Phage-derived peptides as new epitopes of breast cancer

DOI: 10.53660/inter138-s224

Abstract: Epidemiological data on death rates and incidence of Breast Cancer (BC) are still alarming, highlighting the need for new strategies of diagnosis and therapy. Our group has previously described a new antibody in Fab format, FabC4, for diagnostics, staging, and prognosis of BC. Objectives: To map the FabC4 epitopes and evaluate those for their diagnostic potential. Methods: A Phage Display-based assay against FabC4 was performed to identify and characterize peptides as new epitopes of BC. The selected peptides were also evaluated for their reactivity to patient sera through ELISA assay. We finally conducted molecular docking to assess the best conformation of FabC4-epitopes. Results: Four peptides-displaying phages differentiated sera samples from 50 patients with BC, benign disease, and from healthy women. The corresponding peptides were chemically synthesized (pA5, pA7, pC4 and pD6) and bound to FabC4. The peptide pD6 differentiated the neoplastic samples from benign and healthy sera. Molecular docking analyzes confirmed the interaction between FabC4 and the selected peptides. Conclusion: We have successfully mapped the
FabC4 epitopes with diagnostic potential, opening new avenues for the understanding and treatment of BC.

**Keywords:** Breast Cancer; Phage Display; Peptides; Diagnosis; Epitopes; Docking.

1. **Introduction**

Breast Cancer (BC) is at an epidemic scale and represents a public health issue with incidence rates increasing in most countries worldwide [1,2]. Four major BC molecular subtypes are defined by the expression of hormonal receptors (Luminal A and B), superexpression of the Human Epidermal growth factor Receptor-type 2 (HER2-enriched) and in the absence of all (triple-negative). They display different therapy responses, distinct phenotypes and intricate transcriptional control [3]. Numerous efforts have focused on the search for new and promising therapeutic approaches, as well as to elucidate the promotion, invasion and progression of BC [4].

Phage Display-based research represents an expanding area. During the centenary of its existence, this method has presented therapeutic perspectives, providing molecules with wide clinical applicability [5]. This strategy has become a key player in the discovery of new drugs by enabling the identification of peptides and engineered antibodies with significant potency, specificity and stability [6–8]. In fact, the Phage Display allows us to learn more about diseases at the molecular level, and due to the pioneering work of great relevance to the biological and pharmaceutical scenario, Smith and Winter ended up sharing the Nobel Prize in Chemistry in 2018 [9].

Our research group had previously selected, by Phage Display, a new antibody in Fab format, FabC4, with clinical relevance in BC diagnosis and triple-negative BC (TNBC) prognosis [10]. FabC4 correlated with the absence of progesterone receptor, higher histological grade and non-luminal phenotype of breast tumors. In addition, FabC4 identified a subgroup of TNBC with better prognosis. However, further characterization of its target is essential for future BC management attempts. The purpose of the present study was to select, through Phage Display, the FabC4 epitopes. Peptides that are the target of FabC4 could be differentially recognized by the serum samples of patients with BC demonstrating their diagnostic value.
2. Materials and Methods

2.1 Sample collection

This project was carried out in conjunction with the Obstetric Service of the University Hospital of UFU. The study protocol was approved under No. 176/2008 by the Local Research Ethics Committee in accordance with the Declaration of Helsinki of 1975, revised in 2008, and a written informed consent was obtained from all participants.

For protein extraction, liquid nitrogen was added firstly to tissues which were grinded separately, then 1 ml extraction buffer was added (20 mM Tris-HCl pH 7.2, 10 mM EDTA, 2 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM Benzamidine), followed by centrifugation for 20 minutes at 20,000 × g at room temperature (RT). The supernatant was recovered and total proteins were estimated by the Bradford method [11]. To preserve the protein integrity, 100 mM Benzamidine and 150 mM Phenylmethylsulfonyl fluoride were added. Tissues from patients with BC, benign breast disease (BBD) and healthy women (CO), five from each group, were used for the biopanning process. For ELISA, serum samples were collected from another 150 patients, 50 for each group (Table 1)

Table 1. Clinicopathological data of 150 participants.

