Gasdermin B expression predicts poor clinical outcome in HER2-positive breast cancer

Marta Hergueta-Redondo¹, David Sarrio¹, Ángela Molina-Crespo¹, Rocío Vicario², Cristina Bernadó-Morales², Lidia Martínez¹, Alejandro Rojo-Sebastián³, Jordi Serra-Musach⁴, Alba Mota¹,⁵, Ángel Martínez-Ramírez⁶, María Ángeles Castilla⁷, Antonio González-Martín⁸, Sonia Pernas⁴, Amparo Cano¹, Javier Cortes⁹,¹⁰, Paolo G. Nuciforo¹¹, Vicente Peg¹², José Palacios⁷,¹³, Miguel Ángel Pujana⁴, Joaquín Arribas²,⁹,¹¹, Gema Moreno-Bueno¹,⁵

¹Biochemistry Department, Universidad Autónoma de Madrid (UAM), Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), IdiPAZ, Madrid, Spain
²Preclinical Oncology Program, Vall d’Hebron Institute of Oncology (VHIO), Universitat Autònoma de Barcelona, Barcelona, Spain
³Pathology Department, MD Anderson Cancer Center, Madrid, Spain
⁴Breast Cancer and Systems Biology Unit, ProCURE, Catalan Institute of Oncology, IDIBELL, L’Hospitalet del Llobregat, Barcelona, Spain
⁵Translational Research Laboratory, MD Anderson Internacional Foundation, Madrid, Spain
⁶Cytogenetics Department, MD Anderson Cancer Center, Madrid, Spain
⁷Pathology Department, Hospital Universitario Virgen del Rocío, Sevilla, Spain
⁸Oncology Department, MD Anderson Cancer Center, Madrid, Spain
⁹Clinical Oncology Program, Vall d’Hebron Institute of Oncology (VHIO), Universitat Autònoma de Barcelona, Barcelona, Spain
¹⁰Oncology Department, Hospital Universitario Ramón y Cajal, Madrid, Spain
¹¹Molecular Oncology Program, Vall d’Hebron Institute of Oncology (VHIO), Universitat Autònoma de Barcelona, Barcelona, Spain
¹²Pathology Department, Hospital Vall d’Hebron University, Barcelona, Spain
¹³Pathology Department, Hospital Universitario Ramón y Cajal, Madrid, Spain

Correspondence to: Gema Moreno-Bueno, email: gmoreno@iib.uam.es
Marta Hergueta-Redondo, email: mhergueta@cnio.es

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ABSTRACT

Around, 30–40% of HER2-positive breast cancers do not show substantial clinical benefit from the targeted therapy and, thus, the mechanisms underlying resistance remain partially unknown. Interestingly, ERBB2 is frequently co-amplified and co-expressed with neighour genes that may play a relevant role in this cancer subtype. Here, using an in silico analysis of data from 2,096 breast tumours, we reveal a significant correlation between Gasdermin B (GSDMB) gene (located 175 kilo bases distal from ERBB2) expression and the pathological and clinical parameters of poor prognosis in HER2-positive breast cancer. Next, the analysis of three independent cohorts (totalizing 286 tumours) showed that approximately 65% of the HER2-positive cases have GSDMB gene amplification and protein over-expression. Moreover, GSDMB expression was also linked to poor therapeutic responses in terms of lower relapse free survival and pathologic complete response as well as positive lymph node status and the development of distant metastasis under neoadjuvant and adjuvant treatment settings, respectively. Importantly, GSDMB expression promotes survival to trastuzumab in different HER2-positive breast carcinoma cells, and is associated with trastuzumab resistance phenotype in vivo in Patient Derived Xenografts. In summary, our data identifies the ERBB2 co-amplified and co-expressed gene GSDMB as a critical determinant of poor prognosis and therapeutic response in HER2-positive breast cancer.
INTRODUCTION

The identification of molecular cancer drivers has considerably improved clinical outcomes in oncology. As such, inhibition of the human epidermal growth factor receptor 2 (HER2; ERBB2 gene) represents a paradigm in breast cancer oncology. Over-expression of ERBB2 occurs in approximately 20% of breast tumours [1, 2]. Thus, the amplification of chromosome 17q12-q21, which includes ERBB2, defines an intrinsic molecular cancer subtype associated with relatively aggressive tumour behaviour and poor clinical outcome [3]. However, the development of targeted anti-HER2 therapies based principally on humanized antibodies (such as trastuzumab, trastuzumab emtansine -T-DM1 or pertuzumab) and tyrosine kinase inhibitors (such as lapatinib) has dramatically improved the outcome of women with early and advanced stage HER2-positive breast cancer [4–11]. Several clinical trials have demonstrated that combination with chemotherapy in the neoadjuvant or adjuvant setting significantly improves clinical response without relevant toxicity [5, 13–16]. Despite the clinical benefit obtained from the above therapies, inhibition of HER2 alone is generally not sufficient. In fact, trastuzumab response rate is limited to approximately 15–26% [12, 13], which indicates the involvement of other molecules and/or signalling pathways that influence outcome in this setting [14].

