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PRECLINICAL STUDY

Nottingham prognostic index plus (NPI+) predicts risk of distant metastases in primary breast cancer

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Abstract The Nottingham prognostic index plus (NPI+) is based on the assessment of biological class combined with established clinicopathologic prognostic variables providing improved patient outcome stratification for breast cancer superior to the traditional NPI. This study aimed to determine prognostic capability of the NPI+ in predicting risk of development of distant disease. A well-characterised series of 1073 primary early-stage BC cases treated in Nottingham and 251 cases from Budapest were immunohistochemically assessed for cytokeratin (Ck)5/6, Ck18, EGFR, oestrogen receptor (ER), progesterone receptor, HER2, HER3, HER4, Mucin 1 and p53 expression. NPI+ biological class and prognostic scores were assigned using individual algorithms for each biological class incorporating clinicopathologic parameters and investigated in terms of prediction of distant metastases-free survival (MFS). The NPI+ identified distinct prognostic groups (PG) within each molecular class which were predictive of MFS providing improved patient outcome stratification superior to the traditional NPI. NPI+ PGs, between series, were comparable in predicting patient outcome between series in luminal A, basal p53 altered and HER2+/ER+ ($p > 0.01$) tumours. The low-risk groups were similarly validated in luminal B, luminal N, basal p53 normal tumours ($p > 0.01$). Due to small patient numbers the remaining PGs could not be validated. NPI+ was additionally able to predict a higher risk of metastases at certain distant sites. This study may indicate the NPI+ as a useful tool in predicting the risk of metastases. The NPI+ provides accurate risk stratification allowing improved individualised clinical decision making for breast cancer.

Keywords Breast cancer · Classification · Prognostic index · Molecular · Clinical · Outcome

Introduction

Breast cancer (BC) is remarkably heterogeneous with respect to genotypic, phenotypic and behavioural characteristics and subsequent response to treatment. With an increasing number of treatment options available for BC patients, deciding the most appropriate choice remains challenging. However, accurate personalised BC treatment requires robust and accurate risk stratification based on both outcome prediction and biology of tumours [1], combined with therapeutic modality response and resistance assessment.

The Nottingham prognostic index (NPI) [2–5] is based on histopathological factors (tumour size, lymph node stage and tumour grade) and is used to stratify BC patients

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with operable early-stage primary BC into prognostic groups. The NPI accuracy has been confirmed using long-term patient follow-up [2], validated in large independent multi-centre studies [3, 6], revised in order to stratify patients into additional prognostic groups [7], and is currently adopted in clinical practice in the UK and other parts of Europe and Australia. However, the NPI does not consider the biological heterogeneity of BC and it therefore needs further refinement to support a more sophisticated personalised management of patients.

We have therefore developed a biomarker-based prognostic index, Nottingham prognostic index plus (NPI+) [8], using a large well-characterised series of early-stage BC. NPI+ is based on the well-established clinicopathologic variables used in the NPI but has been refined to integrate with tumour biology. It utilises routine formalin-fixed clinical samples and immunohistochemistry (IHC) thus providing an easy adoption into the current international clinical practice. NPI+ is based on a two-tier evaluation; initially the biological class of the tumour is determined and is then combined with clinicopathologic prognostic variables resulting in bespoke NPI-like formulae for each biological class [8–11]. NPI+ is able to assist in predicting long-term patient survival and to support clinical decision making in BC management [8]. Breast tumours are classified into seven core BC biological classes by the evaluation and combination of 10 BC-related biomarkers using IHC [12]. The molecular classes identified are three luminal classes (Luminal A, N and B), two basal classes (Basal—p53 altered and Basal—p53 normal) and two HER2+ classes (HER2+/ER+ and HER2+/ER−). Each biological class is further stratified using a set of well-defined prognostic clinicopathologic variables which are combined in tailored formulae to stratify each individual molecular class into several prognostic subgroups (NPI+ Groups) which are superior to the classic NPI [8]. Recently, we have further refined the NPI+ algorithms and validated the NPI+ in an independent series of primary BC confirming its reproducibility in providing accurate risk stratification allowing improved individualised clinical decision making for BC [13].

In this study, we aimed to determine the applicability of NPI+ to predict risk of distant metastases in two series of clinically annotated early-stage primary invasive BC.

Patients and laboratory methods

Nottingham series

A series of 1073 patients from the Nottingham-Tenovus Primary Breast Carcinoma Series, aged 70 years or less, presenting with primary operable (stages I, II and IIIa) invasive BC between 1986 and 1998 were previously used to develop the NPI+ [8–11]. This is a well-characterised consecutive series of patients who were uniformly treated according to locally agreed clinical protocols (Abd El-Rehim et al. [9]). All tumours were less than 5 cm diameter on clinical/pre-operative measurement and/or on operative histology (T1 and T2). Women aged over 70 years were not included because of the increased confounding effect of comorbidities/death from other causes and because the primary treatment protocols for elderly patients often differed from those for younger women. Adjuvant systemic therapies were offered according to the Nottingham prognostic index (NPI) and hormone receptor (HR) status [2, 7]. Patients in the Moderate I group (NPI 3.41–4.4) with HR-positive tumours were offered hormonal therapy. Patients in the Moderate II (NPI 4.41–5.4) and Poor (NPI > 5.41) groups received hormone therapy for HR-positive tumours and cytotoxic therapy (classical cyclophosphamide, methotrexate and 5-fluorouracil (CMF)) for HR-negative tumours and if the patient was fit enough to tolerate chemotherapy. Hormonal therapy was given to 396 patients (40.3 %), chemotherapy to 213 (19.9 %). Only 19 patients (1.9 %) received a combination of chemotherapy and endocrine therapy (Table 1). Data relating to survival were collated in a prospective manner for those patients presenting after 1989 only. All samples from Nottingham used in this study were pseudo-anonymised and collected prior to 2006 and therefore under the Human Tissue Act informed patient consent was not needed. Release of data was also pseudo-anonymised as per Human Tissue Act regulations.

