

## ARTICLE

# Comparative Proteome Analysis Revealing an 11-Protein Signature for Aggressive Triple-Negative Breast Cancer

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- Background** Clinical outcome of patients with triple-negative breast cancer (TNBC) is highly variable. This study aims to identify and validate a prognostic protein signature for TNBC patients to reduce unnecessary adjuvant systemic therapy.
- Methods** Frozen primary tumors were collected from 126 lymph node–negative and adjuvant therapy–naïve TNBC patients. These samples were used for global proteome profiling in two series: an in-house training (n = 63) and a multi-center test (n = 63) set. Patients who remained free of distant metastasis for a minimum of 5 years after surgery were defined as having good prognosis. Cox regression analysis was performed to develop a prognostic signature, which was independently validated. All statistical tests were two-sided.
- Results** An 11-protein signature was developed in the training set (median follow-up for good-prognosis patients = 117 months) and subsequently validated in the test set (median follow-up for good-prognosis patients = 108 months) showing 89.5% sensitivity (95% confidence interval [CI] = 69.2% to 98.1%), 70.5% specificity (95% CI = 61.7% to 74.2%), 56.7% positive predictive value (95% CI = 43.8% to 62.1%), and 93.9% negative predictive value (95% CI = 82.3% to 98.9%) for poor-prognosis patients. The predicted poor-prognosis patients had higher risk to develop distant metastasis than the predicted good-prognosis patients in univariate (hazard ratio [HR] = 13.15; 95% CI = 3.03 to 57.07; *P* = .001) and multivariable (HR = 12.45; 95% CI = 2.67 to 58.11; *P* = .001) analysis. Furthermore, the predicted poor-prognosis group had statistically significantly more breast cancer–specific mortality. Using our signature as guidance, more than 60% of patients would have been exempted from unnecessary adjuvant chemotherapy compared with conventional prognostic guidelines.
- Conclusions** We report the first validated proteomic signature to assess the natural course of clinical TNBC.
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Triple-negative breast cancer (TNBC) is one of the most aggressive breast cancer subtypes. To date, there is no clinically available targeted therapy for patients diagnosed with TNBC. Current guidelines for breast cancer treatment recommend that the majority of lymph node–negative (LNN) breast cancer patients, including TNBC patients, be treated with adjuvant chemotherapy (1). Eventually approximately 30% of LNN TNBC patients develop distant metastasis (2) and could thus potentially benefit from adjuvant chemotherapy; this indicates that the majority of patients are currently being overtreated. The lack of highly sensitive and specific prognostic markers is a major obstacle to predicting prognosis of TNBC patients. Moreover, there is an urgent need to identify potentially useful targets for therapy. Some commonly applied approaches for biomarker discovery, such as gene expression profiling, have not yet succeeded in identifying highly sensitive and specific markers related to disease progression of TNBC (3). Although

a few publications have claimed to identify gene signatures that predict prognosis of TNBC (4–8), the reported signatures have limited clinical value because of their experimental design and lack of sufficient sensitivity and specificity. Therefore, it is desirable to identify a highly sensitive and specific prognostic signature for clinical application.

Quantitative proteome analysis using nanoscale liquid chromatography and tandem mass spectrometry (nLC-MS/MS) may complement these efforts because nLC-MS/MS quantitatively profiles a large portion of the human proteome (9). Some protein markers predicting prognosis (10) and therapy resistance (11) of breast cancer have already been identified using nLC-MS/MS–based comparative proteome profiling. In this study, we identified and independently validated a protein signature to predict 5-year metastasis-free survival of LNN patients who did not receive systemic adjuvant therapy, which would allow for the selection

of TNBC patients that can be spared the toxicity of adjuvant chemotherapy.

## Methods

### Study Design

Patient specimens used to develop and validate a prognostic signature were collected in five European institutes between 1985 and 2005. Triple negativity of tumor tissues was confirmed based on mRNA expression using real-time quantitative polymerase chain reaction with a reported criterion of estrogen receptor (*ESR1*) less than 0.2, progesterone receptor (*PGR*) less than 0.1, and human epidermal growth factor receptor (*HER2/ERBB2*) genes BB2 less than 18 (12,13). With this criterion, we identified 271 and 61 TNBC tumor tissues from local tissue bank and European Organization for Research and Treatment of Cancer (EORTC) collaborators, respectively. Tissues used in this study were restricted to LNN patients who had not received systemic adjuvant therapy. Patients who developed distant metastasis as first event within 5 years after removal of the primary tumor were considered to have poor prognosis, whereas patients who remained free of distant metastasis for 5 years were defined as having good prognosis. Patients who had bilateral breast cancer were excluded. In addition, inclusion for microdissection and mass spectrometry (MS) profiling depended on sufficient invasive tumor area for microdissection and morphological quality. With the above-mentioned criteria, samples were subsequently rejected on the basis of clinical reasons [patients were diagnosed with positive lymph-nodes (92), received adjuvant chemotherapy (3), had insufficient clinical follow-up (19), and developed local relapse before distant metastasis (26)] and technical reasons [insufficient tumor

