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REVIEW

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Primed for death: prognostic role of BH3--only proteins in breast cancer therapy: a systematic and meta-analysis review

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Abstract

Background Breast cancer (BC) is the primary cause of cancer-related deaths among women. BH3 only proteins expression profile in BC has been linked to chemotherapy and treatment outcomes. Clinical biomarkers provide insights into disease progression. Therefore, we systematically investigated the prognostic significance of proapoptotic Bcl-2 Homology Domain 3 (BH3) only proteins in Disease-Free Survival (DFS) and Overall Survival (OS) among BC patients.

Methods We explored four databases, screening titles, abstracts, and full articles based on predefined criteria. The quality of cohort studies and randomized clinical trials were assessed. Data of BH3-only gene and proteins' expression were extracted and meta-analysis using random effects model was performed.

Results Of the 3541 studies identified, nine studies met inclusion criteria. The meta-analysis revealed that the BH3-only protein-positive group had a higher chance of 5-year DFS risk ratio (RR = 1.17, 95% CI [0.94, 1.46], $P = 0.16$) and significantly improved 10-year DFS (RR = 1.32, 95% CI [1.15, 1.50], $P = 0.0001$). Subgroup analysis indicated that BCL-2 antagonist of cell death (BAD) positive expression significantly correlated with improved 5-year DFS (RR = 1.34, 95% CI [1.06, 1.70], $P = 0.02$), while p53 upregulated modulator of apoptosis (PUMA) positive expression showed a limited association with improved 5-year OS (RR = 1.13, 95% CI [1.03, 1.25], $P = 0.01$). Conversely, BCL-2 interacting killer (BIK) positive expression was significantly associated with worsened 5-year DFS (RR = 0.84, 95% CI [0.73, 0.97], $P = 0.02$) but not OS.

Conclusion While BAD and PUMA positive expression correlated significantly with patients' improved OS and/or DFS, BIK and BCL-2 interacting mediator of cell death (BIM) high expression were correlated with poor survival outcome. This data suggests, despite being from the same family, these BH3-only proteins induce different tumor survival signaling pathways, that could play role in predicting/leading to a good or poor OS as well as DFS outcomes

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in BC patients after treatment. Limited data suggests further studies are needed to confirm BAD and PUMA BH3-only protein positive expression as independent prognostic variables for 5-year DFS and OS, respectively, in BC patients.

Keywords Mitochondrial priming, Breast Cancer, BC, BH3–only proteins, Systematic review meta-analysis, BAD, BID, PIM, PIK, PUMA

Introduction

Globally, the incidence of breast cancer presents a growing health concern, having risen dramatically over the past decade. In 2008, there were an estimated 1.38 million new cases of breast cancer worldwide [1]. By 2020, this number had surged to 2.3 million, representing a staggering increase of approximately 67% [2] and solidifying breast cancer as the most commonly diagnosed cancer globally. One of the characteristics of cancer is the ability to escape apoptosis through molecular and cellular modifications, and defective induction of apoptosis is a significant contributor to resistance to response to therapy [3]. The molecular pro- and anti-apoptotic machinery provides a pivotal frontline innate response for the early riddance of cancer cells, but ironically becomes effective weapons for the development of resistant generations of those cells. Studies linking the delicate balance between pro- and anti-apoptotic cellular responses with clinical outcomes in cancer therapy are desirable tools for improving current protocols and discovering new therapeutic targets.

Numerous signaling pathways can change the proportion of pro- to anti-apoptotic subfamily members in a cell, sending information reporting cellular stress in the form of protein modifications, available nutrients, and DNA damage [4]. After the executioners are turned on, the molecules join forces to create pores in the mitochondrial outer membrane (MOM), which then causes permeabilization (MOMP), cytochrome c release and apoptosis [5–7]. The B cell CLL/lymphoma-2 (BCL-2) protein family controls apoptosis by regulating the MOMP process. The BCL-2 family is made up of a series of related proteins that are classified into three classes based on the α-helical composition of BCL-2 homology domains (BH): anti-apoptotic BCL-2 proteins that down regulate MOMP, pro-apoptotic BCL-2 effectors that mediate the permeabilization of mitochondrial outer membrane, and pro-apoptotic Bcl-2 Homology Domain 3 (BH3-) only proteins which is another subclass of pro-apoptotic BCL-2 that acts as gear-like regulators of the two arms of the Bcl-2 protein factors in response to damage signals [8–10] (Fig. 1).

The BH3-only proteins family includes BAD (BCL-2 antagonist of cell death), BID (BH3 interacting domain death agonist), BIM (BCL-2 interacting mediator of cell death), BMF (BCL-2 modifying factor), PUMA (p53 upregulated modulator of apoptosis), BIK (BCL-2 interacting killer), HRIC (Harakiri), BMF (Bcl-2-modifying factor) and Noxa. Depending on their capacity to interact with effector and anti-apoptotic proteins, the BH3-only proteins are frequently split into two classes: the direct activators proteins such as BID, BIM, BIK, PUMA, NOXA, and the sensitizers/ de-repressors which include BAD, BMF, and HRIC. Direct activators not only bind and inhibit anti-apoptotic proteins but can also directly activate the effector proteins BAK and BAX, leading to their oligomerization and MOMP. Furthermore, the direct activator proteins (such as BID and BIM) directly activate BAK and BAX inducing permeabilization of the mitochondria. However, the sensitizers/de-pressors have a pro-apoptotic effect by vying with the anti-apoptotic BCL2 family members for specific binding and releasing activators and effectors and thus promote MOMP [8, 11].

It has been postulated that the expression of BH3-only proteins (Fig. 2) could predict cell fate decisions in response to toxic treatments targeting malignancies, which is a step toward customized therapy where the response to a medication can be evaluated prior to administration [12]. A promising approach has since then unfolded based on the assessment of mitochondrial loss of transmembrane potential or mitochondrial priming [13] which conveys the proximity of a cell to MOMP. In this paradigm, mitochondrial priming controls whether a cell undergoes apoptosis in response to an insult, and thus can be extrapolated to predict how tumors will respond to chemotherapy [14–16]. Therefore, BH3-profiling, when used on basic cancer cells, can predict how patients would react to chemotherapy [14, 17].

Studies reported differential expression of BH3 proteins in response to chemotherapy and their association with recurrence, disease-free survival (DFS) and overall survival (OS), in treated BC individuals [18–20]. Considering the available data and the expanding demand for biomarkers that might predict a patient's prognosis and responsiveness to therapy, we aimed to conduct a systematic review and meta-analysis of the role of BH3-only protein expression as prognostic factors in OS and DFS in treated BC patients.

Materials and methods

Registration with PROSPERO website for systematic review has been approved with CRD42021256713 and available from: "https://www.crd.york.ac.uk/prospero/display_record.php? ID=CRD42021256713".