Budapest series

This series comprised 368 screen detected and symptomatic consecutive cases diagnosed with primary breast cancer between 1999 and 2002 and operated at the Buda MÁV hospital, Budapest, Hungary. A total of 251 cases were assembled in TMAs; the remaining cases were not included due to technical reasons or lack of relevant data. The age range of patients was 30–88 years, and pathological size was 14 cm and less. All pT stages and inflammatory breast cancers were included. A total of 42.4 % of the patients received the classical Bonadonna scheme (CMF), at the time the standard chemotherapy, unless comorbidities or advanced age permitted. Patients with hormone receptor positive invasive tumours were treated with Tamoxifen. According to the radiation therapy recommendations valid at the time, loco-regional radiation therapy was added to the postoperative treatment in cases of breast conservation and in those patients undergoing mastectomy who had 4 or more axillary lymph node metastases (134 patients, 57.4 %). Pathological features

Table 1 Pathological characteristics of the Nottingham and Budapest series

	Nottingham *n* (%)	Budapest *n* (%)	*p* value
Grade			
1	158 (14.7)	102 (40.8)	
2	348 (32.4)	72 (28.8)	<0.001
3	567 (52.8)	76 (30.4)	
Tubule formation			
1	53 (5.0)	36 (15.1)	
2	346 (33.0)	57 (23.8)	<0.001
3	651 (62.0)	146 (61.1)	
Pleomorphism			
1	19 (1.8)	57 (23.4)	
2	378 (36.1)	93 (38.1)	<0.001
3	651 (62.1)	94 (38.5)	
Mitosis			
1	349 (33.2)	114 (47.5)	
2	190 (18.1)	73 (30.4)	<0.001
3	511 (47.6)	53 (22.1)	
Size	0.13–10 cm (median 2.0 cm)	0.3–14 cm (median 2.1 cm)	
<1.5 cm	240 (22.4)	68 (27.1)	0.111
≥1.5 cm	833 (77.6)	183 (72.9)	
Stage			
1	654 (61.0)	88 (48.1)	
2	330 (30.8)	55 (30.1)	<0.001
3	88 (8.2)	40 (21.9)	
Lymph nodes positive			
0	608 (48.9)	90 (48.9)	
1–3	318 (31.6)	55 (29.9)	<0.001
4–9	70 (7.0)	29 (15.8)	
>9	11 (1.1)	10 (5.4)	
Nottingham prognostic index			
Excellent	110 (10.3)	27 (14.8)	
Good	200 (18.6)	31 (17.0)	
Moderate 1	293 (27.3)	45 (24.7)	
Moderate 2	277 (25.8)	39 (21.4)	0.008
Poor	140 (13.0)	20 (11.0)	
Very poor	45 (4.2)	20 (11.0)	
Chemotherapy			
No	807 (75.2)	141 (57.6)	
Yes	213 (19.9)	104 (42.4)	<0.001
Metastases			
Yes	363 (33.9)	49 (21.1)	<0.001
No	707 (66.1)	183 (78.5)	
Site of metastases			
Bone	219 (20.5)	–	
Brain	57 (5.3)	–	
Liver	149 (13.9)	–	
Lung	91 (8.5)	–	
Lymph node	50 (4.7)	–	
Pleura	35 (3.3)	–	
Visceral	24 (2.3)	–	
Other	51 (4.8)	–	
Metastases-free survival (months)	0–243 (median 119)	0–123 (median 96)	

were retrieved from the pathology reports and slides were reviewed. Treatment data were collected from patients' medical records. The evaluation was approved by the Institutional Review Board of Semmelweis University (IKEB, #185/2007). No informed consent was required by the Institutional Ethical Committee.

Pathological characteristics of both series are summarised in Table 1. Metastases-free survival (MFS) is defined as the interval (in months) between the primary surgery and occurrence of metastases being scored as an event. Metastases did not include local (ipsilateral) or regional recurrences, contralateral breast cancer. Lymph node metastases did not include regional lymph nodes. Patients who did not have metastatic disease were censored at the time of last follow-up.

This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer'.