tissues (33), indistinguishable morphology, and insufficient tumor area for microdissection (32)]. As a consequence, 63 and 64 tumors from training and test sets were prepared for nLC-MS/MS profiling, respectively. One sample was not successfully measured because of machinery failure. Clinical characteristics of the included (n = 126) and excluded (n = 206) subjects are summarized in [Supplementary Table 1](#) (available online). nLC-MS/MS data from 63 samples were used for development of the protein signature (training set), and data from the other 63 samples were used for independent validation (test set) ([Table 1](#); [Supplementary Table 2](#), available online). This study was approved by the local institutional medical ethics committee (MEC 02.953), and wherever possible we adhered to the Reporting Recommendations for Tumor Marker Prognostic Studies (14).

### Experimental Procedures

The dedicated procedure of sample preparation was performed (15) ([Supplementary Methods](#), available online). Tissue samples were cut into 8- $\mu$ m frozen sections and then microdissected to obtain approximately 4000 breast cancer epithelial cells. Proteins were extracted from microdissected cells, and this was followed by denaturation, reduction, and alkylation. Protein samples were digested at 37°C for 4 hours using MS-grade trypsin (Promega, Madison, WI) at a 1:4 (enzyme/protein) ratio and then acidified for further analysis.

Global proteome profiles of the TNBC samples were recorded on an nLC hyphenated LTQ-Orbitrap-XL MS system (ThermoElectron, Bremen, Germany) ([Supplementary Methods](#), available online). Peptide mixtures were separated on the nLC system with a 3-hour binary gradient (mobile phase A: water; mobile phase B: acetonitrile) in a 3- $\mu$ m C<sub>18</sub> silica-packed 50-cm capillary column with 75- $\mu$ m inner diameter. Mass spectra were acquired

**Table 1.** Clinical and pathological characteristics of patients and their tumors\*

Variables	Training set	Test set (11-protein signature)		
	EMC (n = 63)	Multicenter (n = 63)	EMC (n = 30)	EORTC (n = 33)
Age, y				
Mean (SD)	51 (14)	56 (13)	54 (13)	59 (13)
≤40	13 (20.6)	9 (14.3)	6 (20.0)	3 (9.1)
41–55	25 (39.7)	23 (36.5)	12 (40.0)	11 (33.3)
56–70	19 (30.2)	22 (34.9)	8 (26.7)	14 (42.4)
>70	6 (9.5)	9 (14.3)	4 (13.3)	5 (15.2)
Menopausal status				
Premenopausal	34 (54.0)	26 (41.3)	16 (53.3)	10 (30.3)
Postmenopausal	29 (46.0)	37 (58.7)	14 (46.7)	23 (69.7)
Tumor size, cm				
Mean (SD)†	2.9 (1.5)	2.6 (1.1)	2.9 (1.2)	2.3 (0.9)
pT1, ≤ 2cm	22 (34.9)	22 (34.9)	6 (20.0)	16 (48.5)
pT2–4, >2cm	39 (61.9)	35 (55.6)	20 (66.7)	15 (45.5)
Unknown	2 (3.2)	6 (9.5)	4 (13.3)	2 (6.1)
Grade				
Grade 1	0 (0.0)	2 (3.2)	2 (6.7)	0 (0.0)
Grade 2	4 (6.3)	12 (19.0)	2 (6.7)	10 (30.3)
Grade 3	44 (69.8)	43 (68.3)	21 (70.0)	22 (66.7)
Unknown	15 (23.8)	6 (9.5)	5 (16.7)	1 (3.0)
Metastasis within 5 years				
Yes	25 (39.7)	19 (30.2)	9 (30.0)	10 (30.3)
No	38 (60.3)	44 (69.8)	21 (70.0)	23 (69.7)

\* Data are No. (%) unless otherwise stated. EMC = Erasmus Medical Center; EORTC = European Organization for Research and Treatment of Cancer; SD = standard deviation.

