants from the Prospect-EPIC cohort, from whom serum samples were collected about 15 years ago, at enrollment into the cohort. Our study showed the possibility to detect differences in protein profiles before the lesion caused symptoms. The study also showed the possibility to measure protein profiles in serum samples that were stored at liquid nitrogen for a period longer than 10 years (50). An additional benefit of this study design is the exclusion of potential bias introduced by differential sample collection, handling and storage between cases and controls; factors known to have a major impact on serum protein profiles. These results show the potential to the search for breast cancer biomarkers using a nested case-control study design, which is promising for other cohorts that have serum samples stored for several years.

Other techniques to search for breast cancer biomarkers

Isotope labeled protein quantification

The main purpose of SELDI- and MALDI-TOF MS is the discovery of discriminating proteins through protein profiling, for which it is sufficient to measure relative quantities. However, this limits the precision and the ability to compare protein quantities between studies. Techniques that are more quantitative are Isotope-Coded Affinity Tags (ICAT^{TM}) and isotope Tags for Relative and Absolute Quantification (iTRAQ^{TM}). Both techniques are based on the tagging of the proteins in the sample with stable isotopic labels. Performing ICAT, heavy and light labels (for two different samples; usually a case-control pair) are chemically bound to the cysteine residues of the proteins, while performing iTRAQ four labels of identical mass ('isobaric') are chemically bound to the amine groups of the proteins in four different samples (usually two case-control pairs). Samples are mixed after labeling and proteins are digested using trypsin. Using ICAT, labeled peptides are subsequently isolated by affinity chromatography and analyzed by liquid chromatography coupled to tandem MS (LC-MS/MS). iTRAQ samples are immediately analyzed by LC-MS/MS, which results in samples with higher complexity than

samples analyzed with ICAT. With iTRAQ, however, absolute quantification is possible by using labeled reference peptides with known quantity. A great advantage of both techniques is the direct identification of the biomarkers by tandem MS of the peptides. These techniques, however, are low throughput by measuring only two to four samples per run (51). Moreover, using these techniques, post-translational modified proteins can not be detected since they can not be identified using the human International Protein Index (IPI) database. This may lead to the missing of interesting proteins. In one of our studies we analyzed serum samples of 20 breast cancer cases and 20 healthy controls using iTRAQ and online two-dimensional nano-LC-MS/MS (2D-nanoLC-MS/MS). Using this technique we were able to detect several discriminating proteins. Afamin, apolipoprotein E and, interestingly, isoform 1 of ITIH4 were found to be higher, while alpha-2-macroglobulin and ceruloplasmin were lower (9).

Candidate-directed approaches

Several candidate-directed techniques are available to study the discriminating power of selected proteins. Candidates may be proteins that previously have been found to be related to breast cancer and need validation. Or, they may be proteins with limited sensitivity and specificity for breast cancer by their own, that are potentially valuable when used in combination.

Antibody-based detection techniques are very suitable for candidate-directed approaches as they are specific, sensitive, and, if standards exist, quantitative. Common techniques such as ELISA have the disadvantage that they require relatively large volumes of biological material per marker to be measured (25 to 100 µl). However, recently, several new multiplex techniques have become available that can measure multiple proteins in a relatively small volume in a single assay. Examples of such techniques are the multiplex bead-based immunoassays (52) and antibody-microarrays (53). Both techniques are based on the binding of proteins in serum, plasma or tissue lysate to selected antibodies. The bead-based immunoassay utilizes microparticles for the simultaneous detection of up to 100 different antigens. Microparticles conjugated with different antibodies are differently color coded and in this way spectrally distinguishable (52). Antibody-

arrays are spotted with a different antibody of interest per spot. In both techniques quantities of proteins bound to these antibodies are measured using secondary fluorescently labeled antibodies which recognize the same proteins as the antibodies on the beads or array (52,54). Using antibody-arrays, protein expression levels can also be measured directly, so without using secondary fluorescently labeled antibodies, by tagging a case- and control sample with two different fluorescent dyes (such as Cy3 and Cy5) before application to the antibody spotted array (54). Both techniques offer a powerful and rapid tool to screen for target proteins in complex samples. Using these techniques the serum proteome can be screened for a large number of proteins, in a high throughput fashion without the need for extensive sample preparation. They also enable the investigation of lower abundant proteins. Kim et al. successfully used the bead-based immunoassay to identify breast cancer serum markers (55). They constructed an antibody-bead array of 35 markers using the Luminex^{TM} bead array platform. Several proteins were found to be either significantly increased (epidermal growth factor, soluable CD40-ligand and proapolipoprotein AI) or decreased in breast cancer (high-molecular-weight kininogen, apolipoprotein AI, soluble vascular cell adhesion molecule-1, plasminogen activator inhibitor-1, vitamin-D binding protein and vitronectin). Together, proteins were able to distinguish primary non-metastatic breast cancer patients from healthy controls with high accuracy (55). We also used this technique, but applied it to pre-diagnostic serum samples of breast cancer patients and healthy controls. We included proteins that were previously found to be related with (breast) cancer, but with limited sensitivity and specificity. The panel of 10 markers was not able to detect asymptomatic breast cancer (osteopontin (OPN), haptoglobin, cancer antigen 15-3 (CA15-3), CEA, CA125, prolactin, CA19-9, α-fetoprotein (AFP), leptin and migration inhibitory factor (MIF)) (56).

A disadvantage of immunological assays is that they are limited for low molecular weight proteins, such as small cleaved fragments, and post-translational modified proteins owing to difficult antibody production (51). An LC-MS/MS assay that enables the detection of such fragments and post-translational modified proteins was recently developed (57). With this validated assay specific break-down peptides and a post-translational modified peptide could be quantitatively measured

(i.e. concentrations of bradykinin, des-Arg^9-bradykinin, Hyp^3-bradykinin, and fragments of fibrinogen α-chain (Fib-α $_{[605-629]}$), complement component 4a (C4a $_{[1337-1350]}$) and ITIH4 (ITIH4 $_{[666-687]}$)) (57). We used this assay to validate the discriminative power of these fragments in a set of 62 incident breast cancer cases and 62 healthy controls (58). We confirmed that concentrations of ITIH4 $_{[666-687]}$ and des-Arg^9-bradykinin were higher in breast cancer compared to healthy controls (58). A limitation of the technique is that the development of the assay is very time-consuming, especially to make the assay sensitive enough for the low concentrations in which these proteins appear in serum.

Autoantibody screening

A different approach to detect cancer biomarkers is through investigation of the immune response to cancer. By measuring reactivity in sera of cancer patients against tumor-associated antigens (TAAs), autoantibodies can be identified that have potential as cancer biomarkers (59). Cancer patients appear to produce autoantibodies against mutated tumor proteins and truncated, misfolded or over-expressed proteins. Immune reactions to TAAs magnify the signal of tumor presence, demanding less sensitive detection methods. Different proteomic approaches can be used to identify TAAs and their cognate autoantibodies, for example serological identification of antigens by recombinant expression cloning (SEREX) or serological proteome analysis (SERPA) (59). TAAs are generated by messenger RNAs extracted from a tumor, or directly extracted from tumors or cell cultures, by SEREX and SERPA, respectively. Sera from cancer patients and healthy controls are subsequently tested for antibodies that react against separated proteins (59). The advantages of SERPA over SEREX are its speed and the possibility to identify post-translational modifications and protein isoforms (59). Also protein-microarrays can be used to detect TAAs, which is based on the same principle as SEREX. With this technique, however, a much larger number of targets can be analyzed than with SEREX or SERPA (59). Several studies have identified autoantibodies that could represent diagnostic markers for breast cancer (60-62). One of the detected proteins is Heat Shock Protein 60 (HSP60), which was found to be higher expressed in breast cancer and also observed to increase in expression from normal to DCIS to

invasive tissues (60,63). The some of the detected autoantibodies, however, were previously also found to be related with other types of cancer (60,62). It may be that these techniques are primarily sensitive for detection of cancer specific autoantibodies, and less for cancer-type specific autoantibodies. Detected autoantibodies should be validated using for example immunohistochemistry, next to determination of their breast cancer specificity.