Determination of NPI+ biological class

Immunohistochemical reactivity for the NPI+ biomarkers in the Nottingham series was previously determined using standard immunocytochemical techniques on tumour samples prepared as tissue microarrays (TMAs) [9]. The NPI+ biomarkers used for classification were oestrogen receptor (ER), progesterone receptor (PgR), cytokeratin (CK) 5/6, CK7/8, epidermal growth factor receptor (EGFR; HER1), c-erbB2 (HER2), c-erbB3 (HER3), c-erbB4 (HER4), p53, and Mucin 1 [11]. TMAs of the Budapest series were additionally stained for the NPI+ biomarkers using the same procedures as previously described [9, 11]. The Budapest TMAs consisted of 2 mm tissue cores and were produced as previously described [14]. Each tumour was represented by two cores. Levels of immunohistochemical reactivity were determined by microscopic assessment using the modified Histochemical score (*H* score), giving a semi-quantitative assessment of both the intensity of staining and the percentage of positive cells (values between 0 and 300) [15, 16]. For HER2, the American Society of Clinical Oncology/College of American Pathologists Guidelines Recommendations for HER2 Testing in

Breast Cancer were used for assessment [17]. Equivocal (2+) HER2+ cases were confirmed by FISH/CISH [18]. The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria [19] were followed.

Determination of NPI+ score

For biological classification, a fuzzy logic rule-based method algorithm was used where the cut-offs for each biomarker were previously determined [12]. In particular, the median value of markers was used for ER, PgR, CK7/8, HER3, HER4 and MUC1. The expertise values were used for those markers which had a median equal to zero and for those where clinicians were sure about the value to consider (CK5/6, EGFR, p53 and HER2).

The NPI+ prognostic groups were calculated using bespoke NPI-like formulae, previously developed for each NPI+ biological class of the Nottingham series, utilising the existing available clinicopathological parameters [8]. Briefly, these were established by utilisation of the Beta values generated by Cox regression analysis in predicting breast cancer-specific survival of the well-established histopathologic prognostic factors. These formulae were initially derived from the Biological Classes in Green et al. [11] and were subsequently refined using the improved biological classification used in Soria et al. [12] consisting of number of positive nodes, pathological tumour size,

Table 2 Distribution of NPI+ biological classes within the Nottingham and Budapest Series

NPI+ class	Nottingham ($n = 1035$) n (%)	Budapest ($n = 266$) n (%)	p value
Luminal A	288 (27.8)	84 (31.6)	0.005
Luminal N	205 (19.8)	32 (12.0)	
Luminal B	186 (18.0)	65 (24.4)	
Basal p53 altered	113 (10.9)	18 (6.8)	
Basal p53 normal	96 (9.3)	36 (13.5)	
HER2+/ER+	62 (6.0)	15 (5.6)	
HER2+/ER−	85 (8.2)	16 (5.0)	

Table 3 Clinicopathological parameters of the NPI+ breast cancer biological classes in the Budapest series

	Luminal A ($n = 84$) n (%)	Luminal N ($n = 32$) n (%)	Luminal B ($n = 64$) n (%)	Basal—p53 altered ($n = 18$) n (%)	Basal—p53 normal ($n = 23$) n (%)	HER2+/ER+ ($n = 8$) n (%)	HER2+/ER− ($n = 3$) n (%)	Cramer's V (p value)
Size								
<15 mm	24 (30.0)	10 (30.6)	19 (30.6)	7 (46.7)	1 (3.7)	2 (13.3)	3 (18.8)	0.235 (0.034)
≥15 mm	56 (70.0)	21 (69.4)	43 (69.4)	8 (53.3)	26 (96.3)	13 (86.7)	13 (81.3)	
Grade								
1	44 (55.0)	22 (71.0)	26 (42.6)	1 (6.7)	5 (18.5)	3 (20.0)	0	0.378 (<0.001)
2	20 (25.0)	6 (19.4)	23 (37.7)	3 (20.0)	5 (18.5)	8 (53.3)	5 (31.3)	
3	16 (20.0)	3 (9.7)	12 (19.7)	11 (73.3)	17 (63.0)	4 (26.7)	11 (68.8)	
LN stage								
1	34 (61.8)	8 (42.1)	21 (46.7)	4 (33.3)	12 (52.2)	4 (26.7)	4 (36.4)	0.211 (0.188)
2	12 (21.8)	7 (36.8)	17 (37.8)	2 (16.7)	5 (21.7)	6 (40.0)	4 (36.4)	
3	9 (16.4)	4 (21.1)	7 (15.6)	6 (50.0)	6 (26.1)	5 (33.3)	3 (27.3)	
NPI								
Excellent	13 (23.6)	4 (21.1)	8 (17.8)	0	1 (4.3)	1 (6.7)	0	0.239 (0.010)
Good	13 (23.6)	6 (31.6)	8 (17.8)	0	3 (13.0)	1 (6.7)	0	
Moderate 1	16 (29.1)	5 (26.3)	11 (24.4)	2 (18.2)	5 (21.7)	4 (26.7)	1 (9.1)	
Moderate 2	6 (10.9)	1 (5.3)	7 (15.6)	6 (54.5)	5 (21.7)	6 (40.0)	6 (54.5)	
Poor	4 (7.3)	1 (5.3)	7 (15.6)	2 (18.2)	4 (17.4)	0	2 (18.2)	
Very poor	3 (5.5)	2 (10.5)	4 (8.9)	1 (9.1)	5 (21.7)	3 (20.0)	2 (18.2)	

stage, tubule formation, nuclear pleomorphism and mitotic counts. These were identified as the most significant variables in the Nottingham series impacting on survival, according to their Beta value in Cox regression indicating the magnitude of the influence of the hazard. The Nottingham series was split into the NPI+ biological classes, and Cox regression analyses were performed independently for each class to identify the most significant clinicopathological prognostic factors and their beta value in the context of the individual classes. NPI+ Prognostic Groups for the Budapest series were assigned using the categorical cut-points previously derived from the Nottingham series in each of the NPI+ biological classes [8]. For this purpose, the original pathology assessments on full-face sections for the histopathologic parameters were utilised.