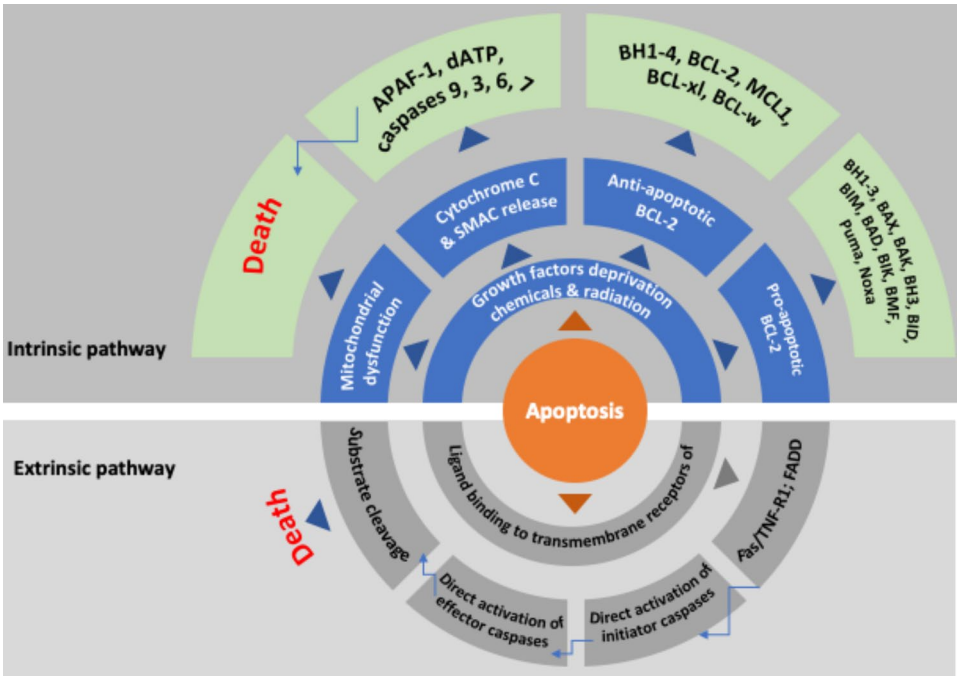

Fig. 1 Apoptotic pathways in the normal cell; the balance between the pro-apoptotic (BH1-3, BAX, BAK, BID, BIM, BAD, BIK, BMF, Puma, and Noxa) and the anti-apoptotic (BH1-4, BCL2, MCL1, BCL-xl, and BCL-w) BCL-2 family members control the mitochondrial apoptotic pathway. Apoptosis-activating factor 1 (APAF-1) binds to cytochrome c and the second mitochondria-derived activator of caspases (SMAC) release to create the apoptosome from activated BAK and BAX go to the mitochondria where they oligomerize and cause permeabilization of the MOM. Caspase-3, Caspase-6, Caspase-7, Caspase-9, and cell death are triggered as a result of this. Apoptosis is triggered by the activation of cell-surface death receptors like FAS by their ligands (the extrinsic pathway) or by pro-apoptotic proteins from the BCL-2 family that permeabilize the MOM. Tumor necrosis factor (TNF) receptor 1 (TNFR1) occupancy can start FAS-associated death domain protein (FADD) apoptosis, which causes necrosis

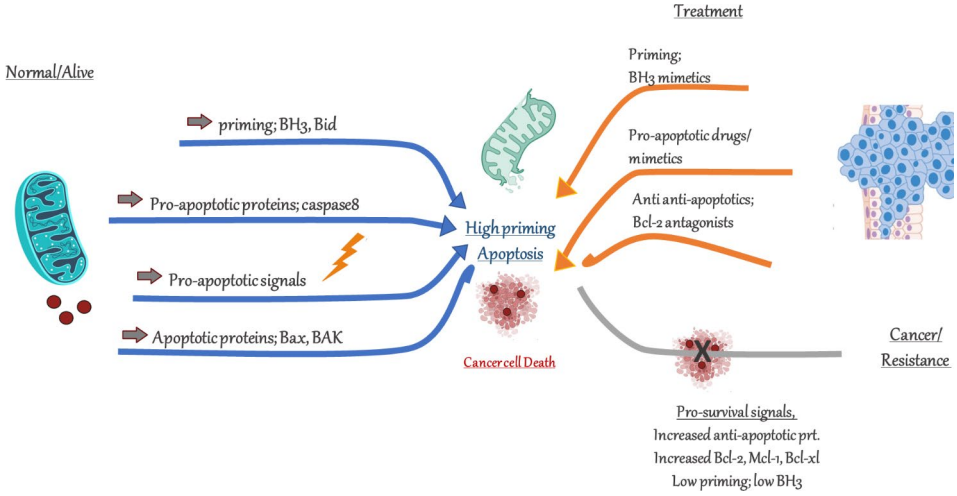

Fig. 2 Mitochondrial priming and cancer cells. Deregulation of apoptosis, which can result in the unintended survival of rogue cells, is a major factor in carcinogenesis. In order to cause apoptosis in cancer cells, medications known as BH3-mimetics target the antiapoptotic members of the BCL-2 protein family. BCL-2 proteins that promote survival also make patients resist to conventional therapies like chemotherapy and radiotherapy, which work by inducing cell death. In multicellular organisms, controlled cell death is a crucial and dynamic process that preserves tissue homeostasis and gets rid of potentially harmful cells. The caspase family of proteases, which are activated by both the intrinsic and extrinsic pathways of apoptosis, are in charge of the final cell death during the so-called execution phase of apoptosis. In addition, by cleaving the Bcl-2 family protein Bid, which then translocate to mitochondria, activating caspase-8, the initiator caspase in the Fast-mediated apoptotic pathway, can also trigger activation of the mitochondrial apoptotic pathway

Literature search strategy

A comprehensive literature search was conducted by two investigators searched four databases of Web of Science, PUBMED, Scopus, and Cochrane using the following keywords and search terms: (((Breast OR Breasts OR "Breast"[Mesh]) AND (cancer OR cancers OR Neoplasm OR Neoplasms OR carcinoma OR Carcinomas OR Tumor OR Tumors OR "Neoplasms"[Mesh] OR "Triple Negative" OR "Triple-Negative" OR "ER-Negative PR-Negative HER2-Negative" OR "ER Negative PR Negative HER2 Negative")) OR (TNBC OR "Triple Negative Breast Neoplasms"[Mesh])) AND ((BH3- OR Trihydridoboron OR Borane OR Borine OR "BH3–interacting domain death agonist" OR "BH3- Interacting Domain Death Agonist Protein"[Mesh]) OR ("Bax-like BH3- protein" OR BID protein OR Bid Protein OR "Bcl-2-Like Protein 11" OR "Bcl-2-Like Protein 11"[Mesh] OR BIM Protein OR BCL2L11 OR "BCL2 associated agonist of cell death" OR "bcl-Associated Death Protein" OR "bcl-Associated Death Protein"[Mesh] OR "bcl2-Antagonist of Cell Death Protein" OR "BCL2 Interacting Killer" OR BIK protein OR BMF protein OR "Bcl2 Modifying Factor" OR hRIC-3 protein OR "human RIC?3" OR RIC?3 OR Puma protein OR "p53 upregulated modulator of apoptosis" OR "Bcl-2-binding component 3" OR Noxa protein OR "Phorbol-12-myristate-13-acetate-induced"))). This comprehensive search strategy yielded a total of 3541 articles across the four databases. Last search update was conducted in November 2023.

