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RESEARCH

Gestational breast cancer: distinctive molecular and clinico-epidemiological features

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Abstract
Gestational breast cancer (GBC), defined as breast cancer (BC) diagnosed during pregnancy or the first-year post-partum, accounts for 6–15% of BC cases in women aged 20–44 years. GBC has worse prognosis than non-GBC, but reasons behind are not clear. The GEICAM/2012–03 Study (Molecular Characterization of Gestational Breast Cancer) is a multicenter prospective/retrospective observational registry of patients diagnosed with GBC. From November 2014 to June 2015 seventy patients diagnosed with GBC were included in the study, 30 diagnosed during pregnancy and 40 after delivery. Our current study was aimed to explore differences in epidemiological, clinico-pathological and gene expression features of GBC tumors, from the GEICAM/2012–03 Study, compared to non-GBC tumors from patients of similar age (< 43 years) from six different GEICAM studies, used as non- GBC control population. As per the main objective, the study found multiple differences showing GBC tumors as a different biological entity. GBC showed a more aggressive biology, with higher Ki67 levels, higher incidence of breast and/or ovarian cancer family history, and germline deleterious *BRCA1/2* mutations, and are enriched in basal-like intrinsic subtype. GBC patients showed a lower number of tumor infiltrating lymphocytes, while specific genetic signatures highlight differences in GBC´s distinctive transcriptome. Our study shows that GBC is potentially a clinically and molecularly different entity, with specific epidemiological, clinical, and histological features, as well as a distinctive altered immune state and genetic signature. Nevertheless, further studies are needed to better understand the biology of GBC and to identify new targets against which develop new, more effective, targeted therapies.

Keywords Gestational breast cancer · Gestation · Breast cancer · Tumor infiltrating lymphocytes (TILs) · PAM50 intrinsic subtypes · Gene expression

Introduction

Gestation is a biological event that is sometimes temporally related to breast cancer. With increasing incidence, the diagnosis of breast cancer is occurring during gestation or one year after delivery (gestational breast cancer) or in the 10-year period after delivery (postpartum breast cancer).

In our work, we focus on the gestational breast cancer (GBC), which is estimated to represent 6–15% of the cases of breast cancer (BC) in women aged 20–44 years [1, 2]. The incidence of GBC varies between 1:3,000–10,000 per number of gestations and it is the 4th most frequent malignancy emerging during pregnancy, after malignant melanoma, cervical cancer, and lymphoma [3]. With more women delaying childbearing in their reproductive years, GBC diagnosis is likely to be seen more commonly in clinical practice [4].

Women diagnosed with GBC are usually older than 30 years (average 32–38 years; median = 35 years). The role of pregnancy in the prognosis of BC remains unclear as, although some studies failed to show any significant association, others attributed a detrimental effect on prognosis based solely on the delayed diagnosis of tumors during pregnancy [5].

Pregnancy, as a modifier of risk for developing BC, confers a ‘‘dual effect’’. Compared to nulliparous women, pregnancy has a protective effect in women who have their first pregnancy in earlier life [6, 7] and is augmented for

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those whose first pregnancy occurs after age 35 [8, 9]. However, all women after pregnancy regardless of their age at first birth, have a subsequent period of years during which they are at increased risk for the development of BC [10].

Tumors in GBC frequently present similar characteristics, as axillary nodes involvement, negative hormone receptors (HR), triple negative phenotype, low differentiation and high histological grade, ultimately having a worse prognosis than non-GBC patients [2, 11–13].

Although some studies have been conducted to better characterize GBC from a molecular point of view [14, 15], little is known about the genomic and immune profiling of these tumors. Our study intends to fill this gap, aiming to assess whether GBC is associated with any specific intrinsic subtype and to explore the correlation between the genomic profile of GBC tumors and the epidemiological and clinico-pathological characteristics of the patients.

Methods

Study design and patients

The GEICAM/2012–03 Study (Molecular Characterization of Gestational Breast Cancer) was a multicenter prospective/retrospective observational registry of patients diagnosed with GBC, defined as BC diagnosed during pregnancy or the first year post-partum (participant institutions are summarized in Supplementary Table 6). The aim of this study was to investigate the distinct clinico-pathological characteristics and genetic profile of this population by comparing them with non-GBC patients of similar age (<43 years) who were recruited in Spain in the studies listed below (Fig. 1):

(i) GEICAM/9906 [16, 17] (NCT00129922) a multicenter randomized phase 3 study evaluating adjuvant chemotherapy for node positive operable BC patients;