Statistical analysis

The association between BC classes and both histopathological and clinical characteristics was assessed using Cramer's V [20] to produce p values. MFS differences between NPI+ biological classes and NPI+ Groups were determined using Kaplan–Meier curves using Log Rank. A $p < 0.01$ was considered significant with Bonferroni adjustment for multiple testing.

Results

NPI+ in the Nottingham and Budapest series

There were significant differences in the distribution of grade and stage (both $p < 0.001$) of the breast tumours between the Nottingham and Budapest series with a larger proportion of the Nottingham series of a higher grade and lower stage (Table 1). The median follow-up for the Nottingham series was 9.9 years and the Budapest series was 8.1 years. A total of 363 (33.9 %) and 49 (21.2 %) patients developed distant metastases during the follow-up period in the Nottingham and Budapest series, respectively. There was, however, no difference in the MFS between the two series ($p = 0.236$, Supplementary Fig. 1). The classic NPI significantly predicted MFS in both the Nottingham (Supplementary Fig. 1B, $p < 0.001$) and Budapest (Supplementary Fig. 1C, $p < 0.001$) series.

There was either very good (ER, PgR, CK7/8, EGFR, p53 and MUC1) or good (CK5/6 and HER4) concordance between the expression for the majority of markers across the two stained TMA cores whereas HER3 showed only moderate concordance (Supplementary Table 1). NPI+ biological class was determined in the Budapest series using the immunohistochemical data for the 10 NPI+ biomarkers. There was a difference in the distribution between each of the seven NPI+ biological classes (luminal A, luminal N, luminal B, basal p53 altered, basal p53 normal, HER2+/ER+ and HER2+/ER−) in Budapest series compared with the Nottingham series ($p < 0.001$, Table 2). This was mainly due to the higher proportion of luminal B tumours in the Budapest series. A total of 5 cases (2.0 %) were not assigned to any class. There were significant associations between the clinicopathological parameters of the Budapest series and the NPI+ biological classes (Table 3). When comparing the MFS between the Nottingham and Budapest series, there were no significant differences in any of the luminal or basal NPI+ biological classes (Supplementary Fig. 2).

Bespoke NPI+ formulae, based on nodal number, tumour size, stage, and mitosis, for each of the seven NPI+ biological classes Rakha et al. [8] were applied to produce an NPI+ score, and then patients were further stratified into NPI+ Groups using the categorical cut-points derived from the Nottingham series. The NPI+ score for the Budapest and Nottingham series was not significantly different from each other (mean = 2.71 vs. 2.10, $p = 0.179$).

Table 4 Distribution of the NPI+ Groups in the Budapest and Nottingham series

NPI+ Group	Budapest ($n = 178$) n (%)	Nottingham ($n = 828$) n (%)	p value
Luminal A			
Low risk	23 (12.9)	148 (17.9)	<0.001
Moderate risk	24 (13.5)	83 (10.0)	
High risk	8 (4.5)	25 (3.0)	
Luminal N			
Low risk	18 (10.1)	133 (16.1)	
High risk	1 (0.6)	17 (2.1)	
Luminal B			
Low risk	41 (23.0)	77 (9.3)	
High risk	2 (1.1)	58 (7.0)	
Basal—p53 altered			
Low risk	7 (3.9)	86 (10.4)	
High risk	4 (2.2)	10 (1.2)	
Basal—p53 normal			
Low risk	24 (13.5)	44 (5.4)	
High risk	0	28 (3.4)	
HER2+/ER+			
Low risk	4 (2.2)	31 (3.7)	
High risk	11 (6.2)	25 (3.0)	
HER2+/ER−			
Low risk	11 (6.2)	55 (6.6)	
High risk	0	8 (1.0)	

Although there was a significant difference in the distribution of the NPI+ Groups between the Nottingham and Budapest series (Table 4, $p < 0.001$), a similar number of NPI+ Groups were evident in each of the Biological classes in both series Rakha et al. [8]. However, some of the poor prognostic groups were under-represented in the Budapest series due to the relatively lower frequency of highly proliferative tumours in the series.