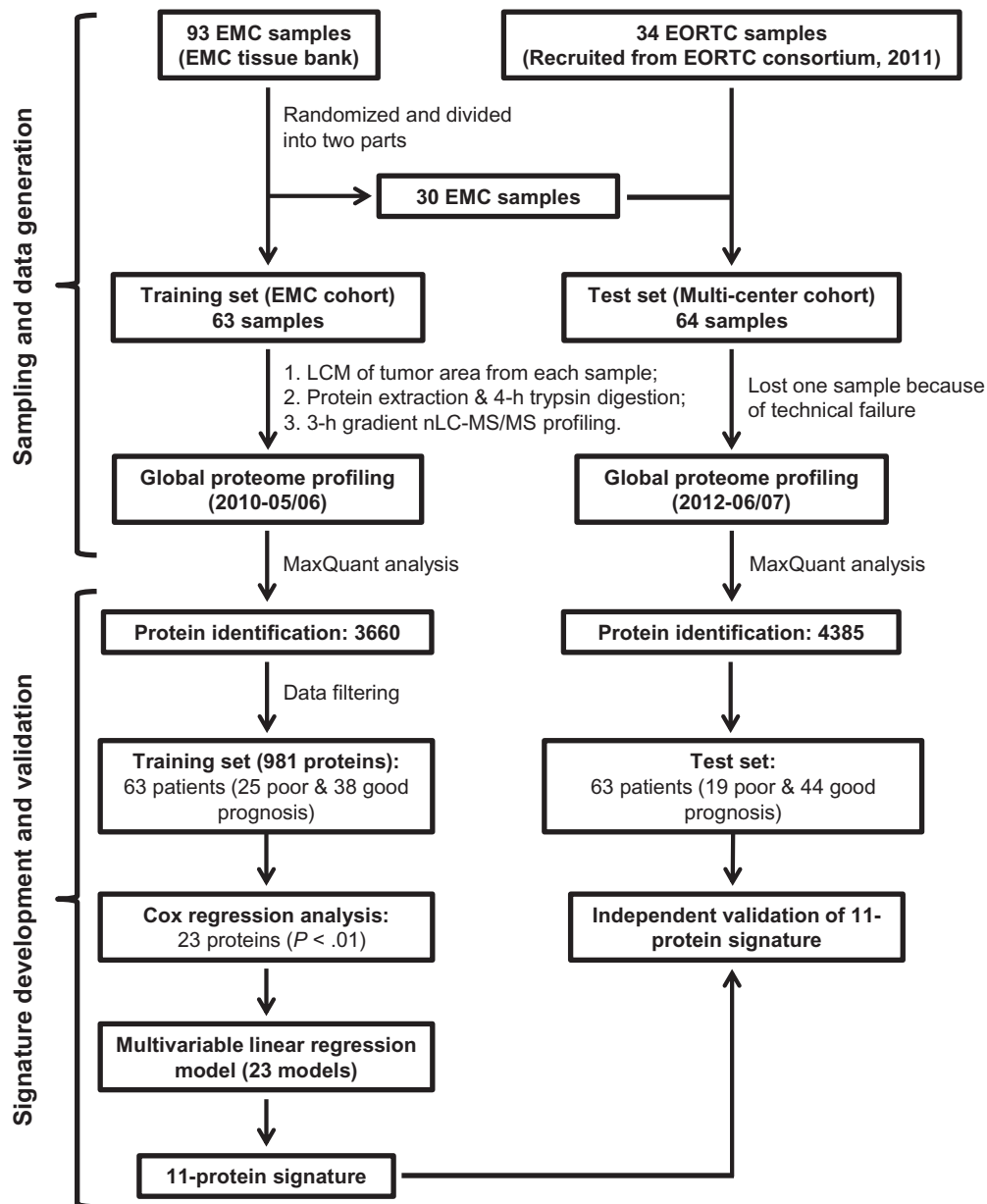
† Samples with recorded tumor size were used for calculation.

over a mass-to-charge ratio ( $m/z$ ) range of 400 to 1800 at a resolving power of 30000 at 400  $m/z$ . The five most intensive parent ions from the full scan were isolated and fragmented by collisional activated dissociation in the linear ion trap. Dynamic exclusion was used to increase the number of parent ions selected for fragmentation. Recorded raw nLC-MS/MS data have been submitted to ProteomeXchange (accession number: PXD000260).

### Statistical Analysis

The recorded MS spectra from the training and test set were separately analyzed in MaxQuant Software (free-ware available from [www.maxquant.org](http://www.maxquant.org), version 1.1.1.36) (16). To identify a prognostic protein signature, Cox regression analysis was performed to associate protein abundance with metastasis-free survival time of patients

in the training set. Cox regression coefficients and corresponding  $P$  values were determined for all tested proteins. The assumption of proportionality was verified by a test based on the Schoenfeld residuals. Prognostic proteins were selected with a  $P$  value cutoff of less than .01. Weighted protein abundance of the prognostic proteins was computed by multiplication of their protein abundance and corresponding Cox regression coefficients. A relapse score was calculated by summation of weighted protein abundance of all protein(s) in a given predictive model, followed by  $z$  score transformation of summed score. Multiple predictive models were constructed from the training set by starting with the protein with the lowest  $P$  value and stepwise adding one more protein in the next model. Efficiency of these models was then assessed by summation of three parameters: 1) area under the curve (AUC) of receiver operating



**Figure 1.** Flow chart of experimental design for the development and validation of an 11-protein signature. EMC = Erasmus University Medical Center; EORTC = European Organization for Research and Treatment of Cancer; LCM = laser capture microdissection; nLC-MS/MS = nanoscale liquid chromatography and tandem mass spectrometry.

characteristic (ROC) curve (17), 2) Youden's index at 100% sensitivity (sensitivity + specificity - 1), and 3) reversed model size ( $1 - n_i/n$ , where  $n_i$  is the number of proteins in a defined model, and  $n$  is the total number of prognostic proteins). The model with the highest efficiency was considered as the best prognostic signature.

