Summary of evidence relating to MammaPrint reported in the Marchionni *et al.* systematic review[33]

Analytical validity	Clinical validity	Clinical utility
Two technical studies[60,61] provided evidence relating to the analytical validity of MammaPrint. Repeated gene expression measurements over time, within and across individual microarrays and across different laboratories, protocols, instruments and operators, provided data on the variability and reproducibility of the test. Buyse *et al.*[61] reported an overall success rate of the assay of 80.9% The systematic review concluded that the studies that used the 70-gene signature provided useful information about the validity of the biological correlations underlying the profile. However, although these studies suggested that MammaPrint could be used in a clinical setting, they could not be considered to be direct validations of the assay. The review also noted that evidence underpinning the analytical validity of the test was obtained from a limited number of patients and a moderate number of replications. The only validation study using the MammaPrint assay (rather than the underlying 70-gene signature) showed that only about 80% of fresh-frozen specimens were analysable	van't Veer *et al.*[63] reported on the development data for the 70-gene panel that formed the basis for the MammaPrint test. Using multivariate analysis, the 70-gene signature was found to be an independent predictor of metastases within 5 years, with an OR = 18 (95% CI 3 to 94) van de Vijver *et al.*[64] reported the first major validation of the 70-gene signature in a young (< 52 years) population with small (< 5 cm) tumours that were heterogeneous with respect to LN positivity, ER status, chemotherapy and tamoxifen treatment. Multivariate analysis showed that the MammaPrint prognosis group, tumour size and adjuvant chemotherapy were the strongest predictors of distant metastases. The 'poor prognosis' MammaPrint group had the largest HR (4.6, 95% CI 2.3 to 9.2). The authors demonstrated the prognostic value of the gene signature using survival curves stratified by conventional clinical indexes. The analyses showed substantial separation between 70-gene prognostic groups that were either low or high risk by clinical indices. **Optimal prediction was achieved when the gene index and conventional clinical predictors were combined** Buyse *et al.*[61] compared the MammaPrint assay with conventional clinical combination risk predictors in an independent, multicentre validation study. The specificity and sensitivity of the MammaPrint assay and the Adjuvant! Online algorithm were compared for prediction of distant metastases within 5 years and for death within 10 years. Similar sensitivities were found in both methods, but a higher specificity was demonstrated for MammaPrint. The areas under the receiver operating characteristic (ROC) curves were comparable for MammaPrint and Adjuvant! Online (0.68 vs. 0.66 for distant metastases at 5 years). However, with ROC values much closer to 0.50 than 1.00 neither prediction was particularly accurate Glas *et al.*[62] compared the commercial MammaPrint assay results with those obtained with a generic 70-gene signature test using the same patients as van't Veer and van de Vijver. The results of the 70-gene signature used in the original cohorts applied equally to the commercial MammaPrint assay based on the signature **Summary:** The authors concluded that, overall, the available published evidence supported MammaPrint as a better predictor of the 5-year risk of distant recurrence than traditional clinical predictors. However, the cohorts used for the development and validation of MammaPrint were considerably more clinically heterogeneous than those used for the OncotypeDX test. Despite this, MammaPrint had an 80% concordance with the OncotypeDX array-based RS classification when applied to the same patients. There was some evidence to suggest that the commercial MammaPrint test and a generic 70-gene signature assay produced comparable results	No studies on clinical utility were reported The systematic review did not identify any published studies evaluating the ability of the 70-gene signature or the commercial MammaPrint test to predict chemotherapy benefit

Systematic review summary

The review found studies that tested the MammaPrint assay, as well as studies about the 70-gene signature that the assay is based on. The studies that use the gene signature cannot be considered as validation of the assay itself. In terms of analytical validity, two recent papers looked at reproducibility between laboratories and found a good degree of agreement. RNA labelling emerged as a possible source of variation, and the question of reproducibility remains open. The only validation study using the MammaPrint assay itself showed that only 80% of fresh-frozen samples were useable, although it is hoped that the success rate would increase with the use of the assay. Studies of clinical validity overall show MammaPrint to be a better predictor of 5-year risk of distant recurrence than traditional algorithms and characteristics, although the validation and derivation cohorts were clinically more heterogeneous than those used for the OncotypeDX test. It remains to be seen how well it predicts in cohorts with greater homogeneity as used in the development of OncotypeDX. No studies that evaluated clinical utility were found