Eligibility criteria and study selection

Inclusion criteria

Studies' titles, abstracts, and full articles were screened, for eligibility, by two authors, independently, using defined inclusion criteria as follow: [1] studies reporting BC association to BH3-only proteins and survival (OS and DFS), [2] studies reporting BH3-only proteins; Bid, Bim, Bad, BIK, BMF, HRIC, Puma, Noxa in BC, [3] retrospective or prospective study investigated the association.

Exclusion criteria

Reviews, conference abstracts, review, letters, commentaries, book chapters, case series, and case reports as well as studies published in languages other than English and studies reporting malignancies other than BC were all excluded. Studies with duplicated or without sufficient data were excluded as well. We carefully screened the references of all excluded review articles to ensure that no relevant studies were missed during our literature search.

Quality assessment and risk of bias (RoB)

Two investigators (NA and TA) assessed the risk of bias and the quality of individual eligible studies independently. Disagreement between the two investigators was resolved by consulting a third investigator (MA). The NewCastle-Ottawa scale assessment (NOS) https://www.ohri.ca/programs/clinical_epidemiology/oxford.asp was used to assess the quality of cohort studies [21]. Thresholds for converting the NOS to express the specific commitments of the Agency for Healthcare Research and Quality (AHRQ) standards (good, fair, and poor). Good quality; 3 or 4 stars in selection domain AND 1 or 2 stars in comparability domain AND 2 or 3 stars in outcome/exposure domain. However, fair quality; 2 stars in selection domain AND 1 or 2 stars in comparability domain AND 2 or 3 stars in outcome/exposure domain. Finally, the poor quality; 0 or 1 star in selection domain OR 0 stars in comparability domain OR 0 or 1 stars in outcome/exposure domain.

Grading and certainty assessments

GRADE technique was used to assess the overall certainty of the body of evidence for the outcomes deemed significant or relevant by clinical professionals. The GRADE approach yields an assessment of the quality of a body of evidence in one of four categories for each outcome: high, moderate, low, or very low [22].

Data extraction

Data from eligible studies was extracted by two independent researchers (NA and TA) and any discrepancies between them were resolved by consulting a third investigator (SO).

The following data were gathered from each included article: study design, name of the first author, year of publication, BC type, sample size, sex, ethnicity, definition of response and non-response, genotype distributions, survival (OS and DFS) data. Data from Kaplan Meier curves of 5-years and 10-years and survival were extracted by two investigators independently using GraphGrabber 2.0.2 https://www.quintessa.org/software/downloads-and-demos/graph-grabber-2.0.2. Data was confirmed using WebplotDigitizer 4.4 software https://automeris.io/WebPlotDigitizer/ [23].

Statistical analysis

Data analysis was performed by one investigator (MA) and confirmed by another investigator (SO). For the purpose of the study "prognostic value of BH3-only proteins' positive expression in BC" meta-analysis was performed using Cochrane Collaboration Review Manager Software (RevMan-computer program version 5.4) https://training.cochrane.org/online-learning/core-software/revman and presented in forest plot at a glance https://uk.cochrane.org/news/how-read-forest-plot. Risk ratio (RR) with 95% confidence intervals (CIs) was used to estimate the association strength between BH3-only proteins expression in BC and the 5-years and 10-years survival

outcomes (OS and DFS). Heterogeneity between studies was assessed using I^2 (with Chi-square (Chi^2) and interpreted following the guidelines outlined in the Cochrane Handbook for Systematic Reviews of Interventions [24] https://training.cochrane.org/handbook and the updates by [25]. The possible explanations for the heterogeneity were investigated using random-effects analysis and subgroup analyses of various members of BH3-only proteins.

Results

This systematic review meta-analysis was designed and followed PRISMA and meta-analysis guidelines [26].

Eligible studies

A total of 3541 articles were retrieved by a literature search using the search strategy previously mentioned. Following the removal of duplicate articles (1293 articles), 2248 items were subjected to title and abstract screening. Around 98 articles were subjected to full text screening. As shown by the flow diagram in (Fig. 3), nine studies met the inclusion criteria and were included in this meta-analysis review.

Study characteristics

The main features of the nine eligible studies are summarized in (Table 1). Collectively, the chosen studies included a total of 3041 patients who were assessed using protein expression and 582 patients who were assessed using mRNA expression method. Around 7/9 studies were conducted in European or North American populations (1775 patients), whereas one was conducted in East Asian populations (275 patient), and one was conducted in south American population (1355 patients). The sample sizes in all the eligible studies ranged from 51 to 1276 patients (mean=276 patients with standard deviation (SD)=333). Data related to patients treated by neoadjuvant chemotherapy comprised 6/9 of the BC trials. Immunohistochemistry (IHC) techniques were used in 8/9 trials to detect the expression of BH3 -only protein. Various antibodies were used to assess BH3-only