NPI+ and risk of distant metastases

In the Nottingham Series, the biological classes were significantly associated with patient MFS where the luminal A (HR 1.57, $p = 0.006$) and luminal N (HR 1.49, $p = 0.024$) classes had a significantly better survival than the luminal B class (Figs. 1, 2). Luminal A tumours also had a significantly better MFS than basal p53 altered (HR 1.49, $p = 0.043$), basal p53 normal (HR 1.80, $p = 0.003$), HER2+/ER+ (HR 2.69, $p < 0.001$) and HER2+/ER− (HR 2.47, $p < 0.001$) tumours. Similarly, luminal N tumours had a significantly longer MFS than basal p53 normal (HR 1.66, $p = 0.014$), HER2+/ER+ (HR 2.47, $p < 0.001$) and HER2+/ER− (HR 2.19, $p < 0.001$) tumours but not the basal-p53 altered class ($p = 0.135$). There was no significant difference in MFS between basal p53 altered and basal p53 normal with the HER2+/ER+ or HER2+/ER− classes.

The NPI+ was used to determine the effect on MFS in the different molecular classes where NPI+ outcome prediction was compared with that achieved by the traditional NPI in each of the biological classes (Fig. 2a–n). In addition to improved outcome prediction using NPI+ compared with the traditional NPI in each class, NPI+ provided more clinically relevant stratification with splitting of each class into two or three prognostic groups compared with the six classes of NPI.

Fig. 2 Patient stratification for MFS with the classic NPI (*left*) ▶ compared with NPI+ (*right*) in each of the biological classes in the Nottingham series. **a–b** luminal A, **c–d** luminal N, **e–f** luminal B, **g–h** basal p53 altered, **i–j** Basal p53 normal, **k–L** HER2+/ER+, **m–n** HER2+/ER−. *EPG* excellent prognostic group, *GPG* good prognostic group, *MPG* moderate prognostic group, *PPG* poor prognostic group, *VPG* very poor prognostic group

When comparing the patient outcome in each of the NPI+ prognostic groups between the Nottingham and Budapest Series, there were no significant differences in MFS in the majority of them (Supplementary Fig. 2). Certain high-risk NPI+ Groups (luminal N; luminal B; basal p53 normal and HER2+/ER) could not be validated due to being under-represented in the Budapest series.

NPI+ Groups were additionally able to predict a higher risk of metastases to certain distant sites, summarised in Table 5. Bone metastases were significantly more likely to occur in the poor prognostic group of the luminal B, basal p53 altered, HER2+/ER+ and HER2+/ER− classes and a lower risk in the good prognostic group of luminal A, basal p53 normal, HER2+/ER+ and HER2+/ER− classes. Lung metastases were associated with the higher risk groups of Basal, HER2+ and luminal N tumours. Metastases to the brain were associated with HER2+ tumours only,