Future perspectives and conclusions

Many proteins have been proposed as candidate breast cancer biomarkers as a result of studies using SELDI- and MALDI-TOF MS. The majority of these proteins are high abundant acute phase reactants which are less likely to confer breast cancer specificity than lower abundant proteins. New discovery-based studies using SELDI- or MALDI TOF MS should more extensively explore the possibilities to eliminate most of the high abundant proteins to allow detection of lower abundant, probably more specific proteins. The greatest advantage of SELDI- and MALDI-TOF MS remains the ability to screen a large part of the proteome and to detect post-translational modified proteins and degradation products of large proteins. To detect these small fragments, MALDI-TOF MS is best suited due to its higher resolution in the very low mass range. Performing these studies, one should keep in mind that identification of detected proteins is of major importance, and that proteins with masses over 4,000 Da, and especially those with low intensity, are difficult to identify. For discovery of high mass breast cancer markers, LC-MS/MS in combination with ICAT or iTRAQ seems very promising, although here too, high abundant proteins should be eliminated to enable detection of lower abundant proteins. Detection of TAAs also seems to have high potential, although questions remain regarding their tumor-type specificity.

Regarding the currently proposed breast cancer biomarkers, research should focus on validation. Techniques that have the highest potential for the validation of candidate biomarkers are assays that enable the simultaneous, quantitative investigation of multiple proteins, such as the bead-based immunoassays. Simultaneous evaluation of multiple potential breast cancer biomarkers may reveal combinations of markers that are together highly sensitive for breast cancer. For

validation of candidate markers that have low mass, such as digested protein fragments, or candidate markers which are post-translational modified proteins, a specific quantitative LC-MS-MS assay as described in a study by Van den Broek et al. could be suitable (62). This test was successfully applied by us for the quantitative measurement of some of the breast cancer specific degradome markers proposed by Villanueva et al. (57). The discriminative power of two of these markers was confirmed (32). This assay can also be used to test the breast cancer specificity of these markers in samples of patients with other tumor types. For further testing of the hypothesis that the expression pattern of fragments of high abundant proteins confers cancer specificity, which lies in its formation by cancer specific exoproteases, the proposed method described by Villanueva et al. seems to have potential as well (58).

In conclusion, an extensive search has been performed for the detection of breast cancer biomarkers. Great advances in the development of techniques yielded many candidate breast cancer biomarkers. Partially due to the lack of identification, many of these proteins have not been validated, and their potential as biomarker for breast cancer is therefore still uncertain. In future discovery research, but also in validation studies, attention should be paid to identical sample handling protocols for cases and controls, to molecular subtypes and early stages of breast cancer, as well as to breast cancer specificity. Ultimately this may lead to the discovery of new and valid breast cancer specific biomarkers.

References

1. Schopper D, de WC. How effective are breast cancer screening programmes by mammography? Review of the current evidence. ***Eur. J. Cancer*** *2009;* 45(11): 1916-1923.
2. Louwman WJ, Voogd AC, van Dijck JA, Nieuwenhuijzen GA, Ribot J, Pruijt JF et al. On the rising trends of incidence and prognosis for breast cancer patients diagnosed 1975-2004: a long-term population-based study in southeastern Netherlands. ***Cancer Causes Control*** *2008;* 19(1): 97-106.
3. Boyd NF, Guo H, Martin LJ, Sun L, Stone J, Fishell E et al. Mammographic density and the risk and detection of breast cancer. ***N. Engl. J. Med.*** *2007;* 356(3): 227-236.
4. Mandelson MT, Oestreicher N, Porter PL, White D, Finder CA, Taplin SH et al. Breast density as a predictor of mammographic detection: comparison of interval- and screen-detected cancers. ***J. Natl. Cancer Inst.*** *2000;* 92(13): 1081-1087.
5. Kolb TM, Lichy J, Newhouse JH. Comparison of the performance of screening mammography, physical examination, and breast US and evaluation of factors that influence them: an analysis of 27,825 patient evaluations. ***Radiology*** *2002;* 225(1): 165-175.
6. National evaluation of breast cancer screening in the Netherlands 1990-2007 Twelfth evaluation report *2010.*
7. Merchant M, Weinberger SR. Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. ***Electrophoresis*** *2000;* 21(6): 1164-1177.
8. Gast MC, Schellens JH, Beijnen JH. Clinical proteomics in breast cancer: a review. ***Breast Cancer Res. Treat.*** *2009;* 116(1): 17-29.
9. van Winden AW, Krop EJM, Kåredal MH, Gast MC, Lindh CH, Jeppson MC et al. Searching for early breast cancer biomarkers by serum protein profiling of pre-diagnostic serum. *Submitted for publication.*
10. Becker S, Cazares LH, Watson P, Lynch H, Semmes OJ, Drake RR et al. Surfaced-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) differentiation of serum protein profiles of BRCA-1 and sporadic breast cancer. ***Ann. Surg. Oncol.*** *2004;* 11(10): 907-914.
11. Belluco C, Petricoin EF, Mammano E, Facchiano F, Ross-Rucker S, Nitti D et al. Serum proteomic analysis identifies a highly sensitive and specific discriminatory pattern in stage 1 breast cancer. ***Ann. Surg. Oncol.*** *2007;* 14(9): 2470-2476.
12. Hu Y, Zhang S, Yu J, Liu J, Zheng S. SELDI-TOF-MS: the proteomics and bioinformatics approaches in the diagnosis of breast cancer. ***Breast.*** *2005;* 14(4): 250-255.
13. Callesen AK, Vach W, Jorgensen PE, Cold S, Tan Q, Depont CR et al. Combined experimental and statistical strategy for mass spectrometry based serum protein profiling for diagnosis of breast cancer: a case-control study. ***J. Proteome. Res.*** *2008;* 7(4): 1419-1426.
14. de Noo ME, Deelder A, van der WM, Ozalp A, Mertens B, Tollenaar R. MALDI-TOF serum protein profiling for the detection of breast cancer. ***Onkologie.*** *2006;* 29(11): 501-506.