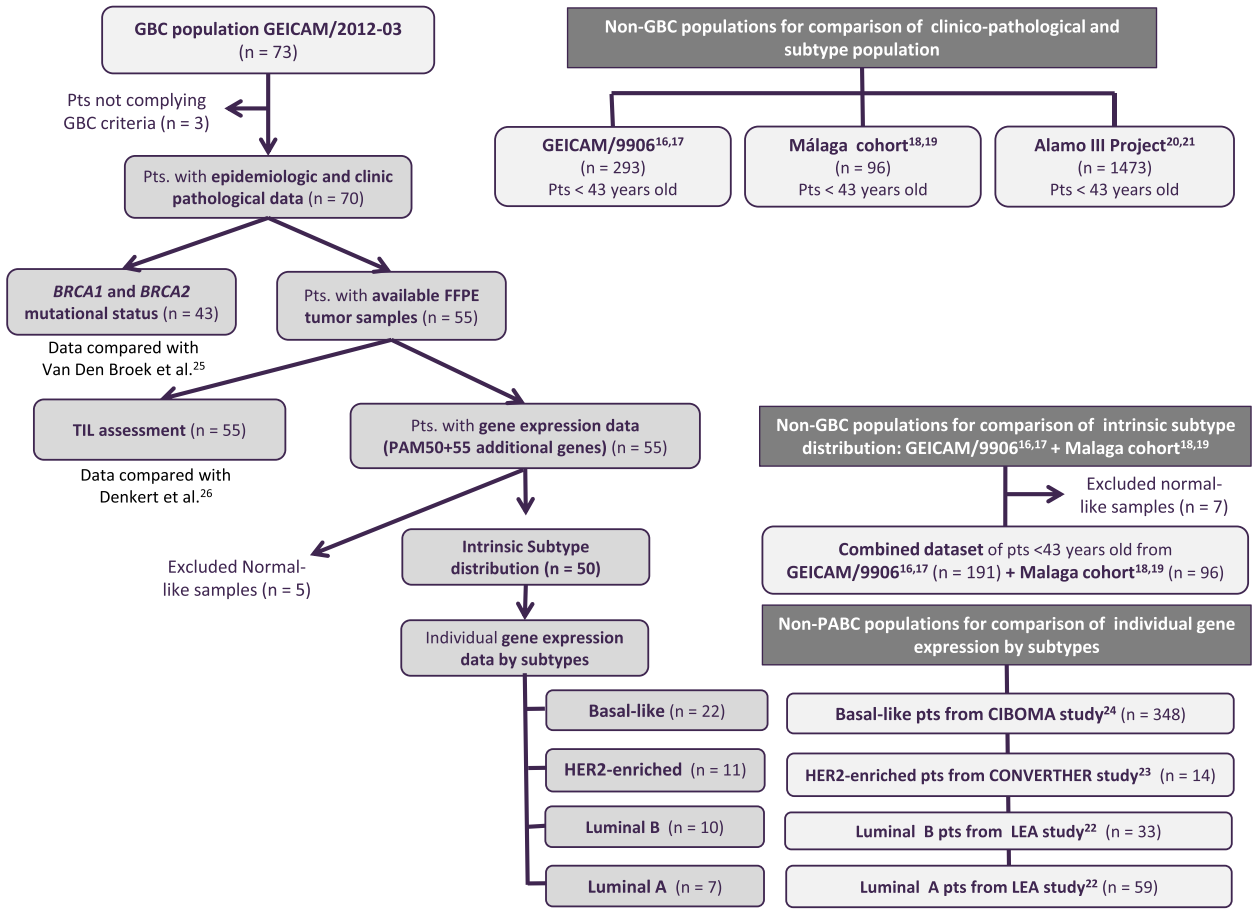

Fig. 1 Consort diagram. Flowchart describing the GBC and non-GBC populations used in the study, the assessments made and the number of patients analyzed in every step. FFPE: Formalin Fixed Paraffin Embedded; n: sample size; Pts: patients; TILs: tumor infiltrating lymphocytes

(ii) the Malaga Cohort [18, 19], a study to predict response to neoadjuvant chemotherapy in early BC patients;
(iii) El Alamo III project [20, 21], a retrospective, observational, multicentric study to describe characteristics and evolution of patients diagnosed with new BC since 1998 to 2001 in Spain;
(iv) GEICAM/2006–11 (LEA) [22] (NCT00545077), a randomized phase III trial comparing endocrine therapy ± bevacizumab in hormonal receptor (HR)-positive/HER2-negative metastatic BC;
(v) GEICAM/2009–03 (ConvertHER) [23], an observational study on HER2 dynamic in paired primary breast tumors and the corresponding metastatic lesions; and
(vi) GEICAM/2003-11-CIBOMA/2004-01 (CIBOMA) [24] (NCT00130533), a randomized phase III trial exploring extended adjuvant capecitabine vs. observation after completion of standard chemotherapy in patients with early triple negative BC.

Previously published data by Van Den Broek et al. [25] ($n = 6294$) and Denkert et al. [26] ($n = 3771$) were used, respectively, in the BRCA1/2 mutational status and tumor-infiltrating lymphocyte analysis.

The study protocol was approved by the applicable Clinical Research Ethics Committees of the participating Spanish clinical sites (Supplementary Table 6). Study procedures were conducted in accordance with the Declaration of Helsinki, as revised in 2013, and good clinical practice guidelines. Informed consent was obtained for the collection of local epidemiological, clinicopathological and biological data, and biomarker assessment according to applicable legislation.

Available archival formalin-fixed paraffin-embedded (FFPE) breast cancer tissue samples were obtained from the participants in the study.

Three biomarker analyses were performed: i) *BRCA1* and *BRCA2* germline mutational status (BRCA1/2: breast cancer susceptibility gene 1 and 2), ii) tumor-infiltrating lymphocyte (TIL) content evaluation and iii) gene expression profiling, including the intrinsic subtype (PAM50) determination (Fig. 1).

Epidemiological and clinico-pathological features of GBC

Main epidemiological and clinicopathological variables in the GBC population were described (Table 1) and compared with those of patients diagnosed up to the age of 42 (the maximum age within the GBC population) from the control studies, (I) GEICAM/9906, (II) the Malaga Cohort and (III) El Álamo III project (Supplementary Table 1).

BRCA1 and BRCA2 mutational status

Germline *BRCA1* and *BRCA2* mutations were recorded based on local reports from participating centers in 43 GBC cases, annotating the presence or absence of deleterious or suspicious deleterious mutations, polymorphisms, variants of unknown significance and gene rearrangements. The prevalence of deleterious *BRCA1*/2 variants in the GBC cohort was compared to previous published data from BC patients diagnosed under the age of 45 [25].

Tumor-infiltrating lymphocyte analysis

TILs were quantified in a central laboratory on hematoxylin and eosin (HE)-stained FFPE tumor sections. Stromal TILs were measured following the guidelines of the international TIL working group [27] as the percentage of stromal area occupied by mononuclear inflammatory cells over total intratumoral stromal area. All mononuclear cells within the borders of the invasive tumor, avoiding necrotic areas, were scored as a continuous variable. The proportion of stromal TILs was classified in three categories: Low-TIL (≤ 10% immune cells in stromal tissue within the tumor), intermediate-TIL (11–59%) and high-TIL content (≥ 60%), as previously suggested by Denkert et al. [26]. Given the lack of TIL data in our non-GBC control populations, the TIL content in the cohort explored by Denkert et al. [26], with age-matched patients at diagnosis (≤ 40 years old), was used as comparator against the TIL content observed in our GBC cohort.