Since the common 17q12-q21 amplicon includes several gene loci, ERBB2-co-amplified genes have been examined for their potential to influence cancer progression and/or therapeutic response [17, 18]. A minimal amplified region around ERBB2 oncogene has been defined in several studies [19, 20], and some of the genes have been found to play a role in tumorigenesis [14, 21, 22]. These genes include STARD3 and GRB7, whose products promote the proliferation of HER2-positive breast cancer cell lines [18]. Thus, GRB7 over-expression has been identified as an independent prognostic factor [23], and the co-amplification of ERBB2 with the non-core gene topoisomerase II (TOP2A) predicts sensitivity to anthracycline therapy [21, 24, 25]. Together, these observations illustrate the importance of additional genes in the biology and clinical evolution of HER2-positive breast cancer.

Previous data demonstrated that GSDMB lies within the evolutionary recombination hotspot closely linked to the ERBB2 amplicon [20, 26]. The gene product belongs to the family of gaseurretsins, which includes three other human members [27–31]. Although these other members have been implicated in the development and progression of some diseases [32, 33], the role of GSDMB in cancer is only now beginning to emerge [30, 31, 34]. We recently demonstrated that GSDMB over-expression promotes cell motility, invasion and metastasis of breast cancer cell lines, and, intriguingly, it was found over-expressed in breast tumour samples [34]; however, the potential link with breast cancer subtypes remained unexplored.

Here, through integrated gene expression and molecular analyses, we show robust associations between GSDMB amplification/expression and HER2-positive status. Most importantly, further analyses show that GSDMB gene and protein expression predicts poor clinical outcome in HER2-positive breast cancer treated, both in the neoadjuvant and adjuvant settings. Importantly, we corroborated that GSDMB expression is associated with trastuzumab resistance phenotype in HER2-positive breast carcinoma cells and in Patient Derived Xenografts. GSDMB increases cell growth and reduces apoptosis after trastuzumab treatment in breast cancer cells. Together, our data reveals GSDMB as a key prognostic and predictive biomarker in HER2-positive breast cancer, highlighting new opportunities for valuable combined therapies.

RESULTS

GSDMB over-expression is associated with poor prognosis of HER2-positive breast cancer

We have previously reported that relative high expression of GSDMB, but no other GSDM genes, in breast tumours is associated with poor survival in unselected breast cancer cases [34]. To assess further the molecular and clinical significance of this observation, two independent microarray expression datasets including a total of 2,096 cases were analysed [35, 36]. Overall, high GSDMB expression was correlated with HER2-positive status; according to the PAM50 classifier [37] or with the reported immunohistochemical results (Supplementary Table 1).

Next, the association between GSDMB over-expression and 17q12-q21 amplification was evaluated. Using copy number estimates from The Cancer Genome Atlas (TCGA) dataset [36], the GSDMB locus was shown to be amplified in 58 out of 526 (11%) tumours. Importantly, this alteration was observed only in tumours that also presented ERBB2 amplification (58/67; 86%); thus, none of the 459 HER2-negative cases was identified as harbouring GSDMB amplification. Consistent with this observation, high GSDMB expression levels were associated with both ERBB2 and GSDMB gene amplification (Supplementary Table 1).

Given the above correlations, and the role of GSDMB in promoting an aggressive breast cancer cell phenotype [34], we next assessed if the over-expression of GSDMB could also influence the prognosis of HER2-high tumours. As shown in Figure 1, GSDMB over-expression in these tumours was significantly associated with poor outcome: shorter disease free survival (DSF) and distant metastasis-free survival (DMFS) in the Ur-Rehman dataset (p < 0.001, Figure 1A–1B); as well as overall survival (OS) in the TCGA dataset (p < 0.01, Figure 1C). Additionally, the prognosis value of GSDMB in these datasets was not independent of tumor grade: the association with poor prognosis was significantly stronger...
(higher Hazard Ratios) in HER2-high cancers than in unselected breast cancers (whole dataset, Figure 1D).

Furthermore, we analysed the prognostic value of other 22 genes located near ERBB2. As shown in Figure 1D, over-expression of GSDMB represented the largest effect relative to poor prognosis (Figure 1D).

Complementarily, to confirm further the association of GSDMB expression with poor prognosis we evaluated an additional series of 58 HER2-positive (by FISH and IHC) breast cancers [38]. Again, higher GSDMB expression associates significantly with shorter OS (log-rank $p = 0.002$) (Supplementary Figure 1).