15. Laronga C, Becker S, Watson P, Gregory B, Cazares L, Lynch H et al. SELDI-TOF serum profiling for prognostic and diagnostic classification of breast cancers. ***Dis. Markers*** *2003;* 19(4-5): 229-238.
16. Lebrecht A, Boehm D, Schmidt M, Koelbl H, Grus FH. Surface-enhanced Laser Desorption/Ionisation Time-of-flight Mass Spectrometry to Detect Breast Cancer Markers in Tears and Serum. ***Cancer Genomics Proteomics.*** *2009;* 6(2): 75-83.
17. Pusztai L, Gregory BW, Baggerly KA, Peng B, Koomen J, Kuerer HM et al. Pharmacoproteomic analysis of prechemotherapy and postchemotherapy plasma samples from patients receiving neoadjuvant or adjuvant chemotherapy for breast carcinoma. ***Cancer*** *2004;* 100(9): 1814-1822.
18. Shin S, Cazares L, Schneider H, Mitchell S, Laronga C, Semmes OJ et al. Serum biomarkers to differentiate benign and malignant mammographic lesions. ***J. Am. Coll. Surg.*** *2007;* 204(5): 1065-1071.
19. Vlahou A, Laronga C, Wilson L, Gregory B, Fournier K, McGaughey D et al. A novel approach toward development of a rapid blood test for breast cancer. ***Clin. Breast Cancer.*** *2003;* 4(3): 203-209.
20. van Winden AW, Beijnen JH, Loof A, van Heerde WL, Vermeulen R, Peeters PH et al. Search for breast cancer biomarkers in fractionated serum samples by protein profiling with SELDI-TOF MS. *Submitted for publication.*
21. Gast MC, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ et al. Serum protein profiling for diagnosis of breast cancer using SELDI-TOF MS. ***Oncol. Rep.*** *2009;* 22(1): 205-213.
22. Solassol J, Rouanet P, Lamy PJ, Allal C, Favre G, Maudelonde T et al. Serum protein signature may improve detection of ductal carcinoma in situ of the breast. ***Oncogene*** *2010;* 29(4): 550-60.
23. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. ***Clin. Chem.*** *2002;* 48(8): 1296-1304.
24. Solassol J, Marin P, Demettre E, Rouanet P, Bockaert J, Maudelonde T et al. Proteomic detection of prostate-specific antigen using a serum fractionation procedure: potential implication for new low-abundance cancer biomarkers detection. ***Anal. Biochem.*** *2005;* 338(1): 26-31.
25. Li J, Orlandi R, White CN, Rosenzweig J, Zhao J, Seregni E et al. Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. ***Clin. Chem.*** *2005;* 51(12): 2229-2235.
26. Mathelin C, Cromer A, Wendling C, Tomasetto C, Rio MC. Serum biomarkers for detection of breast cancers: a prospective study. ***Breast Cancer Res. Treat.*** *2006;* 96(1): 83-90.
27. van Winden AW, Gast MC, Beijnen JH, Rutgers EJ, Grobbee DE, Peeters PH et al. Validation of previously identified serum biomarkers for breast cancer with SELDI-TOF MS: a case control study. ***BMC. Med. Genomics*** *2009;* 2: 4.

28. Habermann JK, Roblick UJ, Luke BT, Prieto DA, Finlay WJ, Podust VN et al. Increased serum levels of complement C3a anaphylatoxin indicate the presence of colorectal tumors. ***Gastroenterology***. *2006;* 131(4): 1020-1029.
29. Ward DG, Suggett N, Cheng Y, Wei W, Johnson H, Billingham LJ et al. Identification of serum biomarkers for colon cancer by proteomic analysis. ***Br. J. Cancer***. *2006;* %19;94(12): 1898-1905.
30. Miguet L, Bogumil R, Decloquement P, Herbrecht R, Potier N, Mauvieux L et al. Discovery and identification of potential biomarkers in a prospective study of chronic lymphoid malignancies using SELDI-TOF-MS. ***J. Proteome. Res***. *2006;* 5(9): 2258-2269.
31. Song J, Patel M, Rosenzweig CN, Chan-Li Y, Sokoll LJ, Fung ET et al. Quantification of fragments of human serum inter-alpha-trypsin inhibitor heavy chain 4 by a surface-enhanced laser desorption/ionization-based immunoassay. ***Clin. Chem***. *2006;* 52(6): 1045-1053.
32. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. ***J. Clin. Invest***. *2006;* 116(1): 271-284.
33. Fung ET, Yip TT, Lomas L, Wang Z, Yip C, Meng XY et al. Classification of cancer types by measuring variants of host response proteins using SELDI serum assays. ***Int. J. Cancer***. *2005;* 115(5): 783-789.
34. Villanueva J, Nazarian A, Lawlor K, Yi SS, Robbins RJ, Tempst P. A sequence-specific exopeptidase activity test (SSEAT) for "functional" biomarker discovery. ***Mol. Cell Proteomics***. *2008;* 7(3): 509-518.
35. Villanueva J, Nazarian A, Lawlor K, Tempst P. Monitoring peptidase activities in complex proteomes by MALDI-TOF mass spectrometry. ***Nat. Protoc***. *2009;* 4(8): 1167-1183.
36. Banks RE, Stanley AJ, Cairns DA, Barrett JH, Clarke P, Thompson D et al. Influences of blood sample processing on low-molecular-weight proteome identified by surface-enhanced laser desorption/ionization mass spectrometry. ***Clin. Chem***. *2005;* 51(9): 1637-1649.
37. Engwegen JY, Alberts M, Knol JC, Jimenez CR, Depla AC, Tuynman H et al. Influence of variations in sample handling on SELDI-TOF MS serum protein profiles for colorectal cancer. ***Proteomics Clin. Appl***. *2008;* 2: 936-945.
38. Gast MC, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ et al. Influence of sample storage duration on serum protein profiles assessed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS). ***Clin. Chem. Lab Med***. *2009;* 47(6): 694-705.
39. Hsieh SY, Chen RK, Pan YH, Lee HL. Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. ***Proteomics***. *2006;* 6(10): 3189-3198.
40. Karsan A, Eigl BJ, Flibotte S, Gelmon K, Switzer P, Hassell P et al. Analytical and preanalytical biases in serum proteomic pattern analysis for breast cancer diagnosis. ***Clin. Chem***. *2005;* 51(8): 1525-1528.

41. Timms JF, Arslan-Low E, Gentry-Maharaj A, Luo Z, T'Jampens D, Podust VN et al. Preanalytic influence of sample handling on SELDI-TOF serum protein profiles. ***Clin. Chem.*** *2007;* 53(4): 645-656.
42. Ransohoff DF. Bias as a threat to the validity of cancer molecular-marker research. ***Nat. Rev. Cancer*** *2005;* 5(2): 142-149.
43. Diamandis EP. Mass spectrometry as a diagnostic and a cancer biomarker discovery tool: opportunities and potential limitations. ***Mol. Cell Proteomics.*** *2004;* 3(4): 367-378.
44. Gast MC, van Dulken EJ, van Loenen TK, Kingma-Vegter F, Westerga J, Flohil CC et al. Detection of breast cancer by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry tissue and serum protein profiling. ***Int. J. Biol. Markers*** *2009;* 24(3): 130-141.
45. Engwegen JY, Helgason HH, Cats A, Harris N, Bonfrer JM, Schellens JH et al. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. ***World J. Gastroenterol.*** *2006;* 12(10): 1536-1544.
46. Engwegen JY, Mehra N, Haanen JB, Bonfrer JM, Schellens JH, Voest EE et al. Validation of SELDI-TOF MS serum protein profiles for renal cell carcinoma in new populations. ***Lab Invest*** *2007;* 87(2): 161-172.
47. Hoffman SA, Joo WA, Echan LA, Speicher DW. Higher dimensional (Hi-D) separation strategies dramatically improve the potential for cancer biomarker detection in serum and plasma. ***J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*** *2007;* 849(1-2): 43-52.
48. Perou CM, Sorlie T, Eisen MB, van de RM, Jeffrey SS, Rees CA et al. Molecular portraits of human breast tumours. ***Nature*** *2000;* 406(6797): 747-752.
49. Weigelt B, Baehner FL, Reis-Filho JS. The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: a retrospective of the last decade. ***J. Pathol.*** *2009;* 220(2): 263-80.
50. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. ***Proc. Natl. Acad. Sci. U. S. A*** *2001;* 98(19): 10869-10874.
51. Engwegen JY, Gast MC, Schellens JH, Beijnen JH. Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. ***Trends Pharmacol. Sci.*** *2006;* 27(5): 251-259.
52. Schwenk JM, Gry M, Rimini R, Uhlen M, Nilsson P. Antibody suspension bead arrays within serum proteomics. ***J. Proteome. Res.*** *2008;* 7(8): 3168-3179.
53. Anderson JE, Hansen LL, Mooren FC, Post M, Hug H, Zuse A et al. Methods and biomarkers for the diagnosis and prognosis of cancer and other diseases: towards personalized medicine. ***Drug Resist. Updat.*** *2006;* 9(4-5): 198-210.
54. Kopf E, Zharhary D. Antibody arrays--an emerging tool in cancer proteomics. ***Int. J. Biochem. Cell Biol.*** *2007;* 39(7-8): 1305-1317.