Gene expression analysis in GBC

A HE staining of the FFPE tumor tissue was initially assessed to confirm the diagnosis and determine the tumor surface area. For total RNA purification, 10 µm tumor sections from 55 GBC patients were macrodissected to avoid normal tissue contamination. A minimum of 100 ng of total RNA was then used to centrally analyze the expression of 105 BC-related genes, containing the research-based PAM50 classifier for intrinsic subtype prediction, the proliferation signature, the Risk of Recurrence (ROR score) based on subtype and proliferation (ROR-P), and the claudin-low classifier using the nCounter platform (Nanostring Technologies, Seattle, WA, US), as previously described [23].

Intrinsic subtypes distribution in GBC vs. non-GBC patients

Tumors from GBC patients were categorized into intrinsic subtypes of BC (luminal A, luminal B, HER2-enriched and basal-like) and the normal-like group using the PAM50 classifier. Normal-like samples were excluded from further analysis. Additionally, claudin-low subtype was identified

Table 1 Epidemiological and clinico-pathological characteristics of the GBC patients

Patient information			
Age at diagnosis	**median (range)**		
Age (years)	35 (24–42)		
Gestational moment at breast cancer diagnosis (*n* = 70)	**n (%)**		
Pregnancy	30 (42.9)		
First year after birth	40 (57.1)		
Child delivery (Diagnosis during pregnancy) (*n* = 30)	**n (%)**		
Full-term	16 (53.3)		
Premature	8 (26.7)		
Abortion	6 (20.0)		
Child delivery (Diagnosis after delivery) (*n* = 40)	**n (%)**		
Full-term	31 (77.5)		
Premature	6 (15.0)		
Abortion	NA		
Unknown	3 (7.5)		
Histological grade (*n* = 70)	**n (%)**		
G1	3 (4.3)		
G2	19 (27.1)		
G3	38 (54.3)		
Gx	10 (14.3)		
Metastasis location[a] (*n* = 6)	**n (%)**		
Liver	3 (50)		
Bone	1 (16.7)		
Bone-Breast-Liver	1 (16.7)		
Lymph nodes-Liver-Other	1 (16.7)		
Staging (*n* = 70)	**n (%)**		
I	10 (14.3)		
IIA	16 (22.9)		
IIB	11 (15.7)		
IIIA	13 (18.6)		
IIIB	5 (7.1)		
IIIC	6 (8.6)		
IV	6 (8.6)		
Unknown	3 (4.3)		
Subtype classification—IHC (*n* = 70)	**n (%)**		
RH + / HER2-	13 (18.57)		
HER2 + (RH ±)	29 (41.43)		
TN (HER2-/RH-)	21 (30)		
Unknown	7 (10)		
Biomarkers (*n* = 70)	**Positive n (%)**	**Negative n (%)**	**Unknown n (%)**
Hormonal Receptors	40 (57.1)	30 (42.9)	0
• Estrogen receptors	34 (48.6)	36 (51.4)	0
• Progesterone receptors	33 (47.1)	35 (50.0)	2 (2.9)
HER2	12 (17.1)	50 (71.4)	8 (11.4)
Ki67			
• ≥ 20%	43 (61.4)		
• < 20%	5 (7.1)		
• Unknown	22 (31.4)		
First- and/or second-degree family history of breast and/or ovarian cancer (*n* = 70)			
Yes	34 (48.6)		

Table 1 (continued)

Patient information	
No	31 (44.3)
Unknown	5 (7.1)

[a]Metastatic lesions presented only in 6 out of 70 GBC patients

as described by Cejalvo *et al* [18]. As control cohort, we used age-matched non-GBC patients (diagnosed at or below 42 years of age) from the GEICAM/9906 clinical trial and the Malaga Cohort.

Differential gene expression by intrinsic subtypes in GBC vs. non-GBC patients

Gene expression analysis by intrinsic subtypes included, as control cohorts, the following non-GBC patients: (i) luminal A/B BC patients from the study GEICAM/2006–11 (LEA); (ii) HER2-enriched BC patients from the GEICAM/2009–03 (ConvertHER); and (iii) basal-like BC patients from the GEICAM/2003–11-CIBOMA/2004–01 (CIBOMA).

Cohort statistical analyses

A descriptive analysis of the epidemiological, clinicopathological and biological characteristics was reported for all patients. Continuous variables were summarized using descriptive statistics (e.g., median and range). Categorical variables were summarized using frequencies and percentages.

Contingency tables were used to analyze the relationships between two categorical variables, and the Fisher's exact and chi-square tests were performed for testing the null hypothesis of independence of the variables. Odds ratios were estimated from logistic regression models. Exact binomial test was used for comparison of proportions with previous published data. Continuous variables were compared between two or more groups using the Wilcoxon rank sum test, as variables do not follow a normal distribution. Box plots were used to visualize these data relationships. P-values < 0.05 (two-sided) were considered statistically significant.

A Principal Component Analysis (PCA) was carried out to compare inter-test normalized gene expression values across cancer subtypes in GBC and non-GBC patients. Prcomp and pca3d R packages were used.

Differential expression analysis

For the analysis of differential gene expression in GBC and non-GBC patients by intrinsic subtypes stratification, gene expression values for the 105 analyzed genes were calculated by an intra-test normalization against 5 housekeeping genes followed by an inter-test z-score normalization as in the NanoStringNorm R package. Differential expression was calculated for every intrinsic subtype in both, GBC and non-GBC patients, using SAM (Significance analysis of microarrays) algorithm [28]. P-values were adjusted using FDR (false discovery rate) and FDR < 0.05 was considered statistically significant. Heatmaps of inter-test normalized genes values were generated, and Euclidean distance and unweighted pair-group method with arithmetic mean (UPGMA) applied for clustering.

Pathway enrichment analysis based on differentially expressed genes in GBC and non-GBC patients across different intrinsic subtypes were performed through Gene set enrichment analysis (GSEA) [29, 30] and Metascape [31].