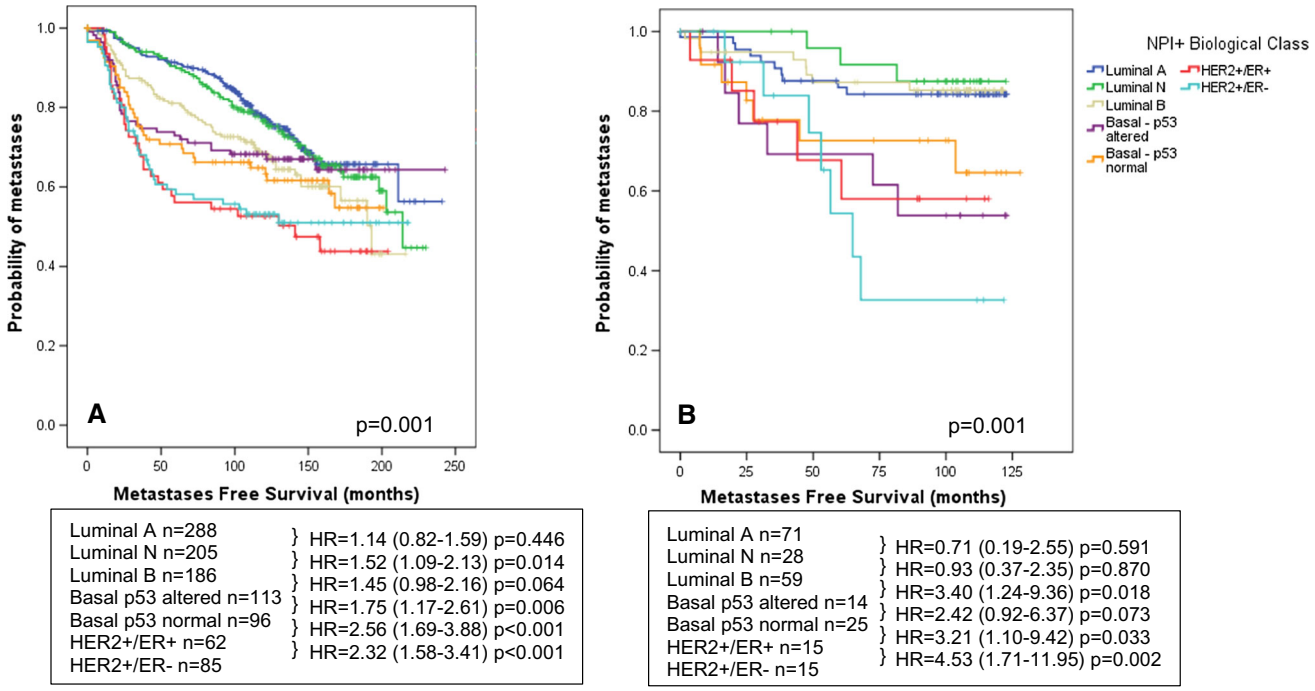
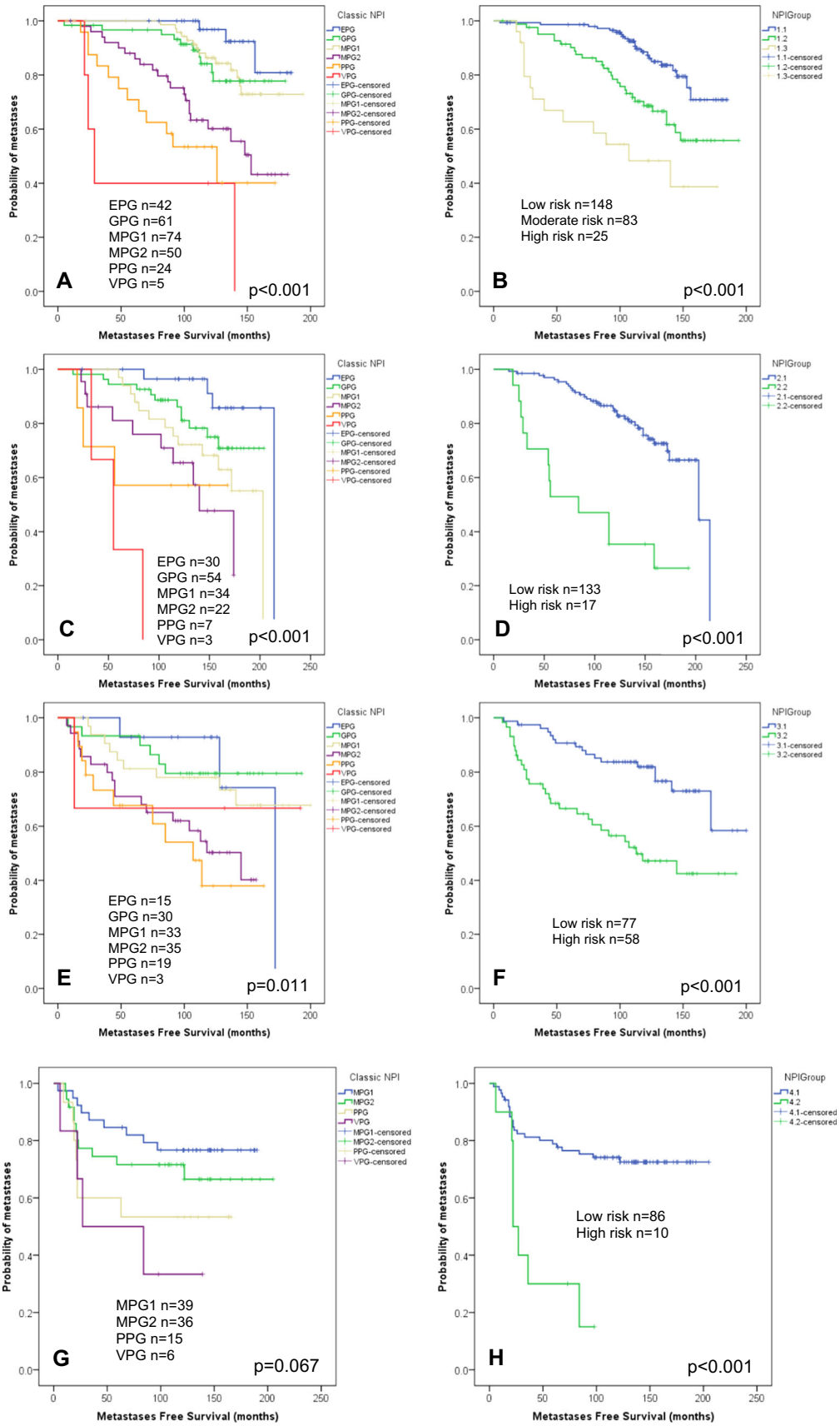


Fig. 1 MFS with respect to NPI+ biological classes. **a** Nottingham and **b** Budapest series


Classic NPI
EPG
GPG
MPG1
MPG2
PPG
VPG
EPG-censored
GPG-censored
MPG1-censored
MPG2-censored
PPG-censored
VPG-censored
Probability of metastases
Metastases Free Survival (months)
EPG n=42
GPG n=61
MPG1 n=74
MPG2 n=50
PPG n=24
VPG n=5
A
p<0.001
NPIGroup
1.1
1.2
1.3
1.1-censored
1.2-censored
1.3-censored
Low risk n=148
Moderate risk n=83
High risk n=25
B
p<0.001
EPG n=30
GPG n=54
MPG1 n=34
MPG2 n=22
PPG n=7
VPG n=3
C
p<0.001
2.1
2.2
2.1-censored
2.2-censored
Low risk n=133
High risk n=17
D
p<0.001
EPG n=15
GPG n=30
MPG1 n=33
MPG2 n=35
PPG n=19
VPG n=3
E
p=0.011
3.1
3.2
3.1-censored
3.2-censored
Low risk n=77
High risk n=58
F
p<0.001
MPG1 n=39
MPG2 n=36
PPG n=15
VPG n=6
G
p=0.067
4.1
4.2
4.1-censored
4.2-censored
Low risk n=86
High risk n=10
H
p<0.001