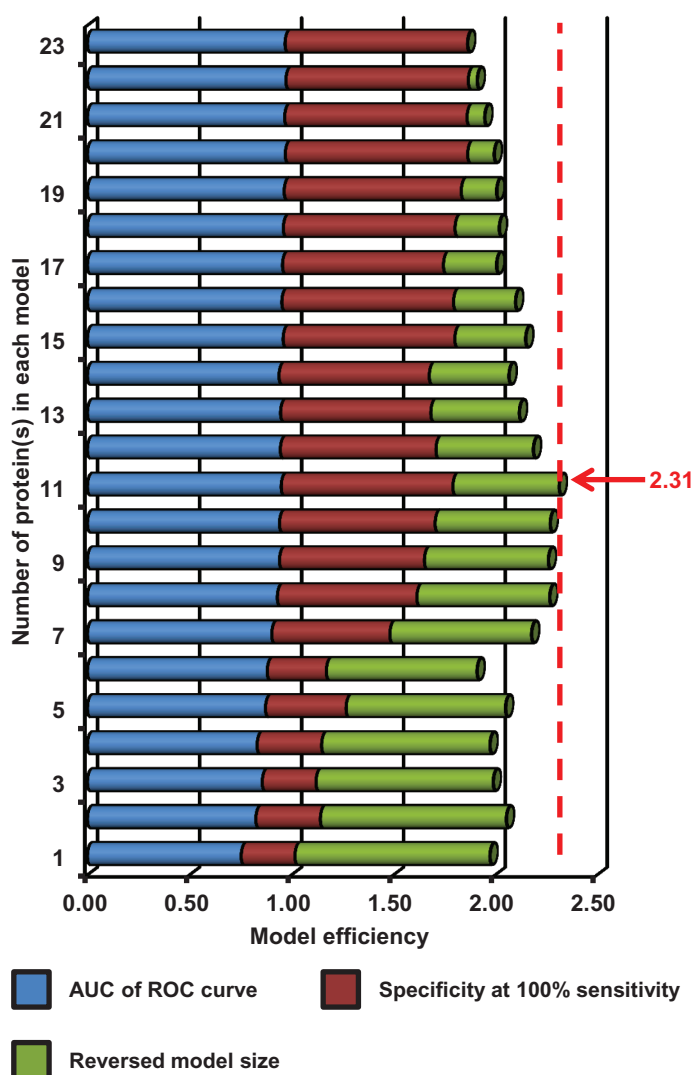
The cutoff of the prognostic protein signature was determined from the ROC curve of the training set to ensure 100% sensitivity and highest specificity. The protein signature was validated in the test set at the determined cutoff. Kaplan–Meier survival curves and log-rank test were performed to evaluate the differences in the time to distant metastasis of predicted good and poor prognosis groups. Univariate and multivariable analyses with Cox proportional hazard model were used to assess the prognostic value of the protein signature with and without consideration of the individual clinical prognostic variables.

Pathway analysis was performed to interpret biological function of signature proteins using gene set enrichment analysis (18). The

Supplementary Methods (available online) describe detailed information about MaxQuant settings, statistical software packages, and pathway analysis.

## Results

The training and test samples were profiled by nLC-MS/MS in 2010 and 2012, respectively (Figure 1). The baseline clinical features of patients were similar between the Erasmus University Medical Center (EMC) training and multicenter test set, although patients were slightly older and more patients were classified with grade 2 tumors in the test set (Table 1). The median follow-ups for the good-prognosis patients in the training and test set were 117 (range = 61–257) and 108 (range = 61–234) months, respectively. Patients were recruited in five EORTC institutes between 1985 and 2005. Clinical data used for data analysis were updated until October 2012.



**Figure 2.** Selection of the best predictive signature from 23 prognostic models developed in the training set. The different models were created from 23 prognostic proteins (Cox regression analysis:  $P < .01$ ), starting with the protein with the lowest  $P$  value and gradually adding one more protein at a time, thereby constructing 23 different prognostic models. Model efficiency was considered by three aspects: 1) area under

the curve (AUC) of the receiver operating characteristic (ROC) curve, 2) specificity at 100% sensitivity, and 3) reversed model size ( $1 - 1/n$ , where  $n$  is number of used protein[s] for the model). The model with the highest model efficiency was considered as the best model, resulting in selection of the 11-protein signature (model efficiency = 2.31) for validation.

## Development of a Proteomic Prognostic Signature for TNBC Patients

Proteome profiles for training and test samples were independently generated by nLC-MS/MS (see [Supplementary Methods](#), available online). A stringent filtering procedure resulted in a total of 981 proteins for development of a prognostic signature ([Figure 1](#); [Supplementary Figure 1](#), available online). Good reproducibility and quantitative precision were observed from quantified proteins in replicate samples by performing the same filtering criteria (see [Supplementary Methods](#), available online).