Predictive models

A neural network (NN) model was applied to every cancer subtype to assess prediction power between GBC and non-GBC patients. Models were built using normalized gene expression values. The NNs were generated using the caret R package [32] and the "nnet" method. We applied a tenfold cross validation (90% training and 10% testing, 70%-30% in the case of luminal A samples due to the small number of GBC instances) to every model. The NN models were trained using automatic selection of i) optimal number of units per hidden layer (1 to 5), and ii) regularization parameter (0.1 to 0.5 with increments of 0.1). The "twoClassSummary" method was applied to compute sensitivity, specificity and the area under the curve (AUC). A receiving operating characteristics (ROC) curve was calculated for every tenfold cross validation process. For subtypes with unbalanced sample size between GBC and non-GBC patients (basal-like, luminal A and luminal B), we generated 10 re-sampled matrices with available GBC samples and a random selection of the same number of samples from non-GBC samples [33, 34]. For each of those 10 re-sampled matrices, NN models were built. The final AUC, accuracy, sensitivity, and specificity were calculated as the mean of the NNs models performed in every case.

Results

Patient and tumor characteristics

From November 2014 to June 2015, 70 patients diagnosed with GBC from 23 April 1987 to 14 January 2015 were included in the study. Of them, 30 (42.9%) were diagnosed during pregnancy and 40 (57.1%) after delivery. Table 1 summarizes the clinicopathological characteristics of the study population.

Main epidemiological and clinicopathological characteristics of GBC population were compared to those of non-GBC patients, from three age equivalent control cohorts: GEICAM/9906 clinical trial ($n = 293$), Málaga cohort ($n = 96$) and El Alamo III project ($n = 1473$). GBC showed a more aggressive clinico-pathological profile at diagnosis with higher proportion of patients having tumors with high histological grade, Ki67 ≥ 20% and HR-negative status, as well as a higher proportion of patients with family history of breast and ovarian cancer (Supplementary Table 1). A sensitivity analysis, excluding those patients with family history of breast and/or ovarian cancer in our GBC and The ALAMO III cohorts showed similar results to those shown in Supplementary Table 1.

BRCA1 and *BRCA2* mutation status was available for 43 (61.4%) GBC patients. *BRCA1* and *BRCA2* deleterious mutations were found in 6 (14%) and 2 (4.6%) patients, respectively. Altogether, deleterious *BRCA1* or *BRCA2* mutations were found in 18.6% of patients, which is significantly higher than the previous published data for BC diagnosed patients under 45 years (5.8%) [25] (Fig. 2A). Six