To conclude, the literature on the 70-gene signature includes numerous studies that focused more on its biological underpinning and less on the clinical implications of the gene expression profile. It is not yet clear which are the optimal patient populations for the use of this test, exactly what its performance is in those populations and how many of its predictions would result in different therapeutic decisions. Larger independent validation studies in therapeutically homogeneous groups are needed. Studies that test MammaPrint alongside standard predictors, develop the use of risk categories rather than a continuous scale and assess the assay's stability in different populations are also needed

Summary of evidence relating to MammaPrint reported in the Smartt systematic review[34]

Clinical validity	Clinical utility
Two studies on clinical validity were reported **Mook *et al.*[65]** *Rational and objective:* Patients with axillary LN metastases are generally considered to have a poor prognosis and most will be treated with adjuvant chemotherapy; however, up to 30% of these patients would remain free of distant metastases without adjuvant chemotherapy (Early Breast Cancer Trialists' Collaborative Group (EBCTCG),[189] 2005). In this study the authors sought to validate the prognostic value and accuracy of MammaPrint in an independent cohort of 241 patients with axillary LN metastases *Results:* 41% of patients in the independent cohort ($n=241$) had a good prognosis gene signature and 59% had a poor prognosis gene signature. There was a significant difference in DMFS (as the first event) and BCSS between the good and poor prognosis gene signature groups at both 5 and 10 years ($p<0.001$). The poor prognosis signature group was associated with a shorter BCSS (HR 5.70; 95% CI 2.01 to 16.23; $p<0.001$). The probability of distant metastases as the first event was significantly greater in the poor gene signature group (HR 4.13; 95% CI 1.71 to 9.96; $p=0.002$) In univariate analysis significant predictors of BCSS were the number of positive nodes, tumour grade, ER status, HER2 status endocrine treatment and MammaPrint risk group. Only the number of positive nodes, endocrine therapy and MammaPrint risk group remained significant predictors in multivariate analysis. MammaPrint was the most powerful independent predictor in this analysis (HR 7.17; 95% CI 1.81 to 28.43; $p=0.005$) Predictors of DMFS in univariate analysis were the number of positive nodes, tumour size, histological grade, ER and HER2 status, endocrine therapy and MammaPrint risk group. Only endocrine therapy was a significant independent predictor of DMFS in multivariate analysis (HR 0.31, 95% CI 0.12 to 0.80, $p=0.02$). MammaPrint risk group and number of positive nodes tended to be prognostic with HR = 2.99 (95% CI 0.996 to 8.99; $p=0.051$) and HR = 2.29 (95% CI 0.99 to 5.29; $p=0.053$) respectively Adjuvant! Online classified 13% of patients as low risk and 87% as high risk; Adjuvant! Online and MammaPrint risk assessments were discordant for 77 patients (32%); 72 of these discordant patients were assessed as having a high risk of relapse by Adjuvant! Online and a good prognosis gene signature When 209 Adjuvant! Online high-risk patients were stratified by MammaPrint the 10-year BCSS probability was 94% for the good prognosis gene signature group and 76% for the poor prognosis gene signature group (HR 4.12; 95% CI 1.45 to 11.76; $p=0.008$). Subgroup analysis suggested that MammaPrint was predictive for BCSS in patients in different treatment groups and patients with ER+ tumours	One study on clinical utility (indirect) was reported **Bueno-de-Mesquita *et al.*[67]** *Rationale and objective:* In most hospitals tumour samples are routinely fixed in formalin and embedded in paraffin blocks. MammaPrint requires fresh tumour samples and one of the potential difficulties in the implementation of the test in daily clinical practice is the ease with which sample requirements can be met. In this prospective multicentre study the authors set out to evaluate (1) whether or not MammaPrint was suitable for use in routine clinical practice in the Netherlands, (2) the effect of the test on the use of adjuvant systemic treatment, (3) the proportion of patients with 'poor' compared with 'good' prognosis and (4) the concordance between risk predicted by MammaPrint and risk predicted by commonly used clinicopathological tools *The patient population and eligibility criteria:* Patients were enrolled in this prospective multicentre study if they had unilateral primary operable invasive adenocarcinoma of the breast (TNM classification = T1–4, N0, M0) and were < 61 years of age. Sixteen participating Dutch hospitals contributed 812 women to the trial between 2004 and 2006. In total, 81 patients had breast-conserving surgery, 70% had small (< 2 cm) tumours, 81% had ductal histology, 80% had grade II–III tumours, 80% were ER+, 84% *ERBB2* negative and 85% LN–. Adjuvant systemic treatment varied: 39% of patients received no adjuvant treatment, 18% received chemotherapy, 13% received endocrine treatment and 29% received both chemotherapy and endocrine therapy. The median age of patients was 49 years and the median follow-up was 14 months (range 0.3–36.4 months). Hospitals were eligible to participate only if they had structured multidisciplinary breast cancer care, used standard operating procedures, treated at least 100 patients a year and had a dedicated physician as the local co-ordinator *Endpoints and analyses:* Differences between MammaPrint and commonly used histopathological guidelines were assessed using Pearson's chi-squared test and the Cochrane–Armitage test for trends. The level of agreement between different risk assessment techniques was assessed using Cohen's kappa. In addition to MammaPrint, the CBO guidelines,[105] Adjuvant! Online, the NPI and the St Gallen guidelines were used to assess clinical risk. MammaPrint analyses were carried out blinded to clinical data and an initial recommendation for treatment using clinical criteria carried out before disclosure of the MammaPrint results *Results:* Of the original 812 enrolled patients, 585 (72%) were eligible for the study. MammaPrint profiles were obtained in 427 (73%) of eligible patients. During follow-up five patients had distant metastases as the first event. According to MammaPrint, 51% of patients had a good prognosis signature compared with 57%, 31%, 58% and 17%, respectively, for the CBO,[105] Adjuvant! Online, NPI and St Gallen risk assessments