55. Kim BK, Lee JW, Park PJ, Shin YS, Lee WY, Lee KA et al. The multiplex bead array approach to identifying serum biomarkers associated with breast cancer. ***Breast Cancer Res***. *2009;* 11(2): R22.
56. van Winden AW, Rodenburg W, Pennings JL, Oostrom CT, Beijnen JH, Peeters PH et al. Bead-based multiplexed immunoassay to identify early breast cancer biomarkers in pre-diagnostic serum; a nested case-control study. *Submitted for publication*.
57. van den Broek I, Sparidans R.W., Schellens J.H.M., Beijnen JH. Quantitative assay for six potential breast cancer biomarker peptides in human serum by liquid chromatography coupled to tandem mass spectrometry. ***J. Chromatogr. B Analyt. Technol. Biomed. Life Sci***. *2010;* 878(5-6): 590-602.
58. van Winden AW, van den Broek I, Gast MC, Engwegen JY, Sparidans RW, Dulken EJ et al. Serum degradome markers for the detection of breast cancer. *Submitted for publication*.
59. Desmetz C, Cortijo C, Mange A, Solassol J. Humoral response to cancer as a tool for biomarker discovery. ***J. Proteomics***. *2009;* 72(6): 982-988.
60. Hamrita B, Chahed K, Kabbage M, Guillier CL, Trimeche M, Chaieb A et al. Identification of tumor antigens that elicit a humoral immune response in breast cancer patients' sera by serological proteome analysis (SERPA). ***Clin. Chim. Acta*** *2008;* 393(2): 95-102.
61. Desmetz C, Bascoul-Mollevi C, Rochaix P, Lamy PJ, Kramar A, Rouanet P et al. Identification of a new panel of serum autoantibodies associated with the presence of in situ carcinoma of the breast in younger women. ***Clin. Cancer Res***. *2009;* 15(14): 4733-4741.
62. Tamesa MS, Kuramitsu Y, Fujimoto M, Maeda N, Nagashima Y, Tanaka T et al. Detection of autoantibodies against cyclophilin A and triosephosphate isomerase in sera from breast cancer patients by proteomic analysis. ***Electrophoresis*** *2009;* 30(12): 2168-2181.
63. Desmetz C, Bibeau F, Boissiere F, Bellet V, Rouanet P, Maudelonde T et al. Proteomics-based identification of HSP60 as a tumor-associated antigen in early stage breast cancer and ductal carcinoma in situ. ***J. Proteome. Res***. *2008;* 7(9): 3830-3837.
64. Shi Q, Harris LN, Lu X, Li X, Hwang J, Gentleman R et al. Declining plasma fibrinogen alpha fragment identifies HER2-positive breast cancer patients and reverts to normal levels after surgery. ***J. Proteome. Res***. *2006;* 5(11): 2947-2955.

Chapter 8

Breast cancer mortality rates in The Netherlands are among the highest in Europe. To improve breast cancer survival, early detection is of vital importance. The national breast cancer screening program in The Netherlands already has led to an positive change in stage at diagnosis of breast cancer. However, among screened women, 1 out of 3 breast cancers occur in between two screening examinations. Moreover, a quarter of all breast cancers occur in women aged 49 years or younger, and these women are currently not included in the national breast cancer screening program. Younger women have breasts with higher density compared to older women, which decreases the sensitivity of mammography to detect small breast lesions. Both aforementioned groups of women would therefore benefit from early detection techniques other than mammography. One such method with high potential is the use of blood-based protein markers. These biomarkers could originate from the tumor or the tumorigenesis process, but could also be generated as a response to the presence of the tumor. Even a-symptomatic breast lesions that are currently undetectable by mammographic screening may be detectable using such biomarkers.

There are two approaches in the search for new protein biomarkers for cancer. The first method is referred to as the discovery-oriented approach. Using this approach serum protein profiles of cancer cases and controls are generated and compared. These protein profiles visualize a large part of the proteins in a serum sample and these proteins' relative quantity. To find potential cancer markers, protein profiles of cancer cases and controls are compared and investigated for discriminating proteins. Using this technique many proteins are measured and there is no prior hypothesis. The second approach is the candidate-based method. Contrary to the first method, this methods is based on an a-priori hypothesis about de potential of proteins as breast cancer biomarkers. Prior the the study one or more candidate markers are selected, which are subsequently measured in breast cancer samples and samples of controls, for example using specific antibodies that bind the proteins of interest.

The objective of this thesis was to detect biomarkers that indicate the presence of breast cancer in an early stage. In the first part of this thesis we applied the discovery-oriented approach for detection of breast cancer biomarkers. In

Chapter 2 we used surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to profile serum samples of patients with symptomatic and/or mammographically detected breast cancer and of healthy controls. In this study we applied a protocol similar to that used in a previous study enabling detection, and possibly validation, of three previously reported discriminative proteins. Two of the three proteins, a 4.3 kDa fragment of inter-alpha trypsin inhibitor heavy chain H4 (ITIH4) and C3a des-arginine anaphylatoxin (8.9 kDa), also showed in our study statistically significantly different intensities between breast cancer cases and controls. Intensities of both proteins were lower in cases compared to controls. Remarkably, C3a des-arginine anaphylatoxin was statistically significantly higher in breast cancer in previous study. The third protein, C-terminal-truncated form of C3a des-arginine anaphylatoxin (8.1 kDa) was not related with breast cancer in our study. Differences in patient populations and pre-analytical sample handling could have contributed to discrepancies.

In **Chapter 3** we again investigated protein profiles using SELDI-TOF MS in a second set of incident primary breast cancer patients. In this study we performed serum fractionation by anion exchange chromatography before protein profiling, which would facilitate better detection of lower abundant proteins. Previous studies have yielded many potential biomarkers that are high abundant acute phase proteins, which are unlikely to be breast cancer specific. Cancer specific markers are thought to be primarily present among lower abundant proteins. As expected, we detected many more proteins than in previous studies without fractionation. Eight proteins were found to be discriminating between cases and controls after correction for multiple testing. However, these proteins were still high abundant proteins. Failure of detection of discriminative lower abundant proteins means that either lower abundant proteins are less distinctive than high abundant proteins, or fractionation as performed in this study was not sufficient to detect the least abundant, possibly highly discriminative proteins.

It is unclear whether markers detected in symptomatic and/or mammographically detected breast cancer patients can be used for the detection of breast cancer in very *early* stages of the disease. In **Chapter 4** we therefore investigated pre-diagnostic serum samples of women who were diagnosed with

breast cancer a month up to three years after the collection of the samples. For this study we included women enrolled into the Prospect-EPIC (European Prospective Investigation in Cancer and nutrition) cohort. An additional advantage of this nested case-control design is that samples of cases and controls were collected, processed and stored in the exact same way under strictly defined conditions at a time when none of the participants was diagnosed with breast cancer yet. Proteins profiles were investigated using SELDI-TOF MS, as well as two-dimensional nano-liquid chromatography tandem MS (2D-nanoLC-MS/MS). These two methods are complementary to each other in the mass range of proteins they detect. Proteins detected with 2D-nano-C-MS/MS can be immediately identified. Using SELDI-TOF MS, two proteins identified as doubly charged apolipoprotein C-I and C3a des-arginine anaphlyatoxin were found to be statistically significantly higher in pre-diagnostic breast cancer serum samples. With 2D-nanoLC-MS/MS, afamin, apolipoprotein E and isoform 1 of ITIH4 were found to be higher in pre-diagnostic breast cancer, while alpha-2-macrogobulin and ceruloplasmin were lower. Two of these discriminating proteins, C3a des-arginine anaphlyatoxin and ITIH4, have previously been related to symptomatic and/or mammographically detectable breast cancer. These proteins, however, are high abundant proteins which are unlikely to be breast cancer specific, at least on their own. On the other hand, the detection of discriminative serum protein profiles up to three years before the diagnosis of breast cancer in these samples that were stored for more than 10 years in liquid nitrogen is very promising.