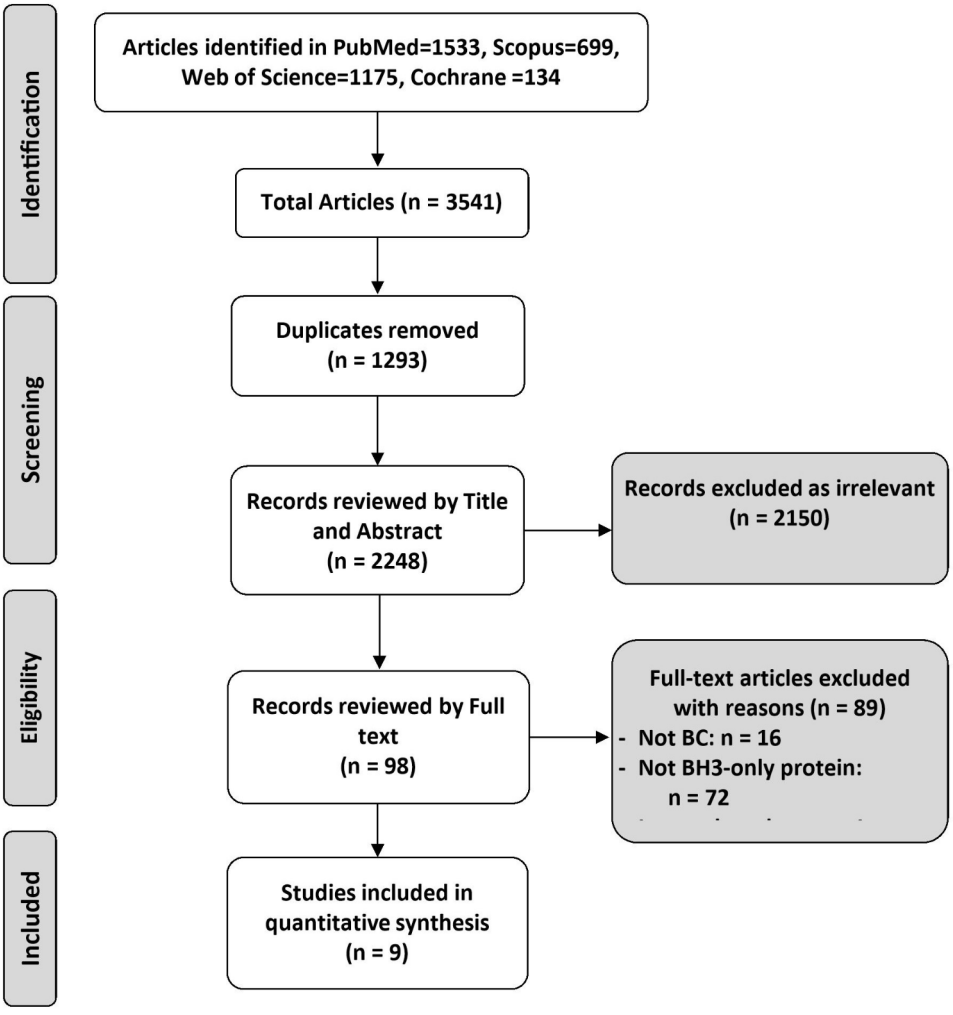

Fig. 3 Flow chart used to select eligible studies (PRISMA)

Table 1 Summary of the studies characteristics included in the systematic review meta-analysis

Author, year, country	Study type	Sample size	Tumor characteristics		Treatment & chemotherapy type	Prognosis	The assessment method	Cutoff value	Mitochondrial priming parameter
			Molecular subtype	Grade					
Al-Bazz 2009, UK	cohort	51 frozen BC samples & 106 fixed BC samples	62 ER positive, 44 ER negative, 42 PR positive & 63 PR negative	in the 51 patients' group; 7 grade 1, 29 grade 2, 15 grade 3 & in the 106 patients' group; 18 grade 1, 57 grade 2, 31 grade 3.	No chemotherapy or radiotherapy before the study	in 51 group: 13 patients died from BC, 3 patients alive with recurrence, 35 patients alive free of disease	Immunohistochemistry	no staining present in any of BC cells (–), slight staining in some cells or in most of the cells (+), moderately strong staining (++) or strong staining present in all cells (+++). Groups stained – & + combined, scored 0, groups stained ++ & +++ scored 1	BAD
Canevari 2016, Brazil	cohort	79 sample & 1276 samples used in tissue microarray validation experiments	931 HER2 negative, 146 positive & 199 not determined. 618 PgR negative, 520 positive & 138 not determined. 374 ER negative, 796 positive & 106 not determined.	locally invasive ductal cell carcinoma	Segmental resection or mastectomy, dissection of axillary LN & radiotherapy & adjuvant systemic therapy. Tamoxifen	metastases	Immunohistochemistry	scores denominated as "negative/weak" (score 0–1) or "positive" (score 2–3).	BAD
Cannings 2007, UK	retrospective cohort	402	402 ER+	99 grade1, 193 grade 2, 99 grade 3, 11 unknowns	Chemotherapy Tamoxifen	74 BC-specific deaths & 100-BC relapses	immunohistochemistry	Intra class correlation coefficients calculated for Bad (0.94), pBad (Ser 112) (0.96), Bax (0.89), Bcl-2 (0.96) & Bcl-xl (0.95)	BAD
Maimaiti 2017, China	cohort	275	119 Luminal A1, 65 Luminal B, 32 HER2 overexpression, 59 TNBC.	251 Grade 1–2 & 24 Grade 3	surgery & no chemotherapy or radiotherapy before surgery	NR	NR	Positive Bim staining; presence of cytoplasmic staining in 20% of malignant cells. ER-positive & PR-positive tumors defined as staining of at least 1% of nuclei	BIM
Craik 2010, USA	retrospective cohort	180	BC cell lines, MCF-7, SKBR-3, T47-D		Chemotherapy Taxane (paclitaxel or docetaxel)	NR	NR	Staining intensity scored as 0 (absent), 1 (weak) or 2 (strong). Cutoff point to dichotomize a continuous variable is 0.57.	BAD

Table 1 (continued)

Author, year, country	Study type	Sample size	Tumor characteristics		Treatment & chemotherapy type	Prognosis	The assessment method	Cutoff value	Mitochondrial priming parameter
			Molecular subtype	Grade					
Jesús 2021, Mexico	retrospective cohort	53	24 luminal A, 29 luminal B HER2/Neu enriched & TNBC	24 grades IIB & IIIA, 29 grades IIIB & IIIC	surgery & preoperative chemotherapy. 37 patients received: 5-Fluorouracil, Epirubicine & Cyclo-phosphamide & Docetaxel 3 patients received in addition Paclitaxel and/or Carboplatin, 1 patient received tamoxifen	23 patients experienced disease recurrence & 28 deaths	immunohistochemistry	Staining intensity was graded as 1 = weak, 2 = moderate, 3 = strong. Staining extent was converted into a number with 1 = 0 to < 10%, 2 = 10 to < 50%, & 3 = 50–100%.	BIK
Pandya 2016, Canada	Retrospective cohort	175 in dataset 1	luminal 116, HER2 amplified 56, TNBC 21	NR	NR	11% recurrence and 16.5% death	mRNA expression	NR	BIK
	cohort	152 in dataset 2	Luminal 112, HER2 amplified 9, TNBC 56	NR	NR	49% recurrence and 27% death	immunohistochemistry	Staining intensity for each core scored on a relative scale between 0–3. ROC analysis identified cutoff ≥1.5	BIK
Roberts 2011, USA	cohort	292	200 ER+, 167 PR+, 54 HER2 amplified.	NR	Chemotherapy & Tamoxifen	NR	immunohistochemistry	Intensity of cytoplasmic staining of PUMA (0: negative, 1þ: weak, 2þ: moderate, 3þ: strong) & % of cells staining positive.	PUMA
	Retrospective cohort	NKI cohort of 295	NR	NR	NR	NR	mRNA expression	NR	PUMA
Karbon 2021, Austria	retrospective cohort	112	NR	NR	microtubule-targeting agents (paclitaxel)	NR	mRNA expression	NOXA mRNA expression more than 12^{th} percentile	NOXA

NR Not Reported

Thresholds for converting the NOS to AHRQ standards (good, fair, and poor): Good quality; 3 or 4 stars in selection domain AND 1 or 2 stars in comparability domain AND 2 or 3 stars in outcome/exposure domain, Fair quality; 2 stars in selection domain AND 1 or 2 stars in comparability domain AND 2 or 3 stars in outcome/exposure domain, Poor quality; 0 or 1 star in selection domain OR 0 stars in comparability domain OR 0 or 1 stars in outcome/exposure domain

proteins (Bid, Bim, Bad, BIK, BMF, HRIC, Puma, Noxa) expression, with different scoring systems to determine the cutoff in the number of positive cells defining a tumor with BH3-only protein overexpression (Table 1). BAD expression was assessed in 4/9 studies, whereas BIM, PUMA and NOXA expression were assessed in three different studies. BIK expression was assessed in two (2/9) studies. In two (2/9) studies, BH3 -only protein expression was measured in two different sets of data at either gene or protein level using mRNA or IHC, respectively. One study (1/9) assessed the validity of BH3 -only protein NOXA expression as predictive factor using gene (mRNA) expression only.