Collectively, consistent results across different datasets demonstrate that high GSDMB expression identifies the most aggressive HER2+ breast tumours.

GSDMB is amplified and over-expressed in HER2-positive breast tumours

Our in silico analyses indicates that GSDMB over-expression is associated with the HER2-positive phenotype in breast cancer. To confirm this observation, the copy number status of the GSDMB locus was determined by FISH (Figure 2A) in a cohort of 53 breast cancer patients including HER2-positive ($n = 29$) and HER2-negative ($n = 24$) tumours classified according to the ERBB2 amplification status (“discovery series” in Supplementary Table 2). Thus, GSDMB amplification was detected in 21 tumours, and 15 of them (71.4%) were HER2-positive cases, therefore confirming the association between the both amplifications ($p = 0.034$, Table 1, Figure 2A, panel d). In the six cases classified as HER2-negative tumours showing GSDMB amplification, the HER2/centromere ratio was 1.9 ± 0.24, suggesting the presence of HER2 aneuploidy [39].

Next, to determine if GSDMB amplification leads to over-expression of the corresponding product, an anti-GSDMB monoclonal antibody was developed. The antibody was able to recognize all the isoforms of GSDMB described [34] by Western blot analysis (Supplementary Figure 2 and Materials and Methods) and by immunohistochemistry (IHC). Thus, IHC assays revealed positive expression of GSDMB in 23 of the 52 cases (44.2%; Figure 2B, Table 1). Importantly, this positivity was significantly correlated with ERBB2 gene amplification (16/23, 69.6%, $p = 0.040$, Table 1) and GSDMB amplification ($p < 0.001$; Table 1). In fact, intense GSDMB staining (Figure 2B, panel f) was found in HER2-positive tumours with clear GSDMB gene amplification (Figure 2A, panel d, more than 10 signals/nucleus); while
undetectable or weak GSDMB staining was detected in normal breast tissue (Figure 2B, panel b) or lesions without ERBB2 or GSDMB amplification (GSDMB gene copy number of < 4 signals/nucleus, Figure 2A, panel c).

Together, these data demonstrate that GSDMB is over-expressed in a subset (~65%) of HER2-positive tumors, with gene amplification being most likely the mechanism responsible of this alteration.

Table 1: Relationship between GSDMB gene amplification/protein expression and HER2 oncogene in breast cancer “discovery series”

|                      | HER2 amplification: *n (%) |
|----------------------|-----------------------------|
|                      | Negative | Positive |
| GSDMB amplification (n = 52) |          |          |
| Negative (n = 31)    | 18 (58.1) | 13 (41.9) |
| Positive (n = 21)    | 6 (28.6)  | 15 (71.4) |
| GSDMB expression (n = 52) |          |          |
| Negative (n = 29)    | 17 (58.6) | 12 (41.4) |
| Positive (n = 23)    | 7 (30.4)  | 16 (69.6) |

*p = 0.034

| GSDMB expression (n = 52) | GSDMB amplification: *n (%) |
|---------------------------|-----------------------------|
| Negative (n = 29)         | 29 (100) | 0 |
| Positive (n = 23)         | 2 (8.7)  | 21 (91.3) |

*p < 0.001

* n (%), number of analyzed cases and (percentage).

Figure 2: GSDMB gene amplification and protein expression in HER2-positive breast cancer. (A) Representative breast tumours with positive and negative HER2 and GSDMB amplification. Panels a, c, e: represent an example of breast carcinoma HER2-positive (red signal) without GSDMB amplification. Panels b, d, f: represent an example of case HER2-positive with GSDMB amplification (blue signal). The lower lane shows the merged image (c of a-c and f of b-d panels). Green signals represent CEP7. Magnification x 63x; insets x 120. (B) Immunohistochemical analysis of HER2 and GSDMB in normal tissue (panel a, b), and in breast carcinoma cases (panels c-f). Low or undetectable GSDMB staining was observed in normal breast tissue (b) or in lesions with GSDMB gene copy number of < 4 signals/nucleus (d) (considered non-amplified tumours). Intense GSDMB staining in a case with > 10 GSDMB copy number (f) considered as GSDMB-positive tumour. Scale bar measures 100 micrometers.
GSDMB gene amplification or expression predict poor clinical response and relapse under neoadjuvant settings in HER2-positive breast tumors

The results of the gene expression analyses indicate that GSDMB over-expression associates with poor prognosis of HER2-positive breast cancer. Next, we assessed the influence of GSDMB gene amplification and/or expression in the therapeutic response of this cancer subtype. First, the analysis of a series of 28 HER2-positive tumours in the discovery series, treated with the neoadjuvant setting revealed an association between GSDMB amplification and the lack of pathologic complete response (pCR) \( (p = 0.001, \text{Table 2a}) \). All of the analysed samples who developed local or distant relapse during the clinical follow-up showed GSDMB gene amplification and/or protein expression, respectively \( (p = 0.030, \text{Table 2a, 2b}) \).