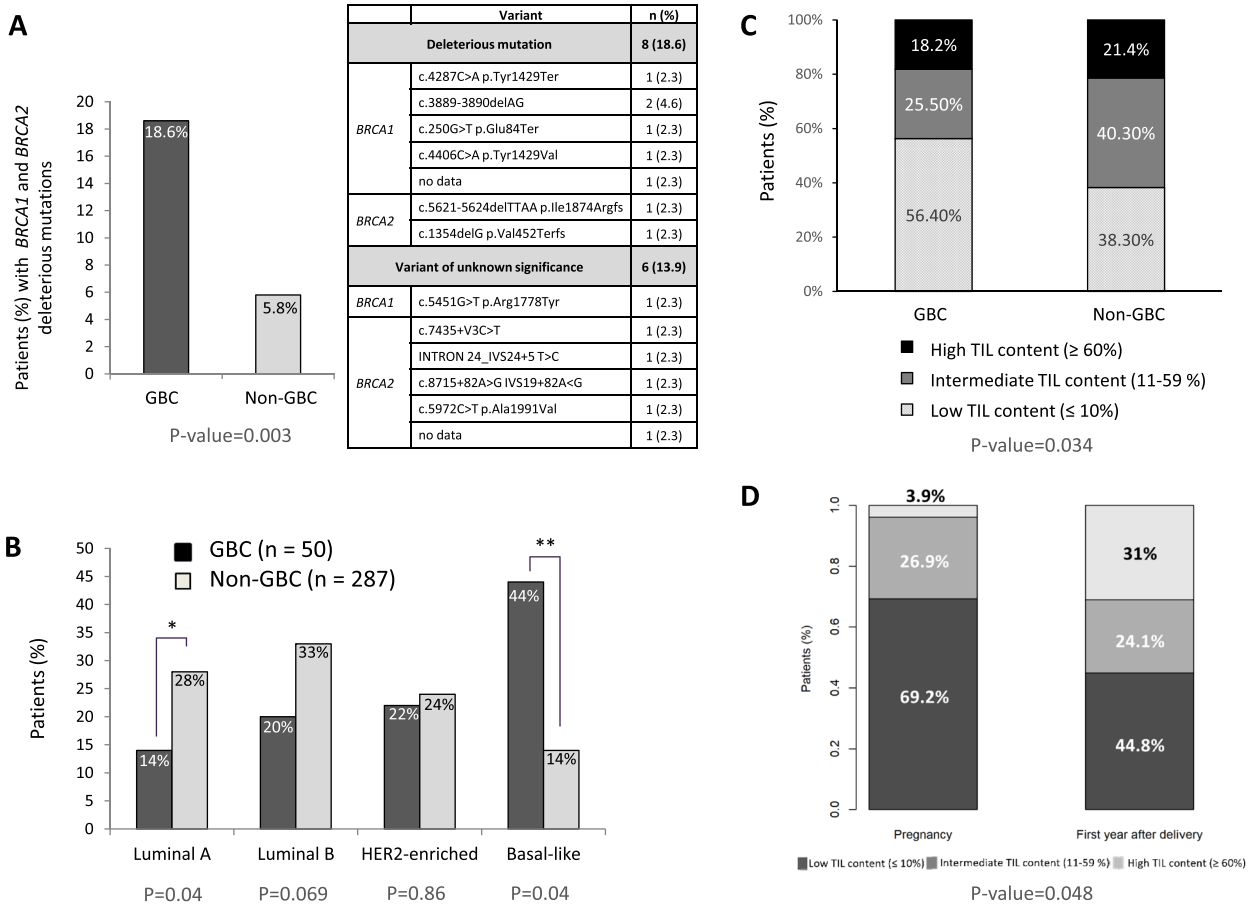

Fig. 2 Comparison of GBC against non-GBC control populations. **A** Presence of deleterious mutations in *BRCA1* and/or *BRCA2* in GBC *vs*. non-GBC control population from Van der Broek et al. (2016) [20]; *exact binomial test for proportions, *p*-value = 0.003; list of *BRCA1* and *BRCA2* deleterious mutations and variants of unknown significance found in GBC patients. **B** Distribution of PAM50 intrinsic subtypes in GBC *vs*. non-GBC control population from GEICAM/9906 [13] and Malaga cohort [14]. **p*-value Fishers' exact test < 0.05; ***p*-value Fishers' exact test < 0.01. Fisher´s exact test for luminal A = 0.04, luminal B = 0.0694, HER2-enriched = 0.86, and basal-like = 0.000039. **C** Tumor-infiltrating lymphocyte (TIL) content in GBC *vs*. non-GBC control population from Denkert et al. (2018) [21] (in both populations patients ≤ 40 years old at diagnosis were considered). Chi. [2] test *p*-value = 0.034. **D** TIL content in GBC population according to the gestational status at the time of breast cancer diagnosis. Fishers' test *p*-value = 0.048

patients (13.9%) showed variants of unknown significance in either *BRCA1* (1 patient) or *BRCA2* (5 patients) (Fig. 2A).

Basal-like subtype is the most prevalent subtype in GBC

Gene expression was available for 50 GBC tumors (71.4%), 31 pre-treatment samples (62%) and 19 (38%) post-treatment (13 of them obtained after neoadjuvant treatment). Basal-like was the most prevalent intrinsic subtype in GBC, observed in 22 cases, (44%). Conversely luminal A subtype was the least prevalent (7 cases (14%) (Fig. 2B). Notably, none of the GBC tumors were classified as claudin-low subtype. In the non-GBC control population (GEICAM/9906 and Malaga Cohort combined dataset; $n=287$), luminal B was the most prevalent subtype (96 cases, 33.4%), whereas basal-like subtype was the least prevalent (40, 13.9%) (Supplementary Table 2).

Basal-like phenotype was significantly enriched in GBC (44.0% vs. 14.0%; $p<0.01$), whereas luminal A subtype was significantly less prevalent in GBC than in the control population (14.0% vs. 28.2%, respectively; $p<0.05$) (Fig. 2B; Supplementary Table 2). These results suggest a particularly aggressive biology of the tumors in GBC patients compared to non-GBC patients of similar age. Additionally, GBC showed a significant higher level of the proliferation marker Ki67 when compared to non-GBC control populations (90% of GBC patients had Ki67 ≥ 20%) (Supplementary Table 1) and a higher rate of patients with high ROR-P score (Risk of Recurrence score based on the proliferation-related genes included in the PAM50 signature) (62.0% vs. 41.4%) ($p=0.011$).

We observed differences among the intrinsic subtype groups of GBC patients in the age at diagnosis ($p=0.016$), being higher in luminal GBC patients than in GBC basal patients ($p=0.02$) (Supplementary Table 3).

TIL content is lower in GBC patients

TIL concentration was available for 55 GBC patients (78.6%). Thirty-one tumors (56.4%) exhibited low-TIL, while 14 (25.5%) showed intermediate-TIL and 10 (18.2%) displayed high-TIL content (Fig. 2C; Supplementary Table 4).

The TIL content from the pooled analysis conducted by Denkert et al. [26] (in patients aged 40 years or younger) was used as group for the comparative analysis against the TIL content in the age-matched population ($n=52$, 94.5% of total) of our GBC series. In their study, Denkert and colleagues found 38.3% of patients had low-TIL, 40.3% intermediate-TIL and 21.4% high-TIL content, a distribution which significantly differs from our population ($p=0.034$). Our results showed that low-TIL content was predominant in GBC patients (56.4%), whereas the control population exhibited more frequently intermediate-TIL content (40.3%) (Fig. 2C).

TIL content varies depending on the gestational status but not on the intrinsic subtype

TIL content analysis according to gestational status at BC diagnosis, showed that TIL content significantly varies depending on the gestational status ($p=0.029$). Women diagnosed during pregnancy showed predominantly low-TIL content, while the TIL content was higher during the first year after delivery (Fig. 2D).

We then analyzed the samples considering the intrinsic subtypes, observing a predominantly low-TIL content in all subtypes, ranging from 45.5% of low-TIL content in basal-like to 72.7% in HER2-enriched tumors. The basal-like subtype showed the higher number of samples with high-TIL content (31.8%), while the rest of subtypes showed none or very limited number of samples with high-TIL content. Although we found no differences in the TIL content when comparing the distribution across all the intrinsic subtypes ($p\text{-}value=0.340$), a trend towards it was observed when comparing basal vs. non-basal-like intrinsic subtypes ($p=0.089$) (Supplementary Table 4).

Gestational BC has specific gene signatures

We subsequently explored the presence of differential gene expression signatures in GBC and non-GBC samples, and by intrinsic subtype stratification. As non-GBC controls, we used 59 luminal A and 33 luminal B tumors from the GEICAM/2006–11 (LEA) clinical trial, 14 HER2-enriched tumors from the GEICAM/2009–03 (ConvertHER) study, and 348 basal-like tumors from the GEICAM/2003–11-CIBOMA/2004–01 (CIBOMA) trial (Fig. 1).

A heatmap of the gene expression data with UPGMA samples clustering is shown in Supplementary Fig. 1, which includes all patients regardless the intrinsic subtype. This analysis showed a general aggregation of the patients with the same type of cancer, a trend confirmed by the principal components analysis (PCA) (Fig. 3A1 and A2).