<table>
<thead>
<tr>
<th>Serum samples Group</th>
<th>N</th>
<th>Median Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>50</td>
<td>46.2 (range 30-80)</td>
</tr>
<tr>
<td>BBD</td>
<td>50</td>
<td>46.8 (range 18-58)</td>
</tr>
<tr>
<td>CO</td>
<td>50</td>
<td>47.6 (range 20-73)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

Clinicopathological parameters for BC patients

<table>
<thead>
<tr>
<th>Stage</th>
<th>N=50</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>19</td>
<td>37.5</td>
</tr>
<tr>
<td>T2</td>
<td>22</td>
<td>45</td>
</tr>
<tr>
<td>T3</td>
<td>4</td>
<td>7.5</td>
</tr>
<tr>
<td>T4</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Histological grade
GI 5 10
GII 28 55
GIII 17 35

**PgR status**
- Positive 29 57.5
- Negative 15 30
- Not defined 6 12.5

**ER status**
- Positive 36 72.5
- Negative 8 15
- Not defined 6 12.5

**HER2 amplification**
- Positive 11 22.5
- Negative 31 62.5
- Not defined 8 15

N: number of patients; PgR: progesterone receptor; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; BC: breast cancer; BBD: benign breast disease; CO: healthy women.

2.2 **Purification of FabC4 and FabC4 epitope mapping**

Expression and purification of FabC4 was performed as described previously [10]. Dot-blotting analysis was conducted to confirm Fab purification [10] and the protein content was estimated using the BCA method [12]. The Ph.D.-12 Phage Display Peptide Library Kit (New England Biolabs, Ipswich, USA), a random 12-mer peptides combinatorial library was used for ligands’ selection. An irrelevant Fab (FabIR) was used as negative control and in subtractive selection (Fig. S1).

For the biopanning, 10 μg of FabC4 and FabIR were coated in a 96-well plate (Nunc, Denmark) for 24 h at 4°C. Blocking was performed for 1 h at 37°C with PBS-BSA 3% (phosphate buffered saline with 3% bovine serum albumin), followed by two washes with TBST 0.1% (Tris-buffered saline containing 0.1% Tween 20). In the subtractive screening strategy, FabIR was incubated with 1.5x10^{11} phage particles from the PhD-12 library in 200 µL of TBST solution at RT for 30 min. The supernatant-recovered phages were submitted to incubation against FabC4 for 1 h at RT (positive selection).
Afterwards, the well containing the FabC4 was washed 10 times with TBS-T 0.1% and the unbound particles were discarded. Then the phages that recognized FabC4 were eluted using a two-step elution in order to select peptides related and specific to BC. Initially, the FabC4-Phage complex was incubated with proteins extracted from CO and benign tissues (30 min at RT), competitively eluting peptides that were similar to non-tumor proteins. This phage solution was discarded. Viruses that remained bound to FabC4 were subjected to a second competitive elution with proteins extracted from BC tissues (30 min at RT) thereby turning off peptides similar to tumor proteins. These were amplified in early-log *Escherichia coli* ER2738 (New England Biolabs, Beverly, MA, USA) and purified with PEG-NaCl (20% Polyethylene Glycol 8000 and 2.5 M NaCl), as already described [13], to the next two additional rounds of biopanning. Clones obtained on the third bioselection were recovered and titrated on LB/IPTG/Xgal plates [14] for further analyses.

2.3 Phage-ELISA screening

To identify individual clones, the selected population was subjected to phage-ELISA experiments. The specificity of these peptides was measured by affinity capture of the phage-clones onto FabC4 coated plates. FabIR was used as experimental control. Twenty-six-well Maxisorp microtiter plates (Nunc, Denmark) were coated with 1 µg/well of the FabC4 and FabIR in carbonate bicarbonate buffer (50 mM pH 9.6) overnight at 4°C. The wells were washed with PBST 0.1% (phosphate-buffered saline plus 0.1% Tween 20) followed by blocking for 1 h at 37°C with 3% BSA in PBS (PBS-BSA 3%).

Subsequently, the plates were washed twice with PBST 0.1% and incubated with culture supernatant containing amplified phage particles (~10^{10} pfu/mL) for 1 h at 37°C. The wells were washed four times with PBST 0.1% followed by incubation with HRP-conjugated anti-M13 (murine host, catalog number: 27-9421-01, GE Healthcare Lifescience) diluted (1:5000) in PBS-BSA 3% for 1 h at 37°C. After four washes with PBST 0.1%, the assay was revealed with O-phenylenediamine dihydrochloride (OPD) SigmaFastTM (Sigma-Aldrich) and read at 492 nm. Wild-type M13, which is identical to the phage clones present in the library, but that did not express foreign exogenous peptides, was used as a negative control and to normalize the data. For the subsequent assays, those phages whose reactivity to the FabIR was less than 0.1 and to the FabC4 was greater than 0.2, were selected.
2.4 Phage-ELISA with patients’ sera