In order to validate the above results, an independent cohort of 95 HER2-positive breast cancer cases treated with the same neoadjuvant regimens ("validation series", Supplementary Table 2) was analysed. As expected, 61.1% and 68.8% of the HER2-positive tumours showed GSDMB amplification and protein expression, respectively (Supplementary Table 3); in fact, a strong association between both variables was observed \( (p < 0.001; \text{Table 2a}) \).

Importantly, the analysis of this series corroborated the association between GSDMB gene amplification/protein expression and poorer therapy response. Among the 37 non-responder tumours, 31 (83.8%) showed GSDMB gene amplification and 33 (89.2%) protein expression \( (\text{Table 2a–2b}) \). Conversely, few cases without GSDMB amplification (16.2%) or protein expression (10.8%) were classified as non-responders \( (p < 0.001, \text{Table 2a–2b}) \).

Next, univariate Cox proportional hazard analyses in the validation series revealed that both variables, GSDMB amplification and GSDMB expression, are significantly associated with reduced DFS in the neoadjuvant setting: HR = 5.60, 95% CI 1.66–18.88, long-rank \( p = 0.0002 \); and HR = 6.75, 95% CI 1.57–28.73, long rank \( p = 0.0004 \), respectively \( (\text{Figure 3, Table 3}) \). The average time to relapse for the GSDMB amplified and non-amplified cases was 37 ± 11 and 67.3 ± 17 months, respectively \( (\text{Table 3, Figure 3A}) \).

Similarly, GSDMB positive and negative cases identified by IHC presented an average time to relapse of 34.5 ± 14 and 66.2 ± 18 months \( (\text{Figure 3B}) \). The Kaplan-Meier and log-rank survival analyses further illustrate the significant difference in DFS between GSDMB-positive and GSDMB-negative groups \( (\text{Figure 3A, 3B}) \). Together, these results indicate that GSDMB expression/amplification, influences both tumour progression and the response to therapy in HER2-positive breast cancer within the neoadjuvant setting.

Clinical association of GSDMB with low therapy response in the adjuvant setting in HER2-positive breast tumours

To evaluate further the influence of GSDMB expression on the clinical response, a series of 138 breast cases treated with adjuvant therapy were analysed, 53 (41.1%) of these tumours were classified as HER2-positive and were treated following standard adjuvant schedules (Supplementary Table 4). In this series, around 51% of these HER2-positive samples also expressed GSDMB \( (\text{Table 4}) \). Next, significant associations between GSDMB expression and positive lymph node status \( (p = 0.013) \) as well as with the existence of distant metastasis \( (p = 0.001) \) were revealed \( (\text{Table 4}) \). Univariate Cox proportional hazard analysis confirmed the association with DFS: HR = 2.85, 95% CI 1.28–6.37, log-rank \( p = 0.007 \) \( (\text{Table 5}) \). Thus, the average time to relapse for the GSDMB-positive and -negative cases was 91.24 + 14.88 and 101.81 + 9.51 months, respectively. The Kaplan-Meier and log-rank survival analyses further illustrate the difference in DFS between GSDMB-positive and GSDMB-negative cases in the adjuvant setting \( (p = 0.007; \text{Figure 3C}) \).

Overall these results and our previous data firmly establishes GSDMB as a negative prognostic marker in HER2-positive breast cancer patients treated (trastuzumab plus chemotherapy) in both the neoadjuvant and adjuvant settings.

GSDMB promotes survival to trastuzumab treatment

Finally, to assess if GSDMB expression was functionally involved in promoting survival to anti-HER2 therapy we performed several complementary experimental approaches. First, we evaluated the potential role of GSDMB expression on trastuzumab response in HCC1954 cells in which endogenous GSDMB has been stably silenced by shRNAs \[34\]. This model is an example of trastuzumab-resistant HER2+ human breast cancer cell line \[40\]. Additionally, we generated GSDMB exogenously expressing cells using SKBR3 \( (\text{Figure 4A}) \), which are trastuzumab-responsive cells \[40\]. In both cell models, trastuzumab inhibition of cell growth (measured by Alamar Blue method) was significantly higher \( (~20\%) \) in cells with low GSDMB expression \( (\text{Expression 4B–4C}) \). Moreover, GSDMB significantly increased cell survival to trastuzumab treatment, as demonstrated by the reduction in apoptosis in GSDMB-overexpressing SKBR3 cells compared to control cells \( (\text{Figure 4D}) \). Given that HCC1954 are intrinsically very resistant to trastuzumab \( (5 \text{ mg/ml}) \) we could not test the effect on apoptosis in this cell line.