The 981 proteins were used to develop a prognostic signature in the training set. Using univariate Cox analysis, a panel of 23 proteins was statistically significantly associated with metastasis-free survival of patients ( $P < .01$ ) ([Figure 1](#); [Supplementary Figure 1](#), available online). Twenty-three prognostic models were derived from these 23 proteins using a multivariable linear regression model, from which an 11-protein model performed fairly comparably with the models with 15 or more proteins based on an AUC of ROC curve and specificity at 100% sensitivity to predict poor-prognosis patients. By further considering model size, we calculated efficiency for all 23 models, and the 11-protein model showed the

highest model efficiency at 2.31 ([Figure 2](#)). Detailed information for these 11 proteins is listed in [Table 2](#) and [Supplementary Table 3](#) (available online). Of these 11 proteins, 10 proteins were upregulated (CMPK1, AIFM1, FTH1, EML4, GANAB, CTNNA1, AP1G1, STX12, AP1M1, and CAPZB), whereas one protein was downregulated (MTHFD1) in good-prognosis patients in the training set. The ROC curve derived from the 11-protein signature showed good sensitivity and specificity with AUC of 0.95 ([Figure 3A](#)). A cutoff was selected with a relapse score of zero to identify good-prognosis patients (negative score) at which maximal specificity was reached at 100% sensitivity ([Figure 3A](#), green dot).

## Validation of the 11-Protein Signature in a Multicenter Cohort of TNBC Patients

The 11-protein signature was validated on the test cohort, resulting in an ROC curve with AUC of 0.83 ([Figure 3B](#)). Using the pre-determined cutoff (relapse score = 0), patients predicted as having a poor prognosis (positive score) had much worse 5-year metastasis-free survival (log-rank  $P < .001$ ) ([Figure 3C](#)) and breast cancer-related survival (log-rank  $P < .001$ ) ([Figure 3D](#)) than predicted good-prognosis patients. In the predicted good-prognosis group,

**Table 2.** Eleven-signature proteins and their prognostic information in training set

Protein ID	Gene name	Protein description	Subcellular localization*	Cox coefficient (Hazard ratio)†	P (FDR)‡
P30085	<i>CMPK1</i>	UMP-CMP kinase	Nucleus/cytoplasm	-0.587 (0.556)	.00 (0.123)
O95831	<i>AIFM1</i>	Apoptosis-inducing factor 1, mitochondrial	Mitochondrion/nucleus/cytoplasm	-0.860 (0.423)	.001 (0.199)
P02794	<i>FTH1</i>	Ferritin heavy chain	Cytoplasm/intracellular matrix	-0.400 (0.670)	.001 (0.199)
P11586	<i>MTHFD1</i>	C-1-tetrahydrofolate synthase, cytoplasmic	Cytoplasm	1.178 (3.247)	.001 (0.199)
Q9HC35	<i>EML4</i>	Echinoderm microtubule-associated protein-like 4	Cytoplasm/cytoskeleton/microtubule	-0.576 (0.562)	.001 (0.267)
Q14697	<i>GANAB</i>	Neutral alpha-glucosidase AB	Endoplasmic reticulum/Golgi apparatus/melanosome	-1.050 (0.350)	.002 (0.312)
P35221	<i>CTNNA1</i>	Catenin alpha-1	Cytoplasm/cytoskeleton/cell junction/Cell membrane	-1.115 (0.328)	.002 (0.312)
O43747	<i>AP1G1</i>	AP-1 complex subunit gamma-1	Golgi apparatus/cytoplasmic vesicle/clathrin-coated vesicle membrane	-0.997 (0.369)	.003 (0.332)
Q86Y82	<i>STX12</i>	Syntaxin-12	Endosome membrane/Golgi apparatus membrane	-0.675 (0.509)	.003 (0.332)
Q9BXS5	<i>AP1M1</i>	AP-1 complex subunit mu-1	Golgi apparatus/cytoplasmic vesicle/clathrin-coated vesicle membrane	-0.846 (0.429)	.004 (0.398)
P47756	<i>CAPZB</i>	F-actin-capping protein subunit beta	Cytoplasm/cytoskeleton	-0.916 (0.400)	.005 (0.398)