Clinical validity	Clinical utility
The second cohort of 106 previously studied patients[64] (with one to three positive nodes) differed significantly from the independent cohort in terms of age (younger), axillary procedures, adjuvant systemic therapy and overall and median survival (10.3 years, range 1.6–21.2 years). The 10-year BCSS probability was 98% for the good prognosis gene profile and 64% for the poor prognosis gene profile. The poor prognosis signature was associated with shorter BCSS (HR 6.60; 95% CI 1.97 to 22.10; $p=0.002$) and a multivariate HR of 3.63 (95% CI 0.88 to 14.76; $p=0.07$)	Clinical and molecular risk assessments were discordant in 27%–39% of patients depending on the clinical assessment tool used. The amount of discordance between the clinical guidelines themselves was between 7% and 40%. Adjuvant treatment was recommended for 48% of patients based on the Dutch guideline alone; this increased to 62% when the guideline was used with the prognostic gene signature. Overall, and once patient preferences had been taken into account, adjuvant systemic treatment was administered to 61% of patients. An increase in systematic therapy occurred in patients whose risk according to the Dutch guidelines and MammaPrint were discordant. In the final analysis, 50 (12%) more patients received endocrine treatment, 54 (13%) patients had endocrine treatment added and 4 (1%) patients had endocrine treatment withheld. Sixteen (4%) more patients had chemotherapy, in 35 (8%) patients chemotherapy was added and it was withheld in 19 (4%) patients
Conclusion: MammaPrint predicted disease outcome better than traditional clinical prognostic factors in patients with one to three positive nodes and was able to accurately identify LN+ patients with an excellent prognosis. The potential clinical utility of MammaPrint was demonstrated in 72 (34%) clinically high-risk patients with a good prognosis signature who had a 10-year BCSS of 94% and therefore might be spared chemotherapy	*Limitations:* There was an early protocol change reducing the age of eligibility to <55 years. It was not clear how representative the hospital sample was and the short follow-up time and low number of events precluded survival analyses
Wittner *et al.*[66]	*Quality:* This was a well-conducted prospective clinical trial that demonstrated the feasibility of conducting the MammaPrint test routinely in Dutch hospitals. As reported, the study fulfilled 35 of 44 (80%) REMARK criteria for the reporting of tumour marker prognostic studies indicating a high level of adherence to the reporting guidelines
Rationale and objectives: Most patients with breast cancer are older and present with smaller early-stage ER+ tumours than the cohorts of patients used to define and evaluate the MammaPrint gene signature. Decisions relating to the use of adjuvant chemotherapy in these older patients may be complicated by comorbidity. To explore these issues the authors carried out a retrospective evaluation of the prognostic value of MammaPrint in 100 older patients diagnosed and treated at the Massachusetts General Hospital (MGH) between 1985 and 1997. The study cohort of 100 patients was compared with the original Dutch cohort (NKI) of 151 LN0 patients used to validate the MammaPrint signature[64]	*Conclusion:* The study demonstrated a lack of congruence between well-known clinical guidelines for risk assessment in breast cancer. In approximately one-third of patients there was discordance between MammaPrint and clinical guidelines in the assessment of risk. The addition of MammaPrint to the standard Dutch clinical assessment of risk (modified by patient preference) increased by 20 the number of patients receiving adjuvant systemic therapy. However, although the study was able to demonstrate that MammaPrint had an impact on clinical decision-making the follow-up was not long enough to provide evidence of its effect on clinical end points such as DMFS or its utility in predicting treatment benefit
The patient population and eligibility criteria: Eligible MGH patients were consecutively diagnosed and treated patients with LN0 breast cancer and frozen primary tumour samples for whom histopathological and clinical information could be retrieved. The median age of the cohort was 62.5 years and the median duration of follow-up was 11.3 years (range 1.2–18.5 years). In total, 72% of patients had small tumours (≤2 cm), 94% were of histological grade II–III. A total of 21% of patients received chemotherapy and 24% hormonal therapy. Surgery included mastectomy (56%) and breast conservation (44%)	One study published as a conference abstract reported on clinical utility
Results: The MGH cohort was significantly older ($p<0.001$) than the original MammaPrint cohort.[64] There were also significant differences ($p<0.005$) in tumour size, histological grade and the proportion of patients undergoing systemic treatment	**Bender *et al.*[68]**
MammaPrint classified 27% of the MGH patients as low risk and 73% as high risk of distant metastases as the first event. The cohort had a significantly lower event rate than the original NKI cohort ($p<0.001$); there was no difference in OS in the older MGH cohort because of death from other causes. Survival analysis discriminated between the high- and low-risk gene signature with non-overlapping CIs; however, because of the low event rate the difference was not significant. This contrasted with the significant difference between the low- and high-risk groups reported for the original Dutch NKI cohort	In this study the authors present the results of a meta-analysis of 1637 patients with MammaPrint outcomes (T1–2, LN–/+ invasive breast cancer and median follow-up 7.1 years) to determine the chemotherapy benefit of patients treated with adjuvant chemotherapy in addition to endocrine therapy. Patient samples were recruited from seven large data sets from multiple institutions across Europe
	MammaPrint assigned 772 patients (47%) to a low-risk category and 865 (53%) to a high-risk category. In total, 349 patients (21%) were treated with endocrine therapy and 226 (14%) were treated with chemotherapy and endocrine therapy. In patients with a poor prognosis MammaPrint profile the 5-year DMFS improved from 69% to 88% (HR 0.28, 95% CI 0.14 to 0.56, $p<0.001$) when chemotherapy was added to hormone therapy. In multivariate analysis patients classified by MammaPrint as having good prognosis had no significant benefit from chemotherapy ($p=0.962$)