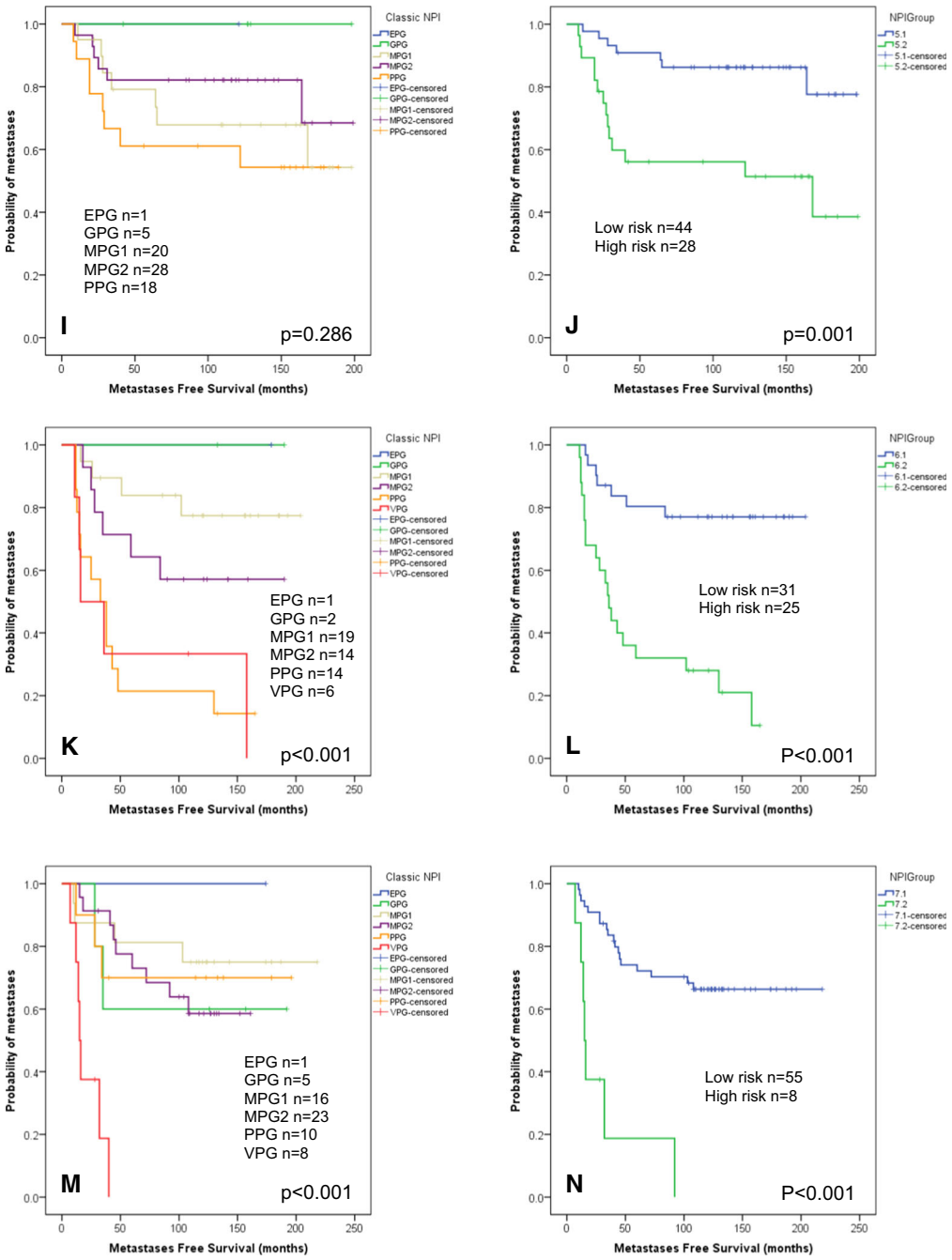


Fig. 2 continued

irrespective of ER status, with poor prognostic tumours having a higher incidence of brain metastases compared with good prognostic tumours. A higher incidence of liver metastases were associated with moderate and poor prognostic luminal A tumours and luminal B poor prognostic tumours compared with luminal A good prognostic tumours which had a significantly lower incidence ($p < 0.001$). Additionally, basal, both p53 altered and normal tumours, in NPI+ high-risk groups had a significantly higher incidence of liver metastases than basal p53 normal tumours in the low risk group. There was no association between NPI+ and number of metastatic sites (data not shown).