Quality assessment

NewCastle-Ottawa scale assessment (NOS) was used to assess the quality of cohort studies. Al-Bazz 2009, Canevari 2016, Pandya 2016, Roberts 2011, Karbon 2021, Craik 2010 (6/9) studies are of fair quality [19, 20, 27–30]. They show low ROB in comparability and outcome assessment, but there is no description of the derivation of cohort and no description of the derivation of the non-exposed cohorts. Cannings 2007, Maimaiti 2017, and Jesús 2021 (3/9) studies are of good quality and showed low risk of bias in the selection, comparability, and outcome assessment domains (Table 2).

Quality of evidence assessment

Table 3 shows a summary of the evidence's quality, the degree of the effect, and the source of information used in the estimated risk. In summary, the GRADE quality assessment approach indicated that the quality of our evidence-based results is very low to low (Table 3).

Data analysis and outcomes

BH3-only protein positive expression was measured by either gene or protein expression, we performed meta-analysis for each data separately.

Protein expression analysis

Immunohistochemistry data from 5-years and 10-years DRS and OS of eight (8/9) of the included studies was subjected to meta-analysis as below.

5-years disease free survival (5-years DFS) This outcome was reported in 6/9 studies. The overall RR of 5-years DFS favored BH3-only protein positive group over BH3-only protein negative group. Random effect model was used with non-significant results (RR = 1.17, 95% CI [0.94, 1.46], $P = 0.16$) (Fig. 4A). Pooled studies were heterogeneous ($I^2 = 85\%$, $P = 0.00001$). However, heterogeneity wasn't resolved by excluding one of the studies or by subgrouping analysis (Fig. 4B).

Table 2 The Newcastle-Ottawa scale for quality assessment of cohort studies

	Selection				Comparability	Outcome			Quality	
Studies	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts based on the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of cohorts follow up	score	level
Al-Bazz 2009			*	*	*	*	*	*	6	Fair
Canevari 2016			*	*	*	*	*		5	Fair
Cannings 2007	*	*	*	*	*	*	*		7	Good
Maimaiti 2017	*	*	*	*	**	*	*		8	Good
Jesús 2021	*	*	*		**	*	*	*	8	Good
Pandya 2016			*	*	*		*	*	5	Fair
Roberts 2011			*	*	*		*	*	5	Fair
Karbon 2021		*	*	*		*	*		5	Fair
Craik 2010			*	*	*	*	*	*	6	Fair

CI = Confidence Interval

[a] Other considerations are publication bias, large effect, dose response, and plausible confounding factors

[b] Only 4/6 studies show Fair risk of bias

[c] As the outcome had significant heterogeneity

[d] 2/3 studies show Fair risk of bias

[e] 5/7 studies show fair risk of bias

[f] 3/4 studies show fair risk of bias

Low indicates that the confidence about the result is limited and the true effect can be different from our result

Very low indicates that confidence about the result is very little and the true effect is more probably to be different from our result

Table 3 The summary of finding

Outcome	Number of included studies	Design of included studies	Risk Ratio, 95% CI	Heterogeneity	No. of patients in Positive expression group	No. of patients in negative expression group	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations[a]	Quality
5-years disease free survival	Six studies with 1220 patients	Observational studies	1.17 [0.94, 1.46]	I2 = 85%, *P* = 0.00001	746	474	Not Serious[b]	serious[c]	Not serious	Not Serious	Not existed	Very low ⊕◯◯◯
10-years disease free survival	Three studies with 715 patients	Observational studies	1.32 [1.15, 1.50]	I2 = 0%, *P* = 0.63	454	261	Not Serious[c]	Not serious	Not serious	Not Serious	Not existed	Low ⊕⊕◯◯
5-years overall survival	Seven studies with 1476 patients	Observational studies	1.06 [0.95, 1.18]	I2 = 71%, *P* = 0.002	877	599	Not Serious[e]	Serious[c]	Not serious	Not Serious	Not existed	Very low ⊕◯◯◯
10-years overall survival	Four studies with 930 patients	Observational studies	1.29 [0.92, 1.80]	I2 = 89%, *P* = 0.00001	551	379	Not Serious[f]	Serious[c]	Not serious	Not Serious	Not existed	Very low ⊕◯◯◯

Subgroup analysis of the 5-years DFS showed BAD positive expression significantly improves the 5-years DFS (RR=1.34, 95% CI [1.06, 1.70], *P*=0.02). On the other hand, BIK positive expression significantly worsened the 5-years DFS (RR=0.84, 95% CI [0.73, 0.97], *P*=0.02) (Fig. 4B).

10-years disease free survival (10-years DFS) This outcome was reported in 3/9 studies. The overall RR of 10-years DFS favored BH3-only protein positive group over BH3-only protein negative group. A fixed effect model was used, and the results were significant (RR=1.32, 95% CI [1.15, 1.50], *P*=0.0001) (Fig. 5). Pooled studies were homogenous (I^2=0%, *P*=0.63).

5-years overall survival (5-years OS) The 5-years OS outcome was reported in 7/9 studies. The overall RR of 5-years OS favored BH3-only protein positive group over BH3-only protein negative group. Random effect model was used with non-significant results (RR=1.06, 95% CI [0.95, 1.18], *P*=0.3) (Fig. 6A). Pooled studies were heterogeneous (I^2=71%, *P*=0.002). Heterogeneity wasn't resolved by either excluding one of the studies or by subgrouping analysis.

Subgroup analysis showed BAD, and PUMA positive expression associated with an improved 5-years OS (RR=1.19, 95% CI [0.95, 1.48], *P*=0.12), and (RR=1.13, 95% Cl [1.03, 1.25], *P*=0.01), respectively. On the other hand, BIK and BIM positive expression worsened the 5-years OS (RR=0.91, 95% CI [0.82, 1.02], *P*=0.1), and (RR=0.92, 95% CI [0.82, 1.02], *P*=0.2), respectively (Fig. 6B).

10-years overall survival This outcome was reported in 4/9 studies. The overall RR of 10-years OS favored BH3-only protein positive group over BH3-only protein negative group. Random effect model was used with non-significant results (RR=1.29, 95% CI [0.92, 1.80], *P*=0.14) (Fig. 7). Pooled studies were heterogeneous (I^2=89%, *P*=0.00001). Again, heterogeneity wasn't resolved by excluding one of the studies.