Clinical validity	Clinical utility
The NPV of MammaPrint in the MGH cohort was 100% (overall and at 5 and 10 years) compared with 88% in the original NKI cohort. The PPV was only 12% in the MGH cohort (because of the large number of patients classified as high risk who did not have distant metastases as the first event) compared with 52% in the NKI cohort. Sensitivity analysis varying the cut-off/classification threshold of MammaPrint did not improve the PPV. In a comparison between the Adjuvant! Online 10-year relapse risk for each MGH patient and MammaPrint, the latter identified an additional 21 patients who did not develop distant metastases as the first event, and an additional five patients when considering DMFS per se *Conclusion:* MammaPrint had a high NPV and provided some information that was additional to that provided by Adjuvant! Online. However, with an extremely low PPV and insignificant differences in OS between MammaPrint high- and low-risk patients the prognostic utility of MammaPrint in this population remained unproven. Moreover, although MammaPrint classified a significant proportion of study patients as high risk, few of these developed metastatic disease Four studies published as conference abstracts reported on clinical validity **Glas *et al.*[70]** Patients with ER+, LN0 from the original validation series[63] were analysed for MammaPrint outcome according to grade. Kaplan–Meier analysis of 106 patients for DMFS at 10 years showed a significant difference between low risk (56 patients, 53%) and high risk (50 patients, 47%) with a HR of 4.7 (95% CI 2.1 to 10.4). Good prognosis (low-risk) patients had a 10-year survival of 86%. In patients with grade II, ER+, LN0 breast cancer a significant separation of patients with good or poor prognosis according to MammaPrint was observed ($p = 0.001$). The probability of developing distant metastasis in the good prognosis group was $<10\%$; in the poor prognosis group it was 44%. MammaPrint provided a significant separation in recurrence risk in these patients, which improved guidance for the requirement of adjuvant therapy **de Snoo *et al.*[69]** A total of 566 tumour samples from women with ER+, LN0, HER2– breast cancer from five previously reported studies were classified using MammaPrint and the NCCN guidelines,[129] and the 10-year BCSS determined according to each MammaPrint classified 380 (57%) samples as having a good prognosis and 186 (33%) as having a poor prognosis. The NCCN guidelines[129] classified 7% as low risk and 93% as high risk. MammaPrint also identified approximately 66% of NCCN high-risk patients as having a good prognosis. There was an overall discordance between the two tools in 62% of cases. In total, 349 (62%) patients received no adjuvant treatment, 17% received hormone treatment only, 2% chemotherapy only and 20% both	It was concluded that MammaPrint poor-prognosis/high-risk patients demonstrated a benefit when adjuvant chemotherapy was added to hormone therapy. Patients classified by MammaPrint as good prognosis/ low risk for recurrence do not appear to benefit from the addition of chemotherapy to hormone treatment