Discussion

Contemporary treatment of BC requires the integration of clinicopathological and biological information to ensure effective stratification of patients with regard to their

Table 5 NPI+ prognostic groups and site of distant metastases

	Site of distant metastases							
	Bone		Liver		Lung		Brain	
	n (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value
Luminal A								
Low risk	15 (10.1)	<0.001	9 (6.1)	<0.001	3 (2.0)	0.002	4 (2.7)	<0.001
Moderate risk	20 (24.1)		17 (20.5)		13 (15.7)		4 (4.8)	
High risk	10 (40.0)		8 (32.0)		3 (12.0)		4 (16.0)	
Luminal N								
Low risk	21 (15.8)		12 (9.0)		13 (9.8)		3 (2.3)	
High risk	8 (47.1)		3 (17.6)		3 (17.6)		1 (5.9)	
Luminal B								
Low risk	11 (14.3)		7 (9.1)		2 (2.6)		2 (2.6)	
High risk	25 (43.1)		13 (22.4)		6 (10.3)		2 (3.4)	
Basal p53 altered								
Low risk	11 (12.8)		7 (8.1)		8 (9.3)		9 (10.5)	
High risk	5 (50.0)		3 (30.0)		2 (20.0)		1 (10.0)	
Basal p53 normal								
Low risk	2 (4.5)		0		3 (6.8)		1 (2.3)	
High risk	24 (14.3)		6 (21.4)		3 (10.7)		3 (10.7)	
HER2+/ER+								
Low risk	4 (12.9)		7 (22.6)		2 (6.5)		0	
High risk	12 (48.0)		11 (44.0)		5 (20.0)		5 (20.0)	
HER2+/ER−								
Low risk	9 (16.4)		11 (20.0)		4 (7.3)		2 (3.6)	
High risk	3 (37.5)		3 (37.5)		3 (37.5)		3 (37.5)	

expected outcome and response to the ever increasing treatment options. Molecular gene assays, such as Oncotype DX® [21] and MammaPrint® [22], are limited in their clinical utility for the management of BC due to factors including reproducibility, validation, cost and targeting only certain BC patients.

We have developed the NPI+ based on the integration of clinical, histopathological and biological data; we determined using routine clinical methodology, which is envisaged to assist clinicians in offering a more personalised adjuvant treatment plan in all early-stage BC patients. Whilst the clinical utility of NPI+ in predicting patient survival has previously been determined [8], we had previously not explored the capability of NPI+ with respect to prediction of risk of metastases. We therefore sought to confirm the prognostic capabilities of NPI+ in predicting risk of metastases in two independent European series of BC (Nottingham and Budapest).

NPI+ uses well-established powerful clinicopathologic variables to stratify each of the biological classes into clinically distinct subgroups using bespoke NPI-like formulae. In the Nottingham series, each biological class was previously stratified into at least two prognostic groups which were predictive of breast cancer-specific survival [8]. Using these prognostic groups, we further show that the NPI+ can predict either a low or high risk of developing metastases after receiving standard adjuvant therapy. The combination of biological class and clinicopathological parameters used in the NPI+ provided enhanced prognostic information for patients with BC into more clinically relevant subgroups compared with the classic NPI. Those patients with an adverse risk of tumour relapse are clearly identified as candidates for additional or alternative forms of therapy as the conventional BC management at the time of diagnosis has failed to minimise metastatic disease. We also confirm that biological subtypes of BC are associated with particular metastatic behaviour where bone metastases were common across all biological subtypes, whereas brain metastases were specifically associated with HER2+ tumours [23].

We further sought to validate and confirm the prognostic capabilities of NPI+ in an independent series of BC from a separate centre (Budapest, Hungary). Although there was some difference in the overall distribution of size, stage and grade of tumours between the Nottingham and Hungary series, the number of NPI+ biological classes was

similar. This is consistent with the proportion of cancer subtypes reported in our previous validation [13] and other studies [9, 11, 22, 24–31], and further provides evidence that the classification of BC into seven biological classes can be achieved using a discrete panel of 10 proteins assessed by immunohistochemistry.

In the Budapest series, the NPI+ Prognostic Groups showed comparable MFS when compared with the Nottingham series in NPI+ biological classes: luminal A and basal p53 altered. The NPI+ Prognostic Groups with a low risk of metastases were similarly validated in the NPI+ biological classes: luminal N, luminal B, basal p53 normal along with the high risk NPI+ Prognostic Group in the HER2+/ER+ class. However, due to very small numbers of patients assigned in the Budapest series in the remaining NPI+ Prognostic Groups of biological classes, Luminal N, Luminal B, Basal p53 normal and HER2+/ER− could not be validated.

In conclusion, the NPI+ can stratify patients with BC of any biological class type into a category of expected low or high risk of developing metastases following conventional therapy and confirmed in an independent series of primary BC. It is envisaged that an NPI+ risk stratification index score could be developed to replace assignment of patients into either a good or adverse outcome group.

We confirm that the NPI+ is a reproducible tool that provides improved individualised clinical decision making for breast cancer by refinement of clinical prediction. Advantages in applying the NPI+ in clinical decision making for BC patients are improved prognostication and risk stratification, and the potential for health economic savings through appropriate targeted treatment. Additionally, NPI+ uses routine clinical samples and robust laboratory methods integrating easily into current international clinical practice. Further validation of the clinical validity of NPI+ using modern eta treatments in randomised clinical trial material is therefore warranted.

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Statement of author contributions ARG, DGP, GRB, JMG, EAR and IOE conceived the study; ARG, CCN, and DGP carried out experiments; ARG, DS, JS, MA, and DGP performed data analysis; JK, AMS, and AMT provided tissue microarrays together with clinicopathological and outcome data for Budapest cases. All authors were involved in data interpretation, writing the paper and had final approval of the submitted and published manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

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