In the second part of this thesis we use candidate-based approaches to evaluate the discriminative value of several candidate biomarkers. In **Chapter 5** we investigated six potential breast cancer biomarkers: bradykinin, des-Arg^{9}-bradykinin, Hyp^{3}-bradykinin, and fragments of fibrinogen α-chain (Fib-α $_{[605-629]}$), complement component 4a (C4a $_{[1337-1350]}$) and inter-alpha trypsin inhibitor heavy chain H4 (ITIH4 $_{[666-687]}$). These peptides were previously proposed to be generated by breast cancer specific exoproteases. Peptide concentrations were measured in symptomatic and/or mammographically detected breast cancer patients and matched controls using a specifically developed and validated LC-MS/MS assay. Our results confirmed the higher concentrations of two of the exoprotease breakdown peptides, ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin in breast cancer cases compared to controls. In this

study we could also analyze serum samples of the same patients collected after surgical removal of the tumor. Comparison of peptide concentrations revealed that levels of ITIH4 $_{[666-687]}$ and des-Arg^{9}-bradykinin significantly decreased in the patients after removal of the tumor and concentrations were not different from concentrations in the controls anymore. These results indicate the potential of degradome markers for the diagnosis of breast cancer.

In **Chapter 6** we investigated the diagnostic capacity of a panel of ten candidate markers in asymptomatic stages of breast cancer. For this study we investigated the same subjects as included in the study described in **Chapter 4**. The ten markers, osteopontin (OPN), haptoglobin, cancer antigen 15-3 (CA15-3), carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), prolactin, cancer antigen 19-9 (CA19-9), α-fetoprotein (AFP), leptin and migration inhibitory factor (MIF), were measured using a quantitative bead-based multiplexed assay, which is based on binding to antibodies specific for these proteins. Investigation of the discriminatory power of this panel of candidate markers revealed that pre-diagnostic cancer cases could not be separated from the controls based on concentrations of this combination of markers. Restriction of the analysis to the cases diagnosed very shortly after enrollment and their matched controls did not change results. Selected markers may be valuable for breast cancer diagnosis in combination with other markers not selected here, but not in described combination.

Finally, in **Chapter 7** we review the many studies searching for blood-based proteins related to the presence of breast cancer using SELDI-TOF MS or its variant matrix assisted laser desorption/ionization (MALDI)-TOF MS and describe what these studies have yielded. We highlight two proteins that frequently and most consistently were reported in relation to breast cancer: C3a des-arginine anaphylatoxin and fragments of ITIH4. However, these proteins are not necessarily the most promising breast cancer biomarkers. Previously described limitations of SELDI- and MALDI-TOF MS for the discovery of cancer biomarkers are the vulnerability of the technique for way of sample collection, sample handling and sample storage and consequently the difficult reproducibility. However, the lack of identification of discriminating proteins also caused that few potential markers

could be validated and their breast cancer specificity could be determined. A major advantage of SELDI- and MALDI-TOF MS remains the ability to screen a large part of the proteome and to detect post-translational modified proteins and degradation products of large proteins. The candidate-directed approach is very suitable for the simultaneous evaluation of multiple potential markers that together may be highly sensitive for breast cancer. In future biomarker research attention should be particularly paid to identification of discriminating proteins, to molecular breast cancer subtypes and early stages of disease, as well as to breast cancer specificity.

Chapter 8

De sterfte als gevolg van borstkanker is in Nederland relatief hoog in vergelijking met andere Europese landen. Een van de belangrijkste manieren om de overlevingskansen van borstkankerpatiënten te verbeteren is het opsporen van de tumor in een vroeg stadium. De invoering van het nationale borstkanker bevolkingsonderzoek in Nederland heeft er al voor gezorgd dat borstkanker vaker in een vroeger stadium wordt ontdekt dan voorheen. Echter, bij 1 op de 3 vrouwen die meedoen aan het bevolkingsonderzoek openbaart de ziekte zich tussen twee screeningsmomenten in. Daarnaast ontstaat een kwart van de borsttumoren bij vrouwen onder de 49 jaar. Deze vrouwen worden momenteel niet uitgenodigd voor het bevolkingsonderzoek omdat de gevoeligheid van mammografie voor het opsporen van kleine tumoren is bij jongere vrouwen lager. Dit komt doordat jongere vrouwen een hogere mammografische borstdichtheid hebben in vergelijking met oudere vrouwen; ze hebben relatief meer klier- en bindweefsel dan vetweefsel in hun borsten. Beide hiervoor genoemde groepen vrouwen zouden dan ook baat hebben bij andere methoden dan mammografie waarmee borstkanker in een vroeg stadium opgespoord kan worden.

Een methode die hiervoor veel potentie lijkt te hebben, is het gebruik van eiwitten in het bloed die de aanwezigheid van een tumor in een vroeg stadium aan kunnen geven. Deze eiwitten kunnen afkomstig zijn van de tumor of het proces van tumorvorming, maar kunnen ook gevormd zijn als reactie op de aanwezigheid van een tumor. Met behulp van deze markers zouden zelfs tumoren die nog niet tot symptomen leiden en die nog niet zichtbaar zijn op een mammogram, gedetecteerd kunnen worden.

Er zijn twee verschillende aanpakken voor de zoektocht naar nieuwe eiwitmarkers voor kanker. De eerste methode is gebaseerd op het ontdekken van onderscheidende eiwitten binnen een grote hoeveelheid eiwitten die in één keer gemeten wordt. Bij deze methode bepaal je vooraf niet welke eiwitten je gaat meten, maar meet je de eiwitten die je ‘tegenkomt’. Per persoon wordt een eiwitprofiel gemaakt welke een groot deel van de eiwitten in het bloedmonster visualiseert en hun relatieve hoeveelheid aangeeft. Eiwitprofielen van borstkankerpatiënten en gezonde vrijwilligers worden vervolgens vergeleken om te onderzoeken welke eiwitten een verschil in concentratie tussen de twee groepen

vertonen en dus potentiële borstkankermarkers zijn. De tweede methode is de kandidaat-gerichte aanpak. In tegenstelling to de eerste methode is deze methode gebaseerd op een vooraf gestelde hypothese over de potentie van een eiwit als borstkankermarker. Voorafgaand aan het onderzoek worden één of meerdere kandidaat-markers gekozen die vervolgens, bijvoorbeeld met behulp van antilichamen die specifiek binden met de geselecteerde eiwitten, gemeten worden in bloedmonsters van patiënten en controlepersonen.

Het doel van het onderzoek beschreven in die proefschrift was het detecteren van bloedmarkers die de aanwezigheid van een borsttumor in een vroeg stadium aan kunnen geven. In het eerste gedeelte van dit proefschrift hebben we gezocht naar borstkankermarkers met behulp van eiwitprofielen, de eerste hierboven beschreven methode. Zo hebben we in **hoofdstuk 2** gebruik gemaakt van "surface enhanced laser desorption/ionization time-of-flight mass spectrometry" (SELDI-TOF MS) voor het maken van eiwitprofielen van bloedmonsters van vrouwen die symptomen van borstkanker vertoonden en/of bij wie een tumor was ontdekt door middel van mammografie. De analyse van de eiwitprofielen voor deze studie hebben we op exact dezelfde manier uitgevoerd als in een eerdere studie waarin drie potentiële borstkankermarkers waren gevonden. Op deze manier beoogden we deze eiwitten ook te detecteren, en mogelijk hun onderscheidende vermogen te kunnen bevestigen. Twee van de drie eiwitten, een fragment van "inter-alpha trypsin inhibitor heavy chain H4" (ITIH4) (moleculaire massa: 4.3 kDa) en C3a des-arginine anafylatoxine (moleculaire massa: 8.9 kDa) vertoonden ook in onze studie statistisch significant verschillende intensiteiten tussen borstkankerpatiënten en gezonde controlepersonen. Beide eiwitten waren in hogere intensiteiten aanwezig bij de patiënten dan bij de gezonde vrouwen. Opvallend genoeg was het eiwit C3a des-arginine anafylatoxine in de eerdere studie juist verlaagd in borstkankerpatiënten. Het derde eiwit, een verkorte vorm van C3a des-arginine anafylatoxine (moeculaire massa: 8.1 kDa) was in onze studie niet gerelateerd aan de aanwezigheid van borstkanker. Verschillen in de resultaten kunnen veroorzaakt zijn door verschillen tussen de onderzochte patiëntpopulaties of door verschillen in de behandeling van de bloedmonsters voorafgaand aan de analyse.