Gene expression analysis

The prognostic value of BH3-only proteins: PUMA, BIK, and NOXA as independent predictor of breast cancer survival were measured using mRNA expression in three studies Robert 2011, Pandya 2016 and Karbon 2021, respectively.

Robert et al., (2011) explored the potential relationship between PUMA mRNA levels and breast cancer outcomes using gene expression data from publicly available datasets of van de Vijver et al. (2002). High PUMA expression correlated with positive estrogen receptor (ER +), low tumor grade, and small tumor size. KM

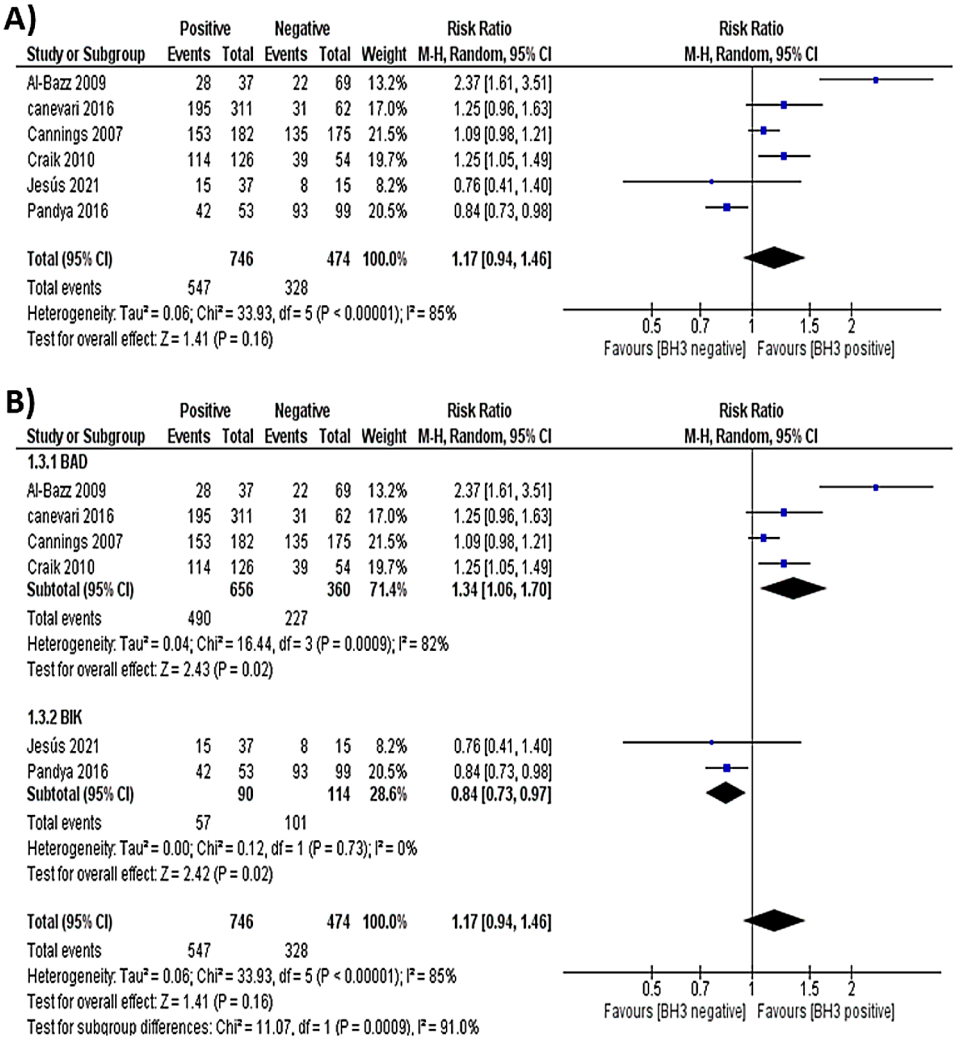

Fig. 4 Forest plot of RR for 5-years DFS comparing BH3-only protein positive and negative expressions in BC. (**A**) pooled studies (6/9), (**B**) subgroup analysis based on proteins type

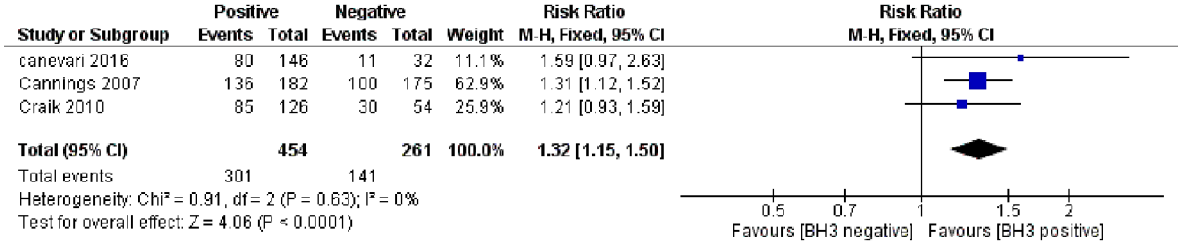

Fig. 5 Forest plot of RR for 10-years DFS comparing BH3-only protein positive and negative expressions in BC

analysis revealed that high PUMA mRNA expression was linked with a favorable prognosis, while low expression was linked to a poor prognosis for breast cancer-specific death (P=0.0014). Subsequent analysis using Cox proportional hazards models confirmed that high PUMA expression was a significant independent predictor of breast cancer-specific death outcome even when accounting for other clinicopathological variables, grade 3 and HER2, in cancer patients (HR 0.534, 95% CI 0.331–0.861, P=0.01). Furthermore, the prognostic significance of PUMA mRNA expression was explored specifically in ER +, endocrine-treated patients, showing that high PUMA expression remained a highly significant predictor of favorable prognosis in this subgroup (RFS outcomes P=0.0000149) [29]. Similarly, NOXA mRNA expression level was found to be the sole BH3-only protein having