Clones that met the absorbance criteria described above were used in a new ELISA-format with patient’s sera to determine the reactivity, binding specificity and diagnostic potential of the selected clones against circulating IgG. Briefly, 96-well Maxisorp microtiter plates (Nunc, Denmark) were coated with $10^{10}$ plaque-forming unit (PFU) of each phage clones in carbonate-bicarbonate buffer (50mM pH 9.6, overnight, 4°C). The plates were washed once with PBST 0.05% (0.05% Tween 20) and blocked with skimmed milk 5% in PBST 0.05% (PBSTM 5%) at 37°C for 1 h. Next, 50 µL/well of serum samples (1:400 in PBSTM 5%) were added and incubated (1 h, 37°C). After incubation, plates were washed five times with PBST 0.1%, and goat-produced anti-human IgG-peroxidase, Fc specific (catalog number: SAB3701270-500UG, Sigma, St. Louis, USA) was added at 1:5000 in PBSTM 5% and incubated for 1 h at 37°C. After washing with PBST 0.1%, the OPD SigmaFastTM (Sigma-Aldrich) substrate was added and the reaction interrupted with H$_2$SO$_4$ (2N).

Optical densities (OD) were determined at 492 nm in an ELISA reader (TP-Reader ThermoPlate). We used a pool of samples of BC, BBD and CO sera from 30 patients, tested in triplicates, to select the promised clones. Each serum sample was tested against wild-type M13 phage as a negative control. Four clones were tested individually in triplicates against a new group of 150 patients, and the final OD was adjusted by the ratio of OD readings for selected phage clones to the OD of the wild-type M13 phage. The optimum point of reaction (cut-off) for each phage clone was determined using the receiver operating characteristic (ROC curve).

2.5 Sequencing and bioinformatics

Single-stranded DNA was extracted from the four phage clones A5, A7, C4 and D6 using iodide buffer method as previously described [13]. The sequencing was performed on ABI3500 automated sequencer (Applied Biosystem, California, USA), following the manufacturer’s recommendations and using the $-96$ M13 primer (5’-CCCTCATTAGTTAGCGCGTAACG-3’).
The exogenous DNA sequence of the phages was translated using the ExPASy translate tool (http://www.expasy.org). The similarity analysis of selected peptides was performed using BLAST search. Peptide sequences were also analyzed with SAROTUP program to identify redundant sequences.

2.6 Peptide design and reactivity to FabC4

After bioinformatics analysis, the four peptides, named pA5, pA7, pC4 and pD6, were chemically synthesized by GenScript USA Inc. coupled to BSA. To determine the peptide affinity to FabC4 and patient’s serum, specific ELISA test was carried out. High affinity microtiter plates (96-well Maxisorp -Nunc, Denmark) were coated with the peptides (2.5 μg/well) in carbonate-bicarbonate buffer, and incubated overnight at 4°C. Microplates were washed once with PBS followed by incubation with FabC4 (1 μg/well) for 1 h at 37°C. After rinsing with PBS, Anti-HA-Peroxidase, High Affinity, clone 3F10, rat monoclonal antibody (catalogue number 12013819001, Roche Applied Science), diluted 1:5000 in PBS-BSA 3%, was added and incubated at 37°C for 1 h. The reaction was terminated with coloration (OPD – SigmaFastTM), and the OD value at a wavelength of 492 nm was measured by a microplate reader (TP-Reader ThermoPlate).

The same conditions were used in ELISA assay with the four peptides against patients’ sera. The FabC4 was replaced by 100 μL/well of 150 patients sera diluted in PBS-BSA 5% containing 0.05% Tween 20 (1:100) and HRP-goat anti-human IgG (Sigma-Aldrich) was added in a dilution of 1:5000 in PBS-BSA 5% containing 0.05% Tween 20. All samples were tested in triplicates.