In **hoofdstuk 3** hebben we opnieuw eiwitprofielen onderzocht met behulp van SELDI-TOF MS in een tweede groep vrouwen die net gediagnosticeerd waren met borstkanker. In deze studie hebben we echter eerst de bloedmonsters gefractioneerd middels "anion exchange chromatografie", wat de detectie van eiwitten aanwezig in lagere concentraties zou faciliteren. In vorige studies zijn namelijk veel potentiële eiwitmarkers gevonden welke veelal een hoge concentratie hadden en betrokken zijn bij acute fase reacties die waarschijnlijk niet specifiek zijn voor borstkanker. Het is waarschijnlijker dat de borstkankerspecifieke eiwitten zich bevinden onder de eiwitten met lagere concentraties. Zoals verwacht detecteerden we in deze studie veel meer eiwitten dan in eerdere studies waarbij we geen fractionering toepasten. Acht eiwitten bleken onderscheidend te zijn tussen patiënten en controlepersonen na correctie voor meervoudig testen. Dit waren echter nog steeds hoog abundante eiwitten. Dat we geen onderscheidende eiwitten hebben gevonden met lagere concentraties kan betekenen dat de laag abundante eiwitten niet zo onderscheidend zijn als de hoog abundante eiwitten, of dat fractionering zoals wij die uitgevoerd hebben niet voldoende was om eiwitten met lagere, en mogelijk zeer onderscheidende concentratie, te detecteren.

Onderscheidende eiwitten die gevonden zijn in onderzoeken met patiënten met klinische symptomen van borstkanker en/of met een duidelijke afwijking op de mammogram zijn niet zonder meer geschikt voor de detectie van borstkanker in een zeer vroeg stadium. In **hoofdstuk 4** hebben we daarom bloedmonsters onderzocht welke *voor* diagnose van de ziekte zijn afgenomen. Voor deze studie hebben we gebruik gemaakt van het Prospect-EPIC ("European Prospective Investigation into Cancer and nutrition") cohort waarin ongeveer 15 jaar geleden meer dan 17.000 gezonde vrouwen zijn geïncludeerd die een bloedmonster hebben gedoneerd en uitgebreide vragenlijsten hebben ingevuld over o.a. hun leefstijl. Uit deze groep hebben we de vrouwen geselecteerd die binnen drie jaar na inclusie zijn gediagnosticeerd met borstkanker en vrouwen van gelijke leeftijd die gezond zijn gebleven. Een extra voordeel van deze onderzoeksopzet (het geneste case-controle design) is dat de monsters van de cases en controles op precies dezelfde en strikte manier zijn verzameld, verwerkt en opgeslagen, op een moment dat nog geen van de deelnemers was gediagnosticeerd met borstkanker. Eiwitprofielen van

bloedmonsters van deze vrouwen zijn zowel gegenereerd met SELDI-TOF MS als met "two-dimensional nano-liquid chromatography tandem MS" (2D-nanoLC-MS/MS). Deze twee methoden meten eiwitten in een verschillend massagebied en zijn daarmee complementair aan elkaar. Eiwitten die gemeten worden met 2D-nanoLC-MS/MS kunnen daarnaast meteen geïdentificeerd worden. Met behulp van SELDI-TOF MS hebben we twee eiwitten gevonden (later geïdentificeerd als dubbel geladen apolipoproteïne C-I en C3a des-arginine anafylatoxine) welke een hogere intensiteit vertoonden in prediagnostische borstkankerbloedmonsters dan in controlebloedmonsters. Met behulp van 2D-nanoLC-MS/MS vonden we dat afamin, apolipoproteïne E en isoform 1 van ITIH4 ook hogere concentraties hadden in prediagnostische borstkankerbloedmonsters, terwijl alpha-2-macrogobuline en ceruloplasmine lagere concentraties vertoonden. Twee van deze discriminerende eiwitten, C3a des-arginine anafylatoxine en ITIH4, zijn eerder ook gevonden in relatie met symptomatisch en/of mammografisch detecteerbaar borstkanker. Het zijn echter twee hoog abundante, acute fase eiwitten welke waarschijnlijk niet specifiek zijn voor borstkanker, in ieder geval niet in hun eentje. Het is daarentegen veelbelovend dat het mogelijk bleek onderscheidende eiwitprofielen te detecteren in bloedmonsters afgenomen tot drie jaar voor de diagnose van borstkanker, en welke meer dan 10 jaar waren opgeslagen in vloeibare stikstof.

In het tweede deel van dit proefschrift hebben we de kandidaat-gerichte aanpak toegepast om het onderscheidende vermogen van diverse kandidaat-borstkankermarkers te onderzoeken. In **hoofdstuk 5** onderzochten we zes potentiële borstkankermarkers: bradykinine, des-Arg^{9}-bradykinine, Hyp^{3}-bradykinine, en fragmenten van fibrinogeen α-keten (Fib-α $_{[605-629]}$), complement component 4a (C4a $_{[1337-1350]}$) en ITIH4 (ITIH4 $_{[666-687]}$). Het is eerder verondersteld dat deze peptiden gegenereerd worden door borstkankerspecifieke exoproteasen. Wij hebben de concentraties van deze peptiden gemeten bij vrouwen met symptomatisch en/of mammografisch detecteerbaar borstkanker en controlepersonen met dezelfde leeftijd, met behulp van een gevalideerde LC-MS/MS analyse welke specifiek voor het meten van deze peptiden ontwikkeld is. Onze resultaten bevestigden de hogere concentraties van twee van de peptiden (ITIH4 $_{[666-687]}$ en des-Arg^{9}-bradykinine) in borstkankerpatiënten vergeleken met controlepersonen. In deze studie hebben we

ook bloedmonsters van dezelfde patiënten geanalyseerd welke na chirurgische verwijdering van de tumor zijn afgenomen. Vergelijking met deze bloedmonsters toonde aan dat de concentraties van ITIH4 $_{[666-687]}$ en des-Arg^{9}-bradykinine significant omlaag gingen in de patiënten na verwijdering van de tumor, en dat de concentraties na operatie niet meer verschilden van de concentraties in de bloedmonsters van de controlepersonen. Deze resultaten tonen de potentie aan van deze degradoom markers voor de diagnose van borstkanker.

In **hoofdstuk 6** onderzochten we de diagnostische capaciteit van een panel van tien kandidaat-markers voor asymptomatische stadia van borstkanker. In deze studie hebben we dezelfde bloedmonsters onderzocht als in de studie beschreven in hoofdstuk 4. De tien markers; osteopontine (OPN), haptoglobine, kanker antigeen 15-3 (CA15-3), carcino-embryonisch antigeen (CEA), kanker antigeen 125 (CA125), prolactine, kanker antigeen 19-9 (CA19-9), α-fetoproteïne (AFP), leptine en migratie inhibitie factor (MIF), hebben we gemeten met een kwantitatieve "bead-based multiplexed assay", welke gebaseerd is op het gebruik van specifieke antilichamen voor de binding van de geselecteerde eiwitten. Resultaten van deze studie toonden aan dat prediagnostische cases met behulp van deze combinatie van kandidaat-markers niet onderscheiden konden worden van controlepersonen. Wanneer we ons bij de data-analyse beperkten tot de vrouwen die zeer kort na het doneren van hun bloedmonster gediagnosticeerd werden met borstkanker veranderden de resultaten niet. De geselecteerde markers kunnen nog steeds waardevol zijn voor de vroege diagnose van borstkanker in combinatie met andere makers die wij niet geselecteerd hebben, maar niet in de hier beschreven combinatie.