Clinical validity	Clinical utility
MammaPrint predicted a 10-year BCSS of 91% vs. 67% for the good and poor prognosis groups respectively (HR 4.0, 95% CI 2.0 to 7.9, $p<0.001$). NCCN guidelines[129] predicted a BCSS of 86% vs. 83% for the low- and high-risk groups respectively (HR 1.11, 95% CI 0.3 to 4.6, $p=0.888$). Median follow-up was 3.5 years (range 0.1–21.1 years). In multivariate analysis (adjusted for known prognostic factors and adjuvant therapy), only MammaPrint and histological grade were independent predictors for 10-year BCSS with HRs of 2.8 (95% CI 1.3 to 6.1, $p=0.008$) and 1.9 (95% CI 1.1 to 3.1, $p=0.015$) respectively. It was concluded that MammaPrint was a strong and independent prognostic indicator in ER+, LN0, HER2– breast cancer	
Knauer *et al.*[71] In this study the authors used MammaPrint to assess prognosis, BCSS and DMFS in 965 pT1 breast cancer tumour samples from seven previous studies. MammaPrint classified 526 patients (55%) as having a good prognosis and 439 (45%) as having a poor prognosis. In total, 562 patients (59%) received no adjuvant treatment, 19% received hormone treatment only, 10% received chemotherapy only and 12% both hormone therapy and chemotherapy. MammaPrint accurately predicted differences in 10-year DDFS (HR 2.7, 95% CI 1.9 to 3.9, $p<0.01$) and BCSS (HR 4.0, 95% CI 2.6 to 6.3, $p<0.01$) for all T1 tumours. Similar results were obtained in multivariate analysis for all patients, adjusted for known prognostic factors and adjuvant therapy, as well as for adjuvant therapy-untreated patients. For the pT1a/b subgroup ($n=140$), 10-year DDFS was 93% vs. 78% for the good and poor prognosis groups (HR 3.9, 95% CI 1.0 to 15.2, $p=0.048$), whereas in the T1c subgroup ($n=825$) DDFS was 86% vs. 72% respectively (HR 2.6, 95% CI 1.8 to 4.0, $p<0.01$). BCSS was 87% vs. 73% for the good and poor prognosis groups in the T1a/b subgroup (HR 2.4, 95% CI 0.8 to 7.7, $p=0.128$) and 92% vs. 72% for the good and poor prognosis groups in the T1c subgroup (HR 4.4, 95% CI 2.7 to 7.1, $p<0.01$) It was concluded that MammaPrint was a strong and independent prognostic indicator in small breast tumours	
Saghatchian *et al.*[72] It has been shown that MammaPrint predicts disease outcome in patients with one to three positive nodes and four to nine positive nodes. In this study the authors report a further analysis of 519 LN+ patients from a consecutive series of patients from two hospitals based on adjuvant treatment received. Female patients diagnosed between 1984 and 1995 with LN+, unilateral T1, T2 or operable T3 primary invasive breast carcinoma who received mastectomy or breast-conserving therapy and for whom fresh-frozen tumour material was available were eligible for the study In total, 346 patients had one to three positive lymph nodes and 173 had four to nine positive lymph nodes. Tumours were classified by MammaPrint as good prognosis/low risk in 212 patients (41%) and poor prognosis/high risk in 307 patients (59%) with strictly equal proportions among the two LN groups. With a median follow-up of 10.3 years, distant metastases occurred in 141 (27%) patients (116 as first event), and 103 (20%) died of their disease. It was concluded that combining nodal status and MammaPrint profiling allowed patients to be stratified for tailored treatment strategies. Patients with an elevated number of LNs and high genomic risk had a very poor prognosis and might need to be considered for stronger treatment combinations	