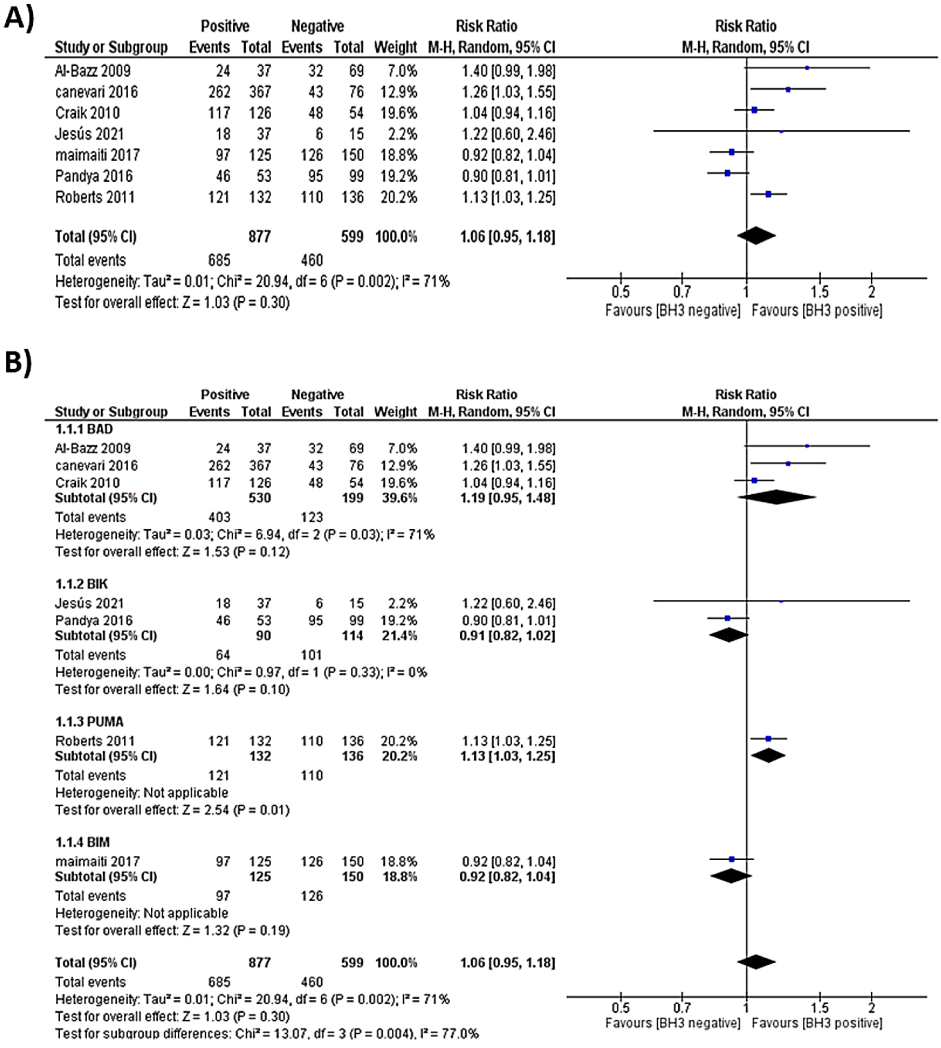

Fig. 6 Forest plot of RR for 5-years OS comparing BH3-only protein positive and negative expressions in BC. (**A**) pooled studies (7/9), (**B**) subgroup analysis based on proteins type

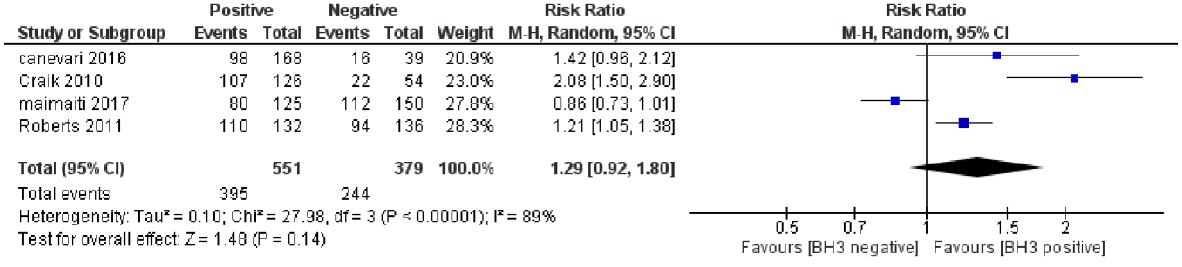

Fig. 7 Forest plot of RR of 10-years overall survival comparing BH3-only protein positive and negative expressions in BC

predictive relevance across all molecular BC subtypes after analyzing a well-characterized group of 92 BC patients' frozen specimens that had later been treated with chemotherapy post-surgery. Using both univariate and multivariate Cox-Regression, high NOXA mRNA expression was strongly linked with improved RFS and OS (P value of <0.001). This finding was confirmed in a second BC patient group from The Cancer Genome Atlas (TCGA) dataset, where NOXA mRNA levels were found to be associated significantly with improved PFI and OS (P=0.002, P=0.028, respectively) in 112 TCGA patients receiving MTAs but no other type of chemotherapeutic

medications [19]. However, data from Pandya (2016) unveiled that high levels of Bik mRNA were observed to be linked with poor DFS (HR = 1.78, 95% CI: 0.99 to 3.20) and OS (HR = 2.05, 95% CI: 0.96 to 4.37) in breast cancer patients [20].

However, findings on mRNA expression from three studies combined in a subgroup meta-analysis were not significant due to inadequate data and considerable heterogeneity (S Fig. 1).

Discussion

BH3-only proteins (BIM, PUMA, BID, BAD, BIK, BMF, NOXA, HRK) are pro-apoptotic members of the broader BCL-2 family, that promote cell death or cell-survival by directly or indirectly activating Bax and Bak [31]. Due to their role in controlling cell death during cancer, BH3-only proteins have been investigated for their impact on BC prognosis. In the current systematic review, we searched and retrieved existing evidence from literature concerning "BH3-only proteins expression as prognostic indicator for BC management". Data were analyzed with the survival outcomes of patients' namely the OS and DFS for 5-years and 10-years. Meta-analysis revealed BH3-only proteins, particularly BAD, positive expression could be considered as a good prognostic predictor of improved long term DFS in treated BC patients.

Meta-analysis data showed that positive expression of BH3-only proteins was associated significantly with the improved 10-years DFS (P=0.0001). Moreover, meta-subgroup analysis of over 700 cases from 6/9 studies revealed that BAD and PUMA BH3-only proteins are the only proteins of this family whose positive expression was statistically significant with improved patients' 5-years DFS (P=0.02) and OS (P=0.01), respectively. This data suggests that BH3-only PUMA and BAD proteins' positivity could be utilized as a predictive marker for disease enhancements in long-term patient prognosis assessment and/or monitoring response-to-therapy. However, these limited studies were heterogenous and conveyed a moderate to high risk of bias. As a result, these findings are subjected to limited evidence and remain uncertain. Our data is comparable to a recent systematic review meta-analysis regarding the usefulness of C-reactive protein as a prognostic biomarker for BC. Although, this review included 11,541 patients from 22 studies, authors were unable to make a conclusive statement either due to the poor quality of evidence or the lack of sufficient data [32].

Systematic reviews are intended to validate existing evidence to formulate evident decision(s) in clinical practice setting, predictive biomarkers measure the likelihood of benefit from treatment, whereas, prognostic biomarkers reflect cancer-related events risk (e.g., recurrence or mortality) irrespective of treatment type [32]. Thus, in the current review, we combined all the available evidence related to BH3-only proteins expression regardless of the patients' treatment. Validation of a potential biomarker requires a substantial sufficient strong analytical and clinical evidence-based studies involving numerous individual research [33]. Despite it is difficult to gather enough high quality data for novel prognostic biomarkers to be stated useful [34], therefor, studies should adhere to "The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK)" by [35, 36] to improve biomarkers research quality-of-reporting and thus, strengthen evidence regarding a biomarker value.