In **hoofdstuk 7** geven we een overzicht van de verschillende studies die tot nu toe uitgevoerd zijn, welke gezocht hebben naar borstkankermarkers met behulp van SELDI-TOF MS en zijn variant "matrix assisted laser desorption/ionization" (MALDI)-TOF MS. Daarnaast bespreken we de stand van zaken in het proteomicsonderzoek naar diagnostische markers voor borstkanker. Twee eiwitten die veelvuldig en meest consistent zijn gerapporteerd in relatie met borstkanker zijn C3a des-arginine anafylatoxine en fragmenten van ITIH4. Deze eiwitten zijn echter niet noodzakelijkerwijs de meest veelbelovende borstkankermarkers. Eerder beschreven beperkingen van het gebruik van SELDI- en MALDI-TOF MS voor het

ontdekken van borstkankermarkers zijn de gevoeligheid van de techniek voor manier van monsterverzameling, -behandeling en –opslag en daarmee de verminderde reproduceerbaarheid. Het gebrek aan identificatie van onderscheidende eiwitten heeft er echter ook voor gezorgd dat weinig potentiële markers gevalideerd konden worden, en hun borstkankerspecificiteit bepaald kon worden. Een groot voordeel van SELDI- en MALDI-TOF MS blijft echter de mogelijkheid om een groot deel van het proteome in één keer te screenen op biomarkers, en om posttranslationeel gemodificeerde eiwitten en degradatieproducten van grote eiwitten te meten. De kandidaat-gerichte aanpak is juist zeer geschikt voor simultane evaluatie van meerdere potentiële markers, die tezamen mogelijk zeer gevoelig zijn voor borstkanker. In toekomstig biomarkeronderzoek moet vooral aandacht geschonken worden aan identificatie van onderscheidende eiwitten, moleculaire borstkankersubtypen en vroege stadia van de ziekte, evenals borstkankerspecificiteit.

Chapter 8

Promoveren is als het maken van een avontuurlijke reis, zeker geen ontspannende reis, maar minstens zo leuk. Er valt zo ontzettend veel te zien, leren en ontdekken. In het begin weet je nog niet goed waar het eindpunt ligt en welke wegen te bewandelen om er te komen. Soms ga je de verkeerde kant op, maar weet je daardoor des te beter hoe verder te gaan en andere keren ontdek je op onverwachte momenten hele mooie dingen. Ik vond het heel leuk! Dit alles was echter niet mogelijk geweest zonder de bijdrage van vele personen die ik dan ook heel hartelijk wil bedanken.

Allereerst wil ik alle patiënten en gezonde vrijwilligers bedanken voor het doneren van bloedmonsters. Zonder jullie was het onderzoek beschreven in dit proefschrift niet mogelijk geweest. Ik heb heel veel bewondering voor jullie kracht en moed.

Mijn promotoren, Prof. dr. J.H. Beijnen en Prof. dr. P.H.M. Peeters, wil ik hartelijk bedanken voor de mogelijkheid om dit onderzoek te doen. Beste Jos, ik heb de vrijheid en het vertrouwen dat je mij gegeven hebt om het onderzoek naar eigen inzicht uit te voeren, zeer gewaardeerd. Jouw 'wat zijn de problemen en wat zijn de mogelijke oplossingen-aanpak' tijdens onze overleggen maakte deze zeer efficiënt en effectief. Beste Petra, met jouw open en klinische blik kon jij mij het onderzoek vanuit een heel ander perspectief laten bekijken. Dit heeft ervoor gezorgd dat ik de puntjes op de i kon zetten. Jouw reactie op mijn aankondiging van mijn promotiedatum is tekenend voor je; je enthousiasme is inspirerend. Ik vond het bovendien heel bijzonder dat ik bij je oratie aanwezig kon zijn.

Ik ben ook veel dank verschuldigd aan mijn copromotoren, Dr. C.H. van Gils en Dr. R. Vermeulen. Beste Carla, wat ontzettend fijn dat jij mijn copromotor was. Ik heb heel veel bewondering voor jou als onderzoeker, je kennis en je manier van begeleiden. Ik heb het als bijzonder prettig ervaren om dit onderzoek met jou te doen. Jouw commentaar legde altijd precies de vinger op de gevoelige plek of onduidelijke zaken in een studie. Dit heeft tot veel verbeteringen geleid. Daarnaast ben ik je heel dankbaar voor je vertrouwen en aanmoedigingen. Ik heb heel veel van je geleerd, op diverse vlakken. Dankjewel! Beste Roel, wat leuk dat ook jij mijn copromotor bent geworden. Met jou had ik nog het beste contact als je ver weg in China of Amerika was, en 'gelukkig' was je dat dan ook vaak. Zo ook in de laatste,

hectische weken voor het afronden van het manuscript. Het voordeel was dat ik jouw heldere, kritische en zeer terechte commentaar op mijn 's avonds laat toegestuurde stukken vaak de volgende ochtend alweer in mijn mailbox aan kon treffen. Ik vraag mij wel eens af waar jij *niet* iets van af weet.

Prof. dr. P.J. van Diest, Prof. dr. K.G.M. Moons, Prof. dr. J.H.M. Schellens en Prof. dr. E.E. Voest ben ik zeer erkentelijk voor het lezen en beoordelen van mijn proefschrift.

Beste Marie-Christine en Judith, wat was het fijn bij jullie op de kamer terecht te komen. Jullie hebben mij wegwijs gemaakt in de wondere wereld van de SELDI-TOF MS en het proteomicsonderzoek. Al snel begreep ik dat het een gecompliceerde wereld is met veel 'uitdagingen'. Het heeft in ieder geval heel veel interessante en leuke discussies opgeleverd! Ik ben jullie daarnaast dank verschuldigd voor het gebruik kunnen maken van de door jullie zo zorgvuldig verzamelde bloedmonsters. MC, ik wil jou vooral ook bedanken voor je hulp bij de 'fractionation', het was fascinerend!

Ik wil de artsen uit het NKI-AVL en het Slotervaartziekenhuis die hebben bijgedragen aan de inclusie van patiënten voor ons onderzoek, heel hartelijk bedanken. Emiel Rutgers, Annemieke Cats, Annekatrien Depla en Erik van Dulken veel dank hiervoor.

Jose Drijvers wil ik hartelijk bedanken voor de hulp bij het lichten van de EPIC-bloedmonsters uit de stikstoftanken op het RIVM. Het was een hele klus om alle samples bij elkaar te zoeken, maar het is gelukt! Bernard Slotboom dank ik voor het aanleveren van het databestand met de gegevens van de EPIC-deelnemers geselecteerd voor ons onderzoek, en de vele aanvullingen in gegevens die daarop gevolgd zijn.

Het leek het beste om de bloedmonsters bij een zo laag mogelijke temperatuur uit de rietjes te halen, in ieder geval voor de monsters. Op het IRAS kreeg ik de mogelijkheid om dit in een koelcel bij 4°C te doen! Mirian Boeve-Wietses en Jack Spithoven wil ik bedanken voor het wegwijs maken op het lab en voor de thee om tussentijds mee op te warmen.