Second, to demonstrate the association of GSDMB with resistance in vivo we utilized six breast cancer Patient Derived Xenografs (PDX), a more clinically relevant model \[41\]. We used four HER2-positive breast cancer PDXs, two sensitive and two primary resistant to trastuzumab
Table 2: Relationship between GSDMB amplification/expression and clinico-pathological features in HER2-positive breast cancer included in the discovery (n = 28) and validation series (n = 95)

|                        | Discovery series | Validation series | Validation series |
|------------------------|------------------|-------------------|-------------------|
|                        | GSDMB amplification: *n (%) | GSDMB amplification: *n (%) | GSDMB amplification: *n (%) |
|                        | Negative | Positive | Negative | Positive | Negative | Positive | Positive |
| (a)                    |          |          |          |          |          |          |          |
| ER expression          |          |          |          |          |          |          |          |
| Negative               | 6/9 (66.7)| 3/9 (33.3)| 11/35 (31.4)| 24/35 (68.6)|          |          |          |
| Positive               | 7/19 (36.8)| 12/19 (63.2)| 26/60 (43.3)| 34/60 (56.7)|          |          |          |
| p                     | 0.142    |          |          |          |          |          |          |
| PR expression          |          |          |          |          |          |          |          |
| Negative               | 10/19 (52.6)| 9/19 (47.4)| 16/49 (32.7)| 33/49 (67.3)|          |          |          |
| Positive               | 3/9 (33.3)| 6/9 (66.6)| 19/44 (43.2)| 25/44 (56.8)|          |          |          |
| p                     | 0.338    |          |          |          |          |          |          |
| GSDMB expression       |          |          |          |          |          |          |          |
| Negative               | 12/12 (100)| 0 (0) | 29/29 (100)| 0 (0) |          |          |          |
| Positive               | 1/16 (6.2)| 15/16 (93.8)| 6/64 (9.4)| 58/64 (90.6)|          |          |          |
| p                     | < 0.001  |          |          |          |          |          |          |
| pCR †                 |          |          |          |          |          |          |          |
| Responders            | 12/16 (75.0)| 4/16 (25.0)| 31/58 (53.4)| 27/58 (46.6)|          |          |          |
| Non-responders        | 1/12 (8.3)| 11/12 (91.7)| 6/37 (16.2)| 31/37 (83.8)|          |          |          |
| p                     | < 0.001  |          |          |          |          |          |          |
| Relapse †             |          |          |          |          |          |          |          |
| Negative               | 12/26 (57.1)| 9/26 (42.9)| 24/47 (51.1)| 23/47 (48.9)|          |          |          |
| Positive               | 0 (0) | 5/5 (100)| 3/19 (15.8)| 16/19 (84.2)|          |          |          |
| p                     | 0.030    |          |          |          |          |          |          |
| (b)                    |          |          |          |          |          |          |          |
| GSDMB expression       |          |          |          |          |          |          |          |
| Negative               | 6/9 (66.7)| 3/9 (79.5)| 9/35 (25.7)| 26/35 (74.3)|          |          |          |
| Positive               | 6/19 (31.6)| 13/19 (68.4)| 20/58 (34.5)| 38/58 (65.5)|          |          |          |
| p                     | 0.090    |          |          |          |          |          |          |
| PR expression          |          |          |          |          |          |          |          |
| Negative               | 9 (47.4) | 10 (52.6)| 13/48 (27.1)| 35/48 (72.9)|          |          |          |
| Positive               | 3 (33.3)| 6 (66.7)| 14/43 (32.6)| 29/43 (67.4)|          |          |          |
| p                     | 0.293    |          |          |          |          |          |          |
| pCR †                 |          |          |          |          |          |          |          |
| Responders            | 11 (68.8)| 5 (31.3)| 25/56 (44.6)| 31/56 (55.4)|          |          |          |
| Non-responders        | 1 (8.3) | 11 (91.7)| 4/37 (10.8)| 33/37 (89.2)|          |          |          |
| p                     | 0.002    |          |          |          |          |          |          |
| Relapse †             |          |          |          |          |          |          |          |
| Negative               | 11 (52.4)| 10 (47.6)| 21/46 (45.7)| 25/46 (54.3)|          |          |          |
| Positive               | 0         | 5 (100)| 2/19 (10.5)| 17/19 (89.5)|          |          |          |
| p                     | 0.033    |          |          |          |          |          |          |

The data refers to the available cases for each markers (a) Statistical analysis of GSDMB amplification in breast carcinoma samples included in discovery and validation series. (b) Statistical analysis of GSDMB IHC study in breast carcinoma samples included in discovery and validation series. *n (%), number of analyzed cases and (percentage). †pCR: pathological complete response when there is no invasive presence of tumour at the breast or ganglia level (< 0.1 mm) and Relapse as local or distant recurrence in HER2-positive tumours.
(42] and data not shown) and two HER2-negative PDX as control (Figure 4E). The PDXs that did not respond to trastuzumab in vivo exhibited higher mRNA (Figure 4E) and protein levels (Supplementary Figure 3) of GSDMB than trastuzumab-sensitive patients and HER2-negative PDXs. Next, one of the trastuzumab-sensitive PDX (PDX118) was treated in vivo (biweekly; 1 mg/ml) with trastuzumab to induce an acquired drug resistance [43]. In most of the resistant PDXs originated from PDX118, we observed a significant increase in GSDMB (but not HER2) levels, thus corroborating that its expression is specifically associated with in vivo resistance to trastuzumab (Figure 4F).