2.7 Data analysis

Data were analyzed by using the GraphPad software package 7.0 (GraphPrism Software Inc., San Diego, USA). One-way ANOVA with post-hoc Bonferroni test was performed to compare the absorbance of pooled sera from BC, BBD and CO. The Mann-Whitney U-test was carried out in comparisons between groups for the ELISA assays with 150 patients. Receiver operating characteristic (ROC) curves and Odds Ratio values were generated to predict the diagnostic potential of phages and peptides. For all analyzes p<0.05 was considered statistically significant.
2.8 Molecular modeling and docking

In silico molecular modeling and docking analyses were performed to predict the three-dimensional structure (3D) of FabC4, and possible interactions between this recombinant antibody and the four selected peptides (pA5, pA7, pC4 and pD6). FabC4 was previously sequenced [10] and its 3D structure was constructed using RaptorX software (http://raptorx.uchicago.edu/StructurePrediction/predict/) [15]. The best model was chosen based on RaptorX scores and evaluation on SAVES v5.0 [16,17], and RAMPAGE: Ramachandram Plot Assessment [18]. PEP-FOLD 2.0- RPBS server was run to de novo model 3D structures of sequenced peptides [19,20]. The interactions (molecular docking) between peptides and FabC4 were predicted using HPEPDOCK software [21], and the best models were chosen based on the higher physical-spatial stability using the Root Mean Square Deviation of atomic positions (RMSD) scores.

3. Results

3.1 Selection of the phage clones

Our biopanning was performed to reach peptides associated with malignant condition with the perspective for diagnostic and characterization of BC subtypes. After the third round, 95 phage clones were randomly selected from individual colonies and evaluated by a phage-ELISA technique (Fig. 1). Five clones (A5, A7, C4, D6 and D12) were successfully selected and showed higher reactivity to FabC4, considering the criteria based on absorbance above 0.2 for FabC4 (Fig. 1a) and below 0.1 for FabIR (Fig. 1b). This strategy allowed the identification of phages with high affinity to FabC4. Considering the clinical significance of the FabC4, we set out to evaluate if the antigens (FabC4 epitopes) are recognized by the serum of patients’ samples.
Fig. 1. Screening of the selected phage-clones against the recombinant FabC4 antibody. In (a) the phages affinity to the FabC4 target and in (b) to the irrelevant Fab (FabIR) are presented. The white bars represent the phages that met the absorbance criteria above 0.2 for FabC4 and less than 0.1 for FabIR.

3.2 Diagnostic profile of selected phages

In order to evaluate the diagnostic performance of A5, A7, C4, D6 and D12 clones, immunoenzymatic tests were carried out in two steps (Fig. 2) using serum samples from patients with BC, BBD and CO. Initially, three pools of sera with 10 samples from each group were used for a preliminary measurement (Fig. 2a). The reactivity of the phagetopes against pool of sera was significantly different between BC and BBD in three (A5, C4 and D6) of the five clones tested. The A7 clone was more reactive to BC serum only when compared to CO group (p <0.001).
Fig. 2. Immunoreactivity of selected phages to serum samples from patients with breast cancer (BC), benign breast disease (BBD) and healthy women (CO). Initial screening against the pool of sera was performed to select phages capable of discriminating BC from the other groups (a). In a second moment, sera from 50 women from each group were evaluated individually. Clones A5 (b), A7 (c), C4 (d) and D6 (e) differentiated BC from CO. The absorbances were normalized with wild-type M13 phage without displayed peptide and the Mann-Whitney U-test was used for comparison between groups. The median values of each group are presented by the horizontal lines. ** p <0.01; *** p <0.0001.

Later, the phages A5, A7, C4 and D6 were used in ELISA assays with a larger number of patients (50 from each group). All samples were analyzed individually, and absorbance values were normalized to the wild-type phage. The results showed a successful affinity selection of four peptides with higher reactivity to serum from BC patients. No significant differences were found between BBD and CO groups.
The absorbance of A5 clone was 0.031 for breast tumor samples, 0.020 for BBD and 0.023 for CO (Fig. 2b). Considering phage A7 (Fig. 2c), the absorbance was 3.10 and 4.25 times higher in tumor samples, when compared to BBD and CO, respectively. The C4 clone (Fig. 2d) presented the highest absorbance for malignant lesions, and phage D6 (Fig. 2e) demonstrated the absorbance profile of 0.032, 0.017 and 0.013 for BC, BBD and CO, respectively. The ROC curve was then constructed to predict the diagnostic power of each phage.

The area under the curve (AUC), an effective measure of accuracy [22] was calculated as well as the sensitivity, specificity and likelihood ratio shown in Table 2. Supporting the ELISA assay, the four clones exhibited similar behavior with AUC above 0.70, except for the C4 phage when BC was compared to CO group (AUC=0.683).

Table 2. Phage displayed selected clones A5, A7, C4 and D6 