UPGMA clustering (Supplementary Figs. 2–5) and PCA analyses (Fig. 3B) were also performed for each intrinsic subtype separately, while comparing GBC and non-GBC samples. All subtypes revealed specific expression profiles for the genes studied that can separate GBC and non-GBC. A gene differential expression analysis was also performed to compare GBC and non-GBC patients, relevant GBC up- and down-regulated genes (FDR < 0.05) are listed in Supplementary Table 5.

Metascape (Fig. 4) and gene set enrichment analysis (GSEA) (Supplementary Fig. 2) from the 105 genes showed

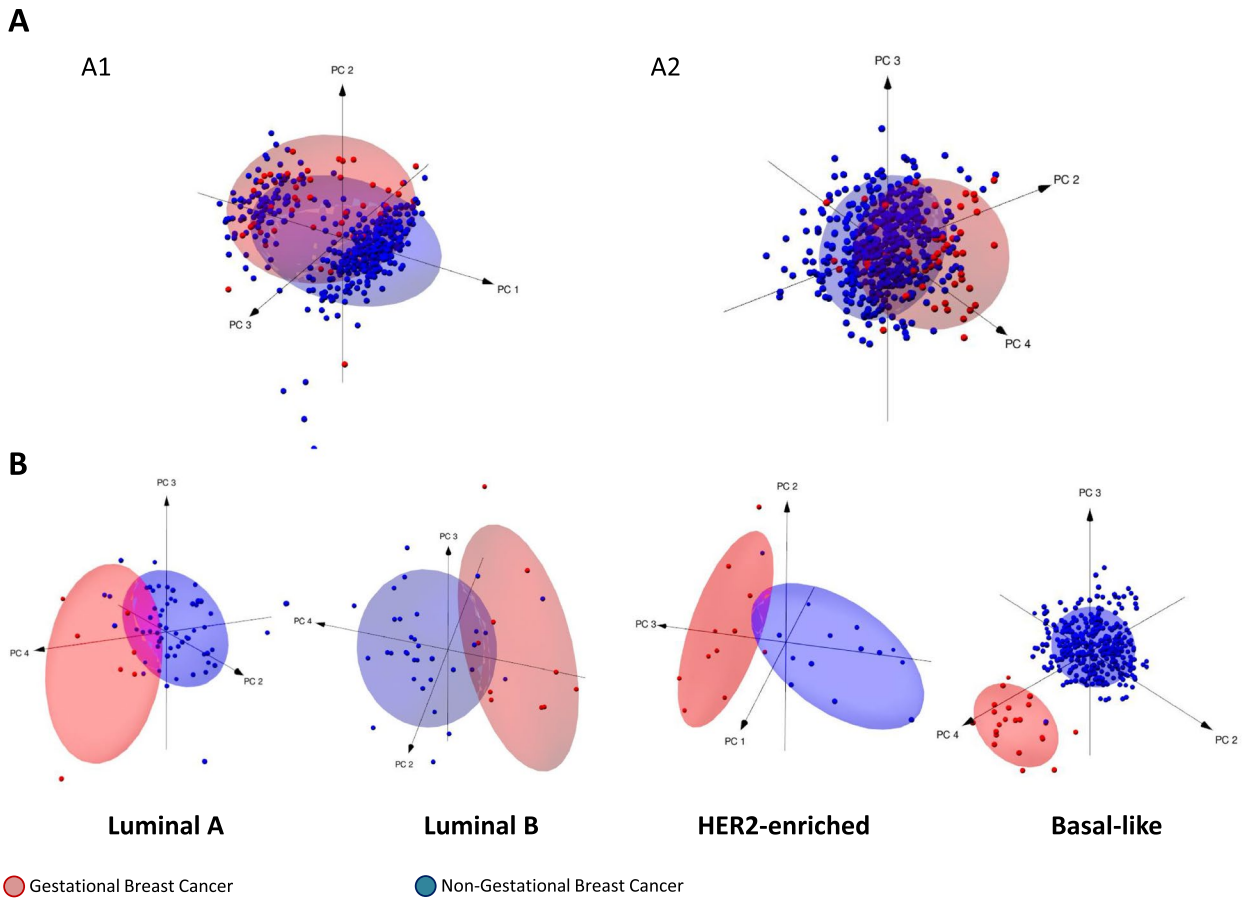

Fig. 3 Principal Component Analysis of GBC vs. non-GBC. Principal Components analysis (PCA) of GBC (red) and non-GBC (blue) samples in (**A**) the global population (PC1, 2 and 3 in figure A1, and PC 2, 3 and 4 in figure A2) and (**B**) stratified by intrinsic subtype

statistically significant differences between GBC and non-GBC tumors using a cutoff FDR ≤ 0.05. All BC intrinsic subtypes showed different dysregulated genes and gene sets related to cell cycle control, DNA repair and genomic stability pathways. Interestingly, *MYBL2* and *UBE2T*, critical genes in DNA repair and genomic stability pathways [35, 36], were dysregulated in GBC tumors, in all BC intrinsic subtypes. Likewise, our results showed the ubiquitin-conjugating enzyme E2T (*UBE2T*) previously found upregulated in basal-like tumors [37], is dysregulated in GBC tumors, in all BC intrinsic subtypes.

A prediction model of GBC vs. non-GBC using a neural network

To assess the prediction capacity of the gene expression data to classify the samples in GBC and non-GBC, we applied a neural network (NN) prediction model to i) all samples, and ii) samples of each subtype. Considering all samples, NN obtained average values of sensitivity and specificity of 0.72 and 0.73, respectively (Supplementary Fig. 3). Predictive capacity for every subtype is showed in Supplementary Fig. 4. In these cases, we obtained average sensitivity and specificity values of 0.7–0.69, respectively, in luminal A tumors; 0.67–0.67 in luminal B tumors; 0.68–0.66 in HER2-enriched tumors and 0.7–0.7 in basal-like tumors.