Arnoud Loof en Waander van Heerde van het UMC St Radboud wil ik hartelijk danken voor de mogelijkheid om een deel van de analyse voor de

fractioneringsstudie bij jullie met de robot uit te voeren. Dit heeft heel veel tijd gescheeld en precisie opgeleverd!

Leo Kruijt van de Animal Science Group in Lelystad ben ik zeer erkentelijk voor zijn enorme gastvrijheid op zijn lab en voor het gebruik kunnen maken van de SELDI. Ook al waren de monsters waarmee we werkten zeer verschillend (is er een groter verschil dan tussen humaan serum en koeienmelk?), toch kon je ons altijd voorzien van adviezen als het weer eens vast leek te lopen. Hartelijk bedankt!

Esmeralda Krop wil ik hartelijk danken voor de prettige samenwerking tijdens de studie met de Prospect monsters. Zonder jou was ik nooit wijs geworden uit de data die jij in Zweden gegenereerd hebt. I am very thankful to Monica Kåredal, Christian Lindh, Marina Jeppsson and Bo Jönsson, from Lund University, for their help with the 2D-nanoLC-MS/MS analysis, and their comments on the paper. Lützen Portengen ben ik zeer dankbaar voor zijn hulp bij het analyseren van de data.

Irene van den Broek, wat een moeite heeft het je gekost om de analyse van de degradoom markers werkend te krijgen. Ik heb er veel bewondering voor dat het je gelukt is. Ik vind het heel leuk dat we deze methode hebben kunnen gebruiken voor het analyseren van de klinische monsters. We hebben elkaar niet vaak gezien, maar door middel van veelvuldig contact via e-mail en telefoon is het ons volgens mij heel goed gelukt samen een paper te schrijven. Nu de ITIH4 fragmenten nog! Heel veel succes met het afronden van jouw proefschrift.

Beste Wendy Rodenburg en Annemieke de Vries van het RIVM, wat een plezier heb ik gehad in het samenwerken aan de multiplex studie, ondanks dat de resultaten niet waren waarop we gehoopt hadden. Bedankt voor de leuke en interessante discussies! Conny, het was een hele klus alle samples te analyseren, maar samen hebben er het beste van gemaakt. Hartelijk dank voor de gezelligheid! Jeroen Pennings, bedankt voor je hulp bij het analyseren van de data.

In het Slotervaart ziekenhuis kwam ik terecht bij een groep OIO's als 'een vreemde eend in de bijt'; als bijna-enige niet-apotheker. Er gingen heel wat 'inside jokes' langs mij heen waar ik niets van begreep, maar wat heb ik een leuke tijd met jullie gehad! Ik heb genoten van alle gezellige lunches, koffiepauzes, borrels, etentjes, feestjes en OIO-weekenden! Elke, jij bent een waar rustpunt. Dankjewel

voor je goede raad en interesse! Ly, na een gesprek met jou was ik van werkelijk alles weer op de hoogte, dankjewel voor de gezelligheid! Rob, Joost, Robbert en Ron, wat heb ik gelachen met jullie. Ik vind het nog steeds ongelooflijk dat ik jullie zo voor de gek heb kunnen houden! Alle analisten van het lab wil ik bedanken voor hun hulp als ik weer eens iets niet kon vinden. Roel, jij ook bedankt voor al je hulp.

Ook alle (oud) collega-promovendi van het Julius Centrum wil ik hartelijk bedanken voor alle gezellige uitjes, borrels, lunches en natuurlijk het promovenski-weekend! Arnoud, Carianne, Peter en Rolf, de discussies tijdens lunches en koffiepauzes waren soms verhit, maar altijd leuk! De pingpong-competitie zal ik niet snel vergeten. Gerrie-Cor en Lotte, wat was het fijn met jullie op de schrijfkamer! Ontzettend bedankt voor het kunnen delen van alle ups en downs van het promoveren, die elkaar in de laatste fase in razend tempo afwisselden. Lotte, jouw lach en stopwoordjes zijn fantastisch! Gerrie-Cor, wat heb ik ontzettend gelachen met jou. Wij hadden aan twee woorden genoeg (maar vaak werden het er véél meer) en dat was heel erg fijn. Ik ben er helemaal voor dat we elkaar nog vaak blijven zien! Marjolein en Caroline, wat waren jullie gezellige buurtjes, bedankt! Ivonne en Florianne, heel veel succes met het afronden van jullie onderzoek!

Ook ben ik mijn vriendinnen, die voor de nodige steun, ontspanning en afleiding hebben gezorgd de afgelopen jaren, heel dankbaar. Een paar in het bijzonder. Simone en Hilde, mijn epi-vriendinnetjes, jullie hoefde ik niets uit te leggen over onderzoek doen en dat was heel fijn. Dankjewel voor jullie luisterend oor en de gezellige dates! Simone, ik vond het heel leuk om 'samen met jou' te promoveren en ik vind het dan ook super dat jij mijn paranimf bent. Als kersverse doctor kun jij mij dadelijk als geen ander ondersteunen! Femke, ontzettend bedankt voor de heerlijk ontspannende theeleutmomenten. Ik wens je een hele mooie zomer! Lieve Iris, wat waardeer ik jouw positiviteit, rust, eerlijkheid, vertrouwen en natuurlijk overheerlijke kookkunsten! Jouw aanmoedigingen en lieve kaartjes hebben mij enorm geholpen. Geweldig dat jij mijn paranimf bent! Igor, heel erg bedankt voor het ontwerpen van de voorkant, ik ben er trots op een echte 'Vermeer' te hebben! Lieve Caroline, ik heb heel veel bewondering voor jouw leven en werk in Zuid-Afrika! En ondanks die enorme afstand heb ik je steun altijd gevoeld. Super dat ik je van de zomer weer zie bij onze bruiloft!

Lieve schoonpap en -mam, dank jullie wel voor jullie interesse en zorgzaamheid, en zeker ook voor al jullie hulp in ons nieuwe huis nu ik daar de afgelopen maanden niet echt prioriteit aan kon geven. Annet, dankjewel voor alle gezelligheid! Lieve Saskia en Bert, Christian en Annika, en Mariëlle en Ard, met jullie is het hilariteit en chaos alom. Mooi dat het recht van de meest luid pratende, in plaats van degene met de beste argumenten, thuis aan de eettafel, steevast telt! Lieve pap en mam, heel veel dank voor jullie steun, zorgzaamheid en vertrouwen. Ik hou veel van jullie.

Lieve Paul, we grapten afgelopen jaren wel eens dat het minstens zo zwaar moest zijn om vriendje van een promovendus te zijn, als promovendus. Jouw liefde, geduld, steun en vertrouwen hebben het voor mij in ieder geval vele malen lichter gemaakt. Ik vind het superleuk met jou!

Annemieke

Utrecht, februari 2010

Chapter 8

Annemieke van Winden was born on February 19th, 1982 in Pijnacker, The Netherlands. In 1998 she finished secondary school at the St. Stanislascollege in Pijnacker. In the same year she started senior secondary school, and the next year grammar school both at the St. Stanislascollege in Delft. In 2001 she graduated and received her VWO degree (cum laude). She started her study in Health Sciences at Maastricht University in the same year. As part of her study she conducted a research project at the Centre for Molecular, Environmental, Genetic and Analytic (MEGA) Epidemiology of the University of Melbourne, Australia on SNPs in the BRCA2 gene and the risk of early onset breast cancer in the Australian Breast Cancer Family Study (ABCFS). The research was performed under supervision of Prof. dr. John Hopper. In 2006 she obtained her Master of Science degree (with honour) with a major in Epidemiology. In the same year she started the work described in this thesis at the Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht and the Department of Pharmacy & Pharmacology of the Slotervaart Hospital / The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam. She carried out her PhD project under supervision of Prof. dr. Jos H. Beijnen, Prof. dr. Petra H.M. Peeters, Dr. Carla H. van Gils and Dr. Roel Vermeulen.