Systematic review summary

This review updates the review by Marchionni *et al.*[33] and found an additional 11 studies, some journal publications and some conference abstracts. Analytical validity remains a weakness of the evidence base for MammaPrint, with no new studies identified. The majority of studies found across the two reviews provide evidence relating to the clinical validity of the test in heterogeneous populations. The additional studies reporting on the clinical validity of the test sought to validate the prognostic value and accuracy of MammaPrint in an independent cohort and to extend previous experience of the test in older patients with small tumours. Four studies reported subset analyses of data reported in previous studies examining the use of the test in very heterogeneous populations. The evidence relating to the clinical validity of MammaPrint was not always conclusive or supportive of the prognostic value of the test. Four studies suggested that the ratio could predict prognosis, one study failed to verify the prognostic utility of the test and in another the methods and results were at variance with those of other studies. Three studies focusing on clinically utility were identified: one journal article and two conference abstracts. The fully reported study provided important evidence of the potential impact of MammaPrint on decision-making in Dutch hospitals and the concordance between the gene profile and commonly used clinicopathological tools for risk prediction. A second study, published as an abstract only, presented initial results of a meta-analysis of 1637 patients from seven large multinational data sets to determine the benefit of adding adjuvant chemotherapy to endocrine therapy. The encouraging results of this study may eventually provide strong enough evidence to provide reasonable justification for the interim use of the test in women in the same population group as the trial patients. One study examined the budgetary impact of MammaPrint. As in the original review, the evidence for the clinical implications of using MammaPrint remains unclear