Discussion

While some series attempt to clinically and molecularly characterize GBC [14, 15], the present study is the first work to address the question of whether gestational cancer is molecularly different by making a comparison with women of the same age diagnosed with BC. Regarding the prevalence of the different intrinsic subtypes and the TIL content analysis, age was chosen as the exclusive matching criterion, due to its inherent ability to control for numerous biological aspects that could potentially introduce bias into our comparison. In addition, as all patients were recruited in Spanish sites, we were not expecting ethnics differences affecting clinico-pathological variables or subtype distribution in our GBC population. This assumption is based on the ethnic homogeneity of the Spanish population, as according

Fig. 4 Metascape pathway enrichment analysis of differentially expressed genes in GBC vs. non-GBC across the different intrinsic subtypes. Multiple gene and gene sets related to cell cycle control, DNA repair and genomic stability pathways showed differential expression between GBC and non-GBC BC. Those gene and gene set are differentially distributed across all BC intrinsic subtypes

to epidemiological and demographic data from Spain, the majority of the population is of European origin and presents low ethnic variability compared to other countries (89.2% of the population in Spain is of European origin, with ethnic minorities representing a small percentage according to the Spanish National Institute of Statistics (INE) 2020 report). The analysis of differential gene expression signatures was performed using subtype as matching criterion.

So, this manuscript showed that GBC has specific features that differentiate it from non-GBC, diagnosed in same age range women. This observation establishes the foundation for the search of a relationship link between pregnancy and BC.

Despite the strength of our design and the results presented in this study, there are also some limitations. Firstly, associated to the GBC cohort, the low number of GBC patients under study could be considered as a limitation, weakening the scope of the study. However, this limitation perfectly reflects the challenges of patients´ recruitment on GBC, a disease with such a low incidence, estimated to represent the 0.2–3.8% of all BC patients [38]. Connected to this, the use of treatment naïve patient samples would be preferred for this type of analysis, but for several patients only post-treatment samples were available, and the omission of such number of samples would have reduced significantly the sample size of the analysis.. Another possible limitation concerns the control group. Among the women included in this group, those diagnosed with postpartum breast cancer were not excluded. Taking into account the published characteristics of these postpartum tumours [39], we consider that they should not negatively influence the observed results.

Our research is aligned with prior findings, as multiple publications in recent decades have shown differential epidemiological and clinical features in GBC tumors [2, 11–13]; although, the scarcity of evidence at the molecular level in previous research [40, 41] serves to highlighted the importance of our study. Some authors have detected a

worse clinical evolution in GBC patients and have linked this poor prognosis not with the pregnancy itself, but with other factors as later diagnoses, or the diagnosis of more aggressive tumors [42]. In our GBC series, we observed a first gestation age (median) above 30 years, 52% of the women had a family history (first and/or second degree) of breast and/or ovarian cancer compared to 27% in El Alamo III series (non-GBC) and, according to this data, we found a higher incidence of deleterious mutations in *BRCA1* and *BRCA2* compared to the incidence previously described in age-matched non-GBC (18.6% vs. 5.8%) [25]. Studies in animal models relate *BRCA1* and *BRCA2* mRNA levels with mammary epithelial proliferation during pregnancy [43]; so, taking all this evidence into account, pregnancy could be a stimulus in the genesis of mammary neoplasms in patients with BRCA mutations. Similarly, another study found a relationship between GBC and mutations in *BRCA1* and *BRCA2* [44].

We also found some differences from the histological characteristics. Poor differentiated tumors with lower expression of hormone receptors and higher Ki67 expression are more frequent in the GBC population. Our findings are concordant with previous published data; Bonnier et al. published 42% of tumors associated with pregnancy with negative HR compared to 21% of those not associated with pregnancy [43]. Similar results are observed in a series of 750 patients diagnosed with BC, in which a direct relationship is observed between the time interval elapsed since pregnancy to BC diagnosis and the expression of hormone receptors [45]; similarly, data related to an increase in the expression of Ki67 in the GBC patients are also coincident with other series [46].

The use of multiple non-GBC control studies to compare our results with, gave our study the strength of a well contrasted hypothesis and well validated results, comparing not only molecular results, but also clinico-pathological features. However, it also introduces a certain amount of heterogeneity due to the use of different technological tools in these studies. Furthermore, this comparison group may not properly represent the universe of BC patients, since some of these studies, such as ConvertHER and LEA cohorts, include metastatic BC patients and, although primary tumor sample was predominantly utilized, they only consider cases with bad prognosis. In our opinion, the undoubted quality of all those control population studies, and the value of the data provided, compensate the possible bias and limitations.

Recent scientific evidence have showed the emergence of an immunotolerance state during pregnancy [47], as the immune system is suppressed to avoid rejection of the fetus; additionally, a higher frequency of infections during pregnancy has been reported as evidence of this immunotolerance state [47, 48]. In accordance with those results, we observed a lower presence of TILs, with 56.5% of GBC patients showing a low-TIL infiltration, while in non-GBC patients, ≤ 40 years, only 38.3% of patients showed a low-TIL infiltration, and more frequently (40.3%) showed an intermediate-TIL infiltration level. Additionally, other series published by Azim et al. confirmed the lower lymphocytic infiltration in pregnant patients (TILs > 50% in 2.3% vs. 9.6% of non-pregnant patients) [49].

In our series, we observed that TIL content varies significantly after delivery, as we found an increase in TILs in those cases diagnosed during the first year after delivery over those detected during pregnancy. This may be due to a pro-inflammatory phenomenon secondary to breast involution during the postpartum period [50], characterized by an increase in programmed death phenomena, by remodeling the extracellular matrix and by an increase in inflammatory infiltrate [51, 52]. In fact, this involution process has been related with the occurrence of postpartum breast cancer [53]. The lower content of TILs in cases diagnosed during gestation may be attributed to a situation of gestational immunosuppression.

A higher presence of TILs has been previously reported in the HER2-positive and triple negative subtypes [26] and, similarly in our GBC series, basal-like intrinsic subtype frequently showed a high inflammatory infiltrate content (31,8%), not common in the remaining intrinsic subtypes. In line with our results, in the series published by Azim et al., the basal-like intrinsic subtype showed the highest percentage of TILs, although with no statistically significant differences against the other subtypes [49].