Altogether, these data demonstrate that GSDMB overexpression is functionally involved in promoting resistance to the anti-HER2 immunotherapy trastuzumab, and this may explain its association with adverse clinical behaviour in HER2+ cancers.

**DISCUSSION**

Despite significant clinical benefits to trastuzumab in HER2-positive breast tumors, not all patients respond well to this therapy, and therefore experience disease recurrence and progression [44, 45], within a few months
of therapy (median of 7.4 months) [8,13]. In this scenario, the mechanisms of resistance to HER2-targeted therapy are not completely understood [46], and this limitation includes the unknown role of 17q12-q21 genes linked to \( \text{ERBB2} \).

Our study demonstrates that gasdermin B (\( \text{GSDMB} \)), mapped 175 kilo bases distal to \( \text{ERBB2} \), is amplified and over-expressed in a subset (~60%) of HER2-positive breast tumours. Importantly, GSDMB gene and protein over-expression is significantly associated with poor clinical outcome, in terms of disease progression, relapse and response after neoadjuvant therapy (Table 2), as well as lymph node positivity and the development of distant metastases.

Figure 3: GSDMB gene amplification and/or protein expression associates with relapse in HER2-positive breast cancer samples under neoadjuvant and adjuvant treatment regimens. Relapse-free survival curves in HER2-positive breast cancer patients (\( n = 95 \)) treated with neoadjuvant settings in relation to (A) GSDMB gene amplification (\( p = 0.0002 \)) and (B) GSDMB expression scored by immunohistochemistry (IHC, \( p = 0.0004 \)). (C) Relapse-free survival curves in HER2-positive breast cancer patients (\( n = 53 \)) treated with adjuvant settings in relation to GSDMB protein expression measured by IHC (\( p = 0.007 \)). Statistical differences, HR and \( p \)-value, were calculated via log-rank test. Red line: GSDMB amplified and expressed patients, green line: patients without GSDMB amplification and expression.

Figure 4: GSDMB expression increases survival to trastuzumab treatment. (A) Analysis of GSDMB expression by western blot in SKBR3 cells (empty vector, Control) and GSDMB-overexpressing cells (GSDMB). GAPDH was used as a loading control. (B) Analysis of cell growth (measured with Alamarblue) in control and GSDMB-overexpressing SKBR3 cells treated with 1 mg/ml trastuzumab for 72 h; (C) Reduction of cell growth in control (shNTC) and GSDMB-silenced (two shRNAs) HCC1954 cells treated with 5 mg/ml trastuzumab. (D) Analysis of cell death (measured with Annexin V and propidium iodide) in control and GSDMB-overexpressing SKBR3 cells 72 h after trastuzumab treatment. Bars represent mean ± SEM of three independent experiments. (E) GSDMB and HER2 mRNA expression in Patient Derived Xenografts (PDX) from HER2-negative (PDX01, 02) and HER2-positive breast carcinomas. PDXs were classified as trastuzumab sensitive (PDX118, 173) or resistant (PDX67, 288) according to \( \text{in vitro} \) patient response [42]. (F) GSDMB and HER2 mRNA expression in a trastuzumab-sensitive PDX (PDX118) and its trastuzumab-resistant derived PDXs (T-R1-R5), obtained by chronic \( \text{in vitro} \) treatment with trastuzumab. mRNA values were normalized using GAPDH as housekeeping gene. Bars represent mean ± SEM expression of three experiments by quantitative PCR.
metastases in the adjuvant context (Table 4) independently of hormone receptors status. Notably, the estimated effect in the neoadjuvant setting is relatively high (HR > 5) and, hence, the average time to relapse is almost halved in cases with GSDMB amplification or GSDMB over-expression.