BH3-only protein expression level was evaluated in (8/9) studies using IHC technique via tissue microarray with relatively similar scoring and cutoff system in most of the studies (except for Cannings et al. 2007 and Maimaiti et al. 2017 studies [37, 38]). However, the antibody used, the investigated protein, and the number of positive cells scored to define a tumor with BH3-only proteins overexpression can vary widely between laboratories. Moreover, studies differed in many other aspects, which could have contributed to our meta-significant analysis's heterogeneity. These variations include patients' age and menopausal status, tumor size, tumor molecular subtype, mitotic grade, the overall grade, vascular invasion, treatment type, and the chemotherapy used before or during patients' follow up as stated in detail in Table 1. Patients in the **Canevari et al. 2016**, **Maimati et el. 2017**, and Jesús **et el. 2021** studies [28, 38, 39], had surgery followed by either radiotherapy or adjuvant systemic therapy, whereas patients in the other studies (6/9) were treated with different chemotherapy such as tamoxifen, taxanes (paclitaxel or docetaxel), 5-fluorouracil, Epirubicin, Cyclophosphamide, and Docetaxel.

Data of mRNA gene expression appears to be correlated with protein expression data from the same study despite using different cohorts [19, 20, 29]. The limited data and the variations in methodologies lead to the high heterogeneity in our metanalysis and prevented us from making a concise conclusion.

The expression patterns of BH3-only protein are distinct and overlapping, pointing out to uniqueness and redundant involvement in cellular processes [40, 41]. In addition to regulating apoptosis, members of BH3-only protein family interact with diverse cellular pathways as autophagy, checkpoint regulation, and metabolism [20, 40].

BAD expression studies (4/9) [27, 28, 30, 37] showed a significant association between the BAD positive expression and the improved OS and DFS, revealing an increase in the survival probability and the decreased risk-of-relapse in patients with BC. BAD expression was found to be associated with DFS in tamoxifen [37], taxane-treated BC patients [30] as well as patients who underwent surgery followed by radio- or chemo-therapy

[28]. In the latter study, BAD expression was lower in patients who developed distance metastasis and positive LN status compared to patients who remained metastasis-free. Reduced BAD expression was significantly associated with shorter systemic DFS and cancer-specific survival (p=0.001) [28]. In addition to BAD's apoptosis-inducing properties, obtained data has anti-metastatic properties and is a useful BC prognostic marker, which corresponds to the finding by **Cekanova et el. 2015** [42]. It is likely that higher Bad expression causes an increased BCL-2 sequestration (heterodimerization), enhancing the amount of Bax accessible to trigger apoptosis [37]. Similarly, BH3-only NOXA and PUMA gene and protein expression was significantly associated with a good prognosis and better OS in patients with BC treated with microtubule-targeting agents (paclitaxel) or tamoxifen, respectively [19, 29]. However, in our meta-analysis, these relationships did not always reach a significant level of confidence.

BIK and BIM BH3-only proteins high expression did not correlate with improved patients' OS or DFS. In contrast, high expression of these proteins found to be associated with poor prognosis [20, 38]. In two different cohorts, **Pandya et al. 2016** showed that BIK positive expression at the gene and protein level was associated with poor/shorter OS and DFS, and clinical outcome, implying that BIK may function as a tumor promoter rather than a tumor suppressor [20]. Therefore, these data created a paradox on BIK protein's role in cell death [20]. On the other hand, the evidence from **Jesús et al., 2021** study did not show a strong link between BIK expression and the OS or DFS. However, the study disclosed BIK expression to be significantly connected to a favorable clinical outcome [39]. This discrepancy between the two studies could be attributed to a variety of factors, including the patients' clinical stage, age, and menopausal status, as well as some other methodological variations. However, several studies have previously shown similar inconsistent results on the significance of BIK in BC [43–45].

The role of the BH3-only protein BIM protein was also subject to controversy. While **Maimaiti et al. 2017** found that high BIM expression was correlated to a considerably (P=0.039) lower OS in BC patients, especially those with luminal A tumors [38], others found BIM was associated significantly (P=0.039) to improved OS in colon cancer patients [46]. However, it is not clear whether BIM and BIK are tumor suppressor or promotor indicators and their exact role in BC patient's clinical outcome requires further exploration, which is beyond the scope of this review.

Limitations and strength

This review has several limitations: small sample sizes, notably for BIK, BIM and NOXA, with the poor quality of some studies, and high heterogeneity between the studies undermined the importance of our findings and rendered them inconclusive. The limited number of studies available for analysis prevented a meaningful assessment of publication bias, which could have potentially influenced our results. Additionally, we didn't include papers written in languages other than English, so we might have missed some data. Furthermore, we focused solely on the association between BH3 proteins expression and 5- and 10-year OS and DFS, ignoring any other clinical outcomes that may have been relevant. However, despite these limitations, our review has several strengths: firstly, we conducted a thorough and in-depth literature search. Secondly, the meta-analysis performed, currently, has added significant strength to the systematic review, as it allowed identifying and validating a BC predictive biomarker, that is strongly connected with long-term DFS in BC patients' cohort. Thirdly, we emphasized the scarcity of clinical evidence on the BIM and BIK proteins, as well as the need for resolving the debate over their role in BC.

Conclusion

While BAD and PUMA positive expression correlated significantly with patients' improved OS and/or DFS, BIK and BIM high expression were correlated with poor survival outcome. This data suggests, despite being from the same family, these BH3-only proteins induce different tumor survival signaling pathways, that could play role in predicting/leading to a good or poor OS as well as DFS outcomes in BC patients after treatment.

Overall, BH3-only proteins' expression (at both gene and protein level) could be a useful prognostic factor in BC. This could have significant clinical implication in BC management. Despite the limited evidence, our findings regarding the BH3-only proteins' expression, particularly BAD expression, contribute to the growing body of evidence linking BAD with an improved long-term patient' DFS. Meanwhile, BH3-only proteins' expression association with BC OS could not be confirmed. The identification and validation of a predictive biomarker will enable us to identify patients with poor prognosis for whom a specific therapy should be designed. However, further studies with larger cohort are still required to strengthen our findings and validate the role of each member of the BH3 only proteins family in BC.

Abbreviations

BIM	Bcl-2 Interacting Mediator
BC	Breast Cancer
DFS	Drug Free Survival
DFS	Progression Drug free survival
OS	Overall Survival

Supplementary Information

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Supplementary Material 1

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Author contributions
SO: confirmed data extraction and meta-analysis, wrote the manuscript method, parts of results, and discussion, and revised the whole manuscript. TA: updated the search and screening, performed data extraction, quality assessment and prepared tables and figures. NA: performed data extraction, quality assessment, and results writing. MA: took part in data extraction, quality assessment, performed the meta-analysis, and revised the manuscript. MK: reviewed the manuscript. NMH: conceptualization, shared in preparing the initial Prospero protocol for registration, supervised the whole process, reviewed, edit the manuscript, and approved the manuscript draft till publication.

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Data availability
All data extracted for the systematic review and meta-analysis is available upon request.

Declarations

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Consent for publication
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Competing interests
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