Regarding the distribution of the different intrinsic subtypes, we found that GBC patients present a significantly lower proportion of luminal A tumors and higher proportion of basal-like tumors than non-GBC patients. Similar results were observed by Azim et al., but although the distribution of the different intrinsic subtypes is very similar, they did not found statistically significant differences between the GBC and control groups [14]. This may be due to the differences in the sample size in the control group of both series.

Lastly, the expression analysis of the genes included in the customized panel highlighted that, the 4 intrinsic subtypes showed a differential gene expression profile between GBC and non-GBC, suggesting a possible genomic signature for GBC.

Supporting the hypothesis of the potential relationship between pregnancy and a specific gene signature, we found an enrichment of genes involved in cell cycle control and DNA repair in GBC. The *MYBL2* gene is essential for regulating vital cellular processes such as cell proliferation, differentiation and DNA repair. Changes in these pathways may facilitate cancer development. MYBL2 upregulation in breast cancer can occur through multiple mechanisms, such as changes in microRNA regulation, amplification of the coding region of the 20q13 gene and elevated *MYBL2*

expression in breast cancer correlates with BC metastasis, worse relapse-free survival and shorter overall survival, providing strong evidence that upregulation of MYBL2 functions contributes to more aggressive disease [54]. The other over expressed gene is *UBE2T* which predicts a poor prognosis and is related to DNA stress phenomena [55].

These results might point these pathways out as a key feature among GBC and non-GBC tumors; however, further investigations are needed, to confirm this hypothesis and to unravel the importance of these pathways in GBC tumorigenesis.

Conclusions

Our study shows that GBC is potentially a clinically and molecularly different entity. Epidemiological, clinical, and histological features highlighted GBC tumors as a particular entity, with a differential altered immune state and a distinctive genetic pattern. Nevertheless, further studies are needed to better understand the biology of GBC and to identify new targets against which develop new, more effective, targeted therapies.

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Author's contributions J.dH., M.M., B.B., A.G. and M.P. designed the study. J.dH., B.B., A.G., J.A., J.G., C.M., A.H.B., S.G.L., M.T.M. and S.B. supervised the data acquisition. P.M., A.P., J.H. performed the formal study analysis. J.dH., M.M., B.B., A.G., M.P. and F.R. supervised the data analysis and results interpretation. J.dH., R.C., P.M., J.H. and N.M. wrote the first manuscript draft. J.dH., M.M., B.B., C.H., S.S., F.R., E.A. and A.RL. coordinate the different studies included as control cohorts. A.P. was the person in charge of central lab (nCounter). J.P. performed the TILs analysis. J.dH. and R.C. worked in funding acquisition. All authors read and approved the final manuscript.

Data availability The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The study protocol was approved by the applicable Clinical Research Ethics Committees of the participating Spanish clinical sites (Supplementary Table 6). Study procedures were conducted in accordance with the Declaration of Helsinki, as revised in 2013, and good clinical practice guidelines. Informed consent was obtained for the collection of local epidemiological, clinico-pathological, and biological data, and biomarker assessment according to applicable legislation.

Consent for publication Not applicable.

Competing interests J. de la Haba-Rodriguez declares having received research grants from Roche and Pfizer, consulting/advisory fees from AstraZeneca, Amgen, Roche/Genentech, Novartis, Eli Lilly and Pfizer and speakers' honoraria from AstraZeneca, Lilly, Amgen, Roche/Genentech, Novartis, and Pfizer, but declares no non-financial competing interests. A. Prat declares stock and Other Ownership Interests from Reveal Genomics; honoraria from Pfizer, Novartis, Roche, MSD Oncology, Lilly, Daiichi Sankyo, Amgen, and Guardant Health; consulting/advisory role from Amgen, Roche, Novartis, Pfizer, Bristol-Myers Squibb, Boehringer, PUMA, Oncolytics Biotech., Daiichi Sankyo, Abbvie, AstraZeneca, and NanoString Technologies; research funding from Roche, Novartis, Incyte, and Puma Biotechnology; travel /accommodation expenses from Daiichi Sankyo. Other relationship with companies as, Oncolytics, and Peptomyc S.L.; stock at Reveal Genomics; and patents/royalties from PCT/EP2016/080056, WO/2018/096191, HER2DX filing, and US 63/023785, but declares no non-financial competing interests. A. Guerrero-Zotano declares institutional grant from Pfizer; advisory role honoraria from Novartis, Palex, Pfizer, AstraZeneca and Pierre Fabre; travel grants from Roche, Pfizer, and Novartis, but declares no non-financial competing interests. M. Martin declares having received research grants from Roche, PUMA and Novartis, consulting/advisory fees from AstraZeneca, Amgen, Taiho Oncology, Roche/Genentech, Novartis, PharmaMar, Eli Lilly, PUMA, Taiho Oncology, Daiichi Sankyo and Pfizer and speakers' honoraria from AstraZeneca, Lilly, Amgen, Roche/Genentech, Novartis, and Pfizer, but declares no non-financial competing interests. F. Rojo declares research grants from Roche, consulting/advisory fees from BMS, MSD, AstraZeneca, Janssen, Roche/Genentech, Novartis, Eli Lilly, and Daiichi Sankyo, but declares no non-financial competing interests. J.A. Perez-Fidalgo declares research grants from Pharmamar, Novartis and GSK consulting/advisory fees from AstraZeneca, Amgen, Abilify Pharma, GSK Tesaro, Pharmamar and Clovis and speakers' honoraria from AstraZeneca, GSK Tesaro, Pharmamar, Roche, Clovis, and Pfizer. Co-inventorship of a European patent of response prediction in triple negative breast cancer, but declares no non-financial competing interests. J. Gavila declares having received advisory role honoraria from Novartis Pfizer, AstraZeneca; travel grants from Roche, Pfizer, and Novartis, but declares no non-financial competing interests. All other authors declare no financial or non-financial competing interests.

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