GSDMB belongs to the gasdermin family of proteins [47]. Four gasdermin (GSDM) gene members have been identified in humans (GSDMA-D) [48]. GSDM genes have been involved in secretion, proliferation and differentiation in various tissues [48], although their major role seems to be the regulation of diverse types of cell death. In fact, GSDMD has been reported to be a substrate of caspase 11, essential for the activation of pyroptosis as anti-bacterial innate immune defence [49–52] and GSDMA promotes cell death through autophagy and mitochondrial damage [49,50]. The cell-death promoting function of GSDMA, C and D, depends on the cleavage of their N-terminal portion [51]. Interestingly, GSDMB does not induce cell death, and its C-terminal region is able to reduce partially the cell death induced by GSDMA [50]. Consistently, our data in different breast cancer cell lines models as well as in tumor Patient Derived Xenografts (PDXs) indicate that GSDMB promotes cell survival to trastuzumab anti-HER2 immunotherapy. Given that GSDMB alone does not affect cell growth [34], these results point out that GSDMB might be a negative regulator of cell death/survival under certain conditions (anti-HER2 therapy). Additionally, we have previously shown that GSDMB over-expression in a breast cancer cell line increased tumorigenic and invasive behaviour [34]. Therefore, GSDMB is functionally involved in promoting aggressive tumour behaviour and reduced clinical response to anti-HER2 therapies. It would be of interest in the future to decipher the exact molecular mechanisms by which GSDMB mediates these effects.

In conclusion, our study has identified GSDMB as a relevant biomarker of poor prognosis that is also able to predict non-response to current standard therapy for HER2-positive breast cancer. Since GSDMB expression promotes cell survival to anti-HER2 treatment, the analysis of GSDMB gene status and/or GSDMB expression may lead to more precise molecular classification of this cancer subtype and, in turn, help to identify cases that may not benefit from the current therapies.

### Table 5: Risk of relapse in HER2-positive breast tumours treated under adjuvant regimens according to GSDMB immunohistochemistry results

| Evaluation | Non-relapsed *n, % | Relapsed *n, % | Average time to relapse (months) | HR 95% CI | p |
|------------|---------------------|---------------|---------------------------------|----------|---|
| IHC GSDMB (n, %) |                      |               |                                 |          |   |
| Positive (28, 45.2%) | 7 (11.3%) | 21 (33.9%) | 91.24 ± 14.88 | 2.85 | 1.28–6.37 | 0.007 |
| Negative (34, 54.8%) | 17 (27.4%) | 17 (27.4%) | 101.8 ± 9.51 |        |     |

### MATERIALS AND METHODS

#### Tumour samples

A total of 286 paraffin-embedded ductal breast carcinoma included in this study was collected between 2003 and 2014 at the Vall d’Hebron Hospital (Barcelona), Virgen del Rocio Hospital (Seville, Spain) and the MD Anderson Cancer Centre (Madrid). This study was approved by the local ethical committee from each institution, and a complete written informed consent was obtained from all patients. Briefly, this study was performed in two independent data samples treated under neoadjuvant treatment: discovery series, which included 29 HER2 positive and 24 HER2 negative breast carcinomas, and validation series composed by 95 HER2 positive breast carcinomas (Supplementary Tables 2 and 3). The median age of patients and tumour size was 53.2 ± 14.9 years and 2.23 ± 1.45 cm, respectively. Additionally, we studied 138 high-grade ductal breast tumours (52 HER2-positive and 86 HER2-negative samples) all of them treated with adjuvant schedules (Supplementary Table 4). The mean age of patient and tumour size at surgery was 59.6 ± 9.1 years and 1.69 ± 0.42 cm, respectively. HER2 positivity was considered when the tumours showed gene amplification. All cases were treated with trastuzumab plus chemotherapy (based on a combination of anthracyclines and taxanes). Hormone therapy was added in those cases with positive hormone receptor expression. The clinical response of HER2-positive cases in the neoadjuvant setting was evaluated using the pathological complete response criteria (pCR). The pCR was assessed at the breast parenchyma and at the ganglia level. A patient will be deemed to be in pCR when there is no invasive presence of tumour at the breast or ganglia level.

#### Immunohistochemistry (IHC) and Fluorescence in situ hybridization (FISH) analysis

HER2, ER (oestrogen receptor) and PR (progesterone receptor) were analysed by IHC in TMAs and in a complete section samples from PDXs, according to standard methods as previously published [40]. GSDMB monoclonal antibody was generated as...
described in Supplementary Information, and GSDMB expression was classified as positive when more than 10% of tumour cells showed positive cytoplasmic staining. All studied tumour sections also included normal breast tissue as an internal control. In negative controls, the primary antibodies were omitted. FISH study was performed as previously described [53]. Briefly, the analysis was carried out simultaneously using red-labelled ERBB2, green-labelled chromosome enumeration probe (CEP17) as control (PathVysion Kit, Abbott Molecular), and aqua-labelled GSDMB. The GSDMB probe was generated from DNA isolated from the bacterial artificial clone (BAC) RP11–387H17 obtained from the BACPAC Resource Centre (BPRC) at the Children’s Hospital Oakland Research Institute (CA, USA) and labelled by nick translation. Fluorescence signals were scored in each sample by counting the number of single copy genes and control probe signals in an average of 180 (100–210) well-defined nuclei. The ASCO CAP 2007 criteria for ERBB2 gene amplification [39] were used to evaluate the status of GSDMB gene. Increased gene copy number and amplification were defined as the presence of 4–6 and > 6 signals, respectively. The GSDMB/CEP17 ratio used to delimit amplification was of ≥ 2.2 [39].