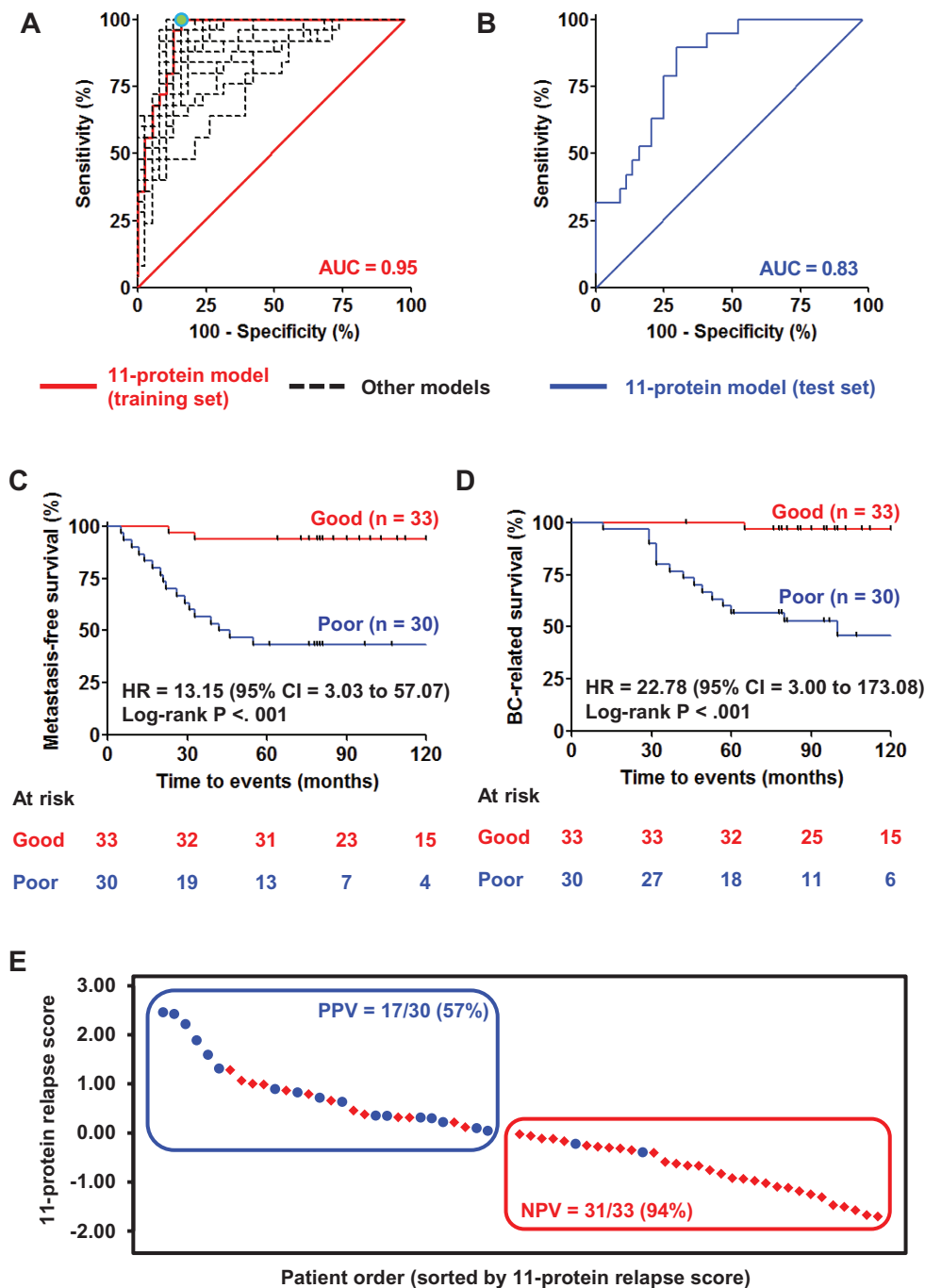
\* Adapted from UniProt Knowledgebase.

† Cox coefficients were calculated by natural logarithm (ln) of hazard ratios.

‡ P values were computed by Cox regression analysis, and the corresponding false discovery rate (FDR) of the proteins is reported in parentheses.

31 of 33 patients did not develop distant metastasis (negative predictive value = 93.9%; 95% confidence interval [CI] = 82.3% to 98.9%) (Figure 3, C and E). In contrast, 17 of 30 patients developed distant metastasis in the predicted poor-prognosis group (positive predictive value = 56.7%; 95% CI = 43.8% to 62.1%) (Figure 3, C and E). Overall, our signature results in a sensitivity of 89.5% (95%

CI = 69.2% to 98.1%) and specificity of 70.5% (95% CI = 61.7% to 74.2%) to predict poor-prognosis patients in the test set. In univariate Cox analyses, the poor-prognosis patients also have statistically significantly higher risk of developing distant metastases (hazard ratio [HR] = 13.15; 95% CI = 3.03 to 57.07;  $P = .001$ ) (Figure 3C; Table 3) and breast cancer–related death (HR = 22.78;

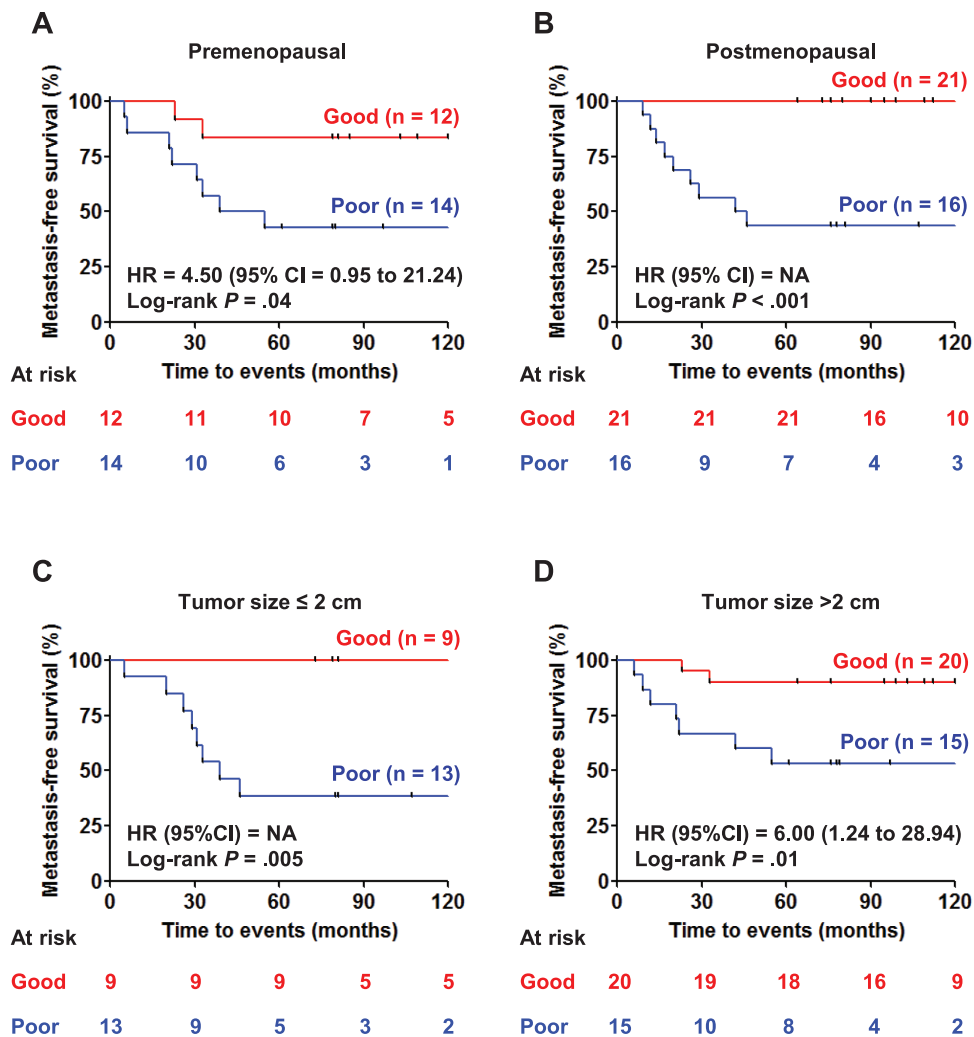


**Figure 3.** Development and multicenter validation of the 11-protein signature. **A)** Receiver operating characteristic (ROC) curve of the 11-protein signature in the training set (red solid line: area under the curve [AUC]). A cutoff was chosen to ensure maximal sensitivity to identify the good-prognosis patients with the highest possible specificity in the training set (green dot). Other models developed from 23 prognostic proteins were also plotted as ROC curves (black dashed lines). **B)** ROC

curve of the 11-protein signature in the test set. Kaplan-Meier analysis shows that the 11-protein signature is strongly predictive of metastasis-free survival (C) and breast cancer (BC)–related survival (D) CI = confidence interval; HR = hazard ratio. **E)** Waterfall plot stratifies two groups of triple-negative breast cancer patients with different predicted prognosis in the test set. NPV = negative predictive value; PPV = positive predictive value. All statistical tests were two-sided.







**Figure 4.** Kaplan–Meier analysis of metastasis-free survival in subgroups of triple-negative breast cancer patients in the test set. **A)** Premenopausal patients. **B)** Postmenopausal patients. Hazard ratio (HR) and 95% confidence interval (CI) could not be computed because of the absence of metastatic events in one of the tested groups. **C)** Patients with tumor size of 2 cm or less. Hazard ratio and 95% confidence interval could not be computed because of the absence of metastatic events in one of the tested groups. **D)** Patients with tumor size greater than 2 cm. All statistical tests were two-sided.

in TNBC patients with poor prognosis and was associated with two metabolic pathways (metabolism of nucleotides and noncoding RNA) with false discovery rate less than 0.25 (Supplementary Table 4, available online).

## Discussion

Prognosis of LNN TNBC patients is extremely heterogeneous and often not associated with conventional prognostic parameters (patient age, tumor size, and tumor grade) (21), concordant with this study. Approximately 30% of TNBC patients eventually experience distant relapse (2). Therefore, a substantial proportion of patients are being overtreated with systemic adjuvant therapy. In this study, we identified and independently validated a clinically relevant prognostic 11-protein signature for TNBC, which could be used to reduce the number of patients that would receive unnecessary adjuvant chemotherapy.

To translate the 11-protein signature into a clinically useful assay, two future steps are essential. First, a quantitative, targeted,

and ready-to-use assay needs to be developed for absolute quantification of the signature proteins. The most promising candidate for such an assay would be a mass spectrometry–based selected reaction monitoring assay (22). Second, such a targeted assay can then be incorporated into future clinical trials and subsequently used

**Table 4.** Comparison of the 11-protein signature with currently applied clinical consensus criteria on treatment of breast cancer

Method	Patients guided to receive adjuvant chemotherapy in the test set	
	Poor prognosis	Good prognosis
St. Gallen*	18/18 (100%)‡	40/44 (91%)
NIH†	14/17 (82%)‡	38/40 (95%)‡
11-protein signature	17/19 (89%)	13/44 (30%)§

\* St. Gallen consensus criteria: tumor  $\geq 2$  cm, ESR1 negative, grade 2–3, patient aged  $< 35$  years (one of these criteria).

† National Institutes of Health (NIH) guideline: tumor  $> 1$  cm.

‡ Patients with missing clinical information were excluded from these analyses.

§ A statistically significant improvement.

and ready-to-use assay needs to be developed for absolute quantification of the signature proteins. The most promising candidate for such an assay would be a mass spectrometry–based selected reaction monitoring assay (22). Second, such a targeted assay can then be incorporated into future clinical trials and subsequently used



for clinical decision making for treatment options. Furthermore, some of the signature proteins may serve as potential targets for novel therapies.

Some signature proteins have been reported to be prognostic in breast cancer or other cancers. The prognostic role of these proteins is summarized in three categories. First, expression of some proteins is directly linked to disease progression. For instance, decreased levels of FTH1 protein have been associated with lymph node metastasis in colorectal cancer (23). Similarly, abnormal expression of CTNNA1 protein has been related with poor patient survival in invasive breast cancer (24). Also, association has been observed between increased expression of GANAB protein and favorable prognosis in head and neck cancer (25). Second, genetic polymorphisms of some proteins showed important prognostic values. Single nucleotide polymorphisms of CMPK1 have been associated with prognosis of non-small cell lung cancer (26) and pancreatic cancer (27) patients treated with gemcitabine-based chemotherapeutics. A correlation has also been reported between a 1958AA single nucleotide polymorphism of MTHFD1 gene and poor clinical outcome of premenopausal breast cancer patients (28). Moreover, genetic mutations of certain protein-coding genes may also be prognostic. EML-ALK fusion gene has been suggested to be associated with relatively favorable prognosis of non-small cell lung cancer patients (29). We speculate that there may be a correlation between expression of these proteins and their genetic variance, which needs to be further investigated.

Pathway analysis showed that immune response, cell death, cell metabolism, and transport of macromolecules are the major underlying molecular mechanisms of the signature proteins related to prognosis of TNBC patients. These findings are in concordance with previous observations. Lehmann and colleagues identified an immunomodulatory subtype that was associated with favorable clinical outcome (30). Rody and colleagues identified a signature of high B-cell and low IL-8 metagenes that predicted good prognosis of TNBC patients (5), and Yau and coworkers reported a 14-gene signature linked to immune/inflammatory cytokine regulation in which the majority of genes were associated with good prognosis of TNBC patients (8). In our prognostic signature, three proteins (FTH1, GANAB, and STX12) are associated with immunomodulation and cell death-associated pathways. FTH1 is an iron storage protein and indirectly regulates the ratio of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells by altering iron distribution (31). Increased CD8<sup>+</sup> T cells may help to eliminate tumor cells (32), which would be favorable for survival of TNBC patients. Molecular functions of GANAB and STX12 related to immune response and cell death have not been well studied. Therefore, further studies are required to understand the functions of these proteins in disease progression. On the other hand, it is known that the luminal androgen receptor subtype of TNBC, with aberrant alteration of cell metabolism, is related to adverse clinical outcome of TNBC patients (30). In our signature, MTHFD1 is associated with poor prognosis and is involved in folate metabolism. It has been suggested that increased concentration of folate resulted in a dose-dependent downregulation of tumor suppressor genes in breast cancer cell lines due to increased DNA methylation of their promoter region (33), which may indicate the importance of MTHFD1 in TNBC progression.

Our study has some limitations. First, microdissection-based sample preparation is tedious and may introduce additional biases between diagnostic individuals and laboratories. Second, the nLC-MS/MS-based platform is difficult to apply in routine clinical practice. Therefore, our future work will focus on developing simple sampling strategies and high-throughput selected reaction monitoring assay to reliably measure the 11-protein signature.

In summary, we have developed an 11-protein signature to predict TNBC patients who develop a distant metastasis with high sensitivity, specificity, positive predictive value, and negative predictive value (82.3% to 98.9%). Therefore, our signature could aid in clinical practice to avoid unnecessary treatment with adjuvant chemotherapy of LNN TNBC patients. Future prospective clinical trials are needed to further consolidate the validity of the 11-protein signature.

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## Notes

The authors are solely responsible for the study design, data generation, analysis and interpretation of the data, writing the manuscript, and decision to submit the manuscript for publication.

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