**GSDMB expression analysis in breast cancer microarray datasets**

To evaluate the expression of GSDMB in breast tumours, several independent datasets were analyzed [35, 36, 38] Microarray and clinical data were obtained from the ICR database (www.rock.icr.ac.uk). For each gene, normalized expression was categorized as “high” when it was above the third quartile (top 25% expression) of all tumour samples; otherwise, it was categorized as “low”. Kaplan-Meier estimates of GSDMB expression (assessed separately for each stratum) were plotted and compared to overall survival (OS), disease-free survival (DFS) and metastasis-free survival (MFS) curves using log-rank and Chi-square tests.

**Cell lines and Patient Derived Xenografts (PDXs)**

For the study of the role of GSDMB in response to trastuzumab two well-studied HER2-positive breast cancer cell lines [40], HCC1954 and SKBR3, were used. Both cell lines were obtained from the American Type Cell Culture (ATCC). Cells were grown as monolayer cultures at 37°C in an atmosphere with 5% CO2 and authenticated by STR-profiling according to ATCC guidelines. For the analysis of GSDMB over-expression, SKBR3 cells were stably transfected using Lipofectamine (Invitrogen) with pEZ-M61-GSDMB (Genecopoeia) or the empty vector. Cells containing the plasmids were selected with G418 (0.8 mg/ml) treatment. GSDMB-silenced HCC1954 cells were obtained as previously reported [34]. HER2-positive and trastuzumab resistant Patient Derived Xenografts (PDXs) were previously described in [42, 43]. HER2-negative PDXs were obtained from primary triple-negative breast tumours after engrafting orthotopically in the mammary fat of female athymic (nu/nu) mice (Harlan™). The patients provided written informed consent and the study was approved by IDIBELL Ethics committee. All ortho-xenograft models were established for > 3 passages. Their triple negative status was confirmed following standard immunohistochemical assays.

**Cell-based assays**

Trastuzumab anti-proliferative effects were measured in 2 × 10^3 cells plated on 96-well-plates using alamarBlue assay (Thermo Scientific) according to the manufacturer’s protocol. HCC1953 and SKBR3 cells were daily treated up to 72 h with 5 mg/ml and 1 mg/ml trastuzumab (Roche Diagnostics GmbH, Penzberg, Germany), respectively, or were left untreated. For detection of the death rate, SKBR3 cells were pre-treated in 0,1% FBS-containing medium during 24 h, and then (50 × 10^3,000) cells were cultured in 6-well plates and treated with or without trastuzumab (1 mg/mL dissolved in medium) for 72 hours. Cell death was measured by staining with Annexin V and propidium iodide (PI) using PE-Apoptosis Detection kit (Immunostep) according to the manufacturer's instructions, and analyzed on a FACSCANTO II flow cytometer (BD Biosciences). Three independent experiments were performed for Alamar blue and Cell death measurements.

**Gene expression assays**

Gene expression was performed after extraction of total RNA from PDXs using RNA miniprep system, ReliaPrep™ FFPE total (Promega) following manufacturer recommendations. cDNA was obtained from 1 µg of total RNA using random primers and M-MLV reverse transcriptase (Amresco) as manufacturer recommendations. GSDMB, HER2, and GAPDH gene expression levels were measured by quantitative real time RT-PCR (qRT-PCR) using pre-designed TaqMan probes (Thermofisher) and PerfeCTa FastmixII (Quanta BioSciences, Inc), on an iQ5 iCycler Realtime PCR Detection System (BioRad), according to the manufacturer’s recommendations. All qRT-PCR were performed in triplicate. Relative GSDMB and HER2 expression was normalized to GAPDH.

**Statistical analysis**

The χ^2 or Fisher’s exact tests were used to test associations between categorical variables. All tests were two-tailed and 95% confidence intervals (CIs) were used.
Values of $p < 0.05$ were considered statistically significant. Hazard ratios (HRs) and 95% CIs were estimated from univariate or multivariate Cox proportional hazards models. Disease-free survival was defined as the time from the date of diagnosis to the date of recurrence or development of novel distant metastasis. Survival curves were generated using the Kaplan-Meier method. The $p$-values are based on the Wald test for Cox proportional hazards models, and the log-rank test for Kaplan-Meier analysis. Statistical analysis were performed in R and using the Survival package and SPSS Statistics 17.0 (SPSS Inc., Chicago, IL).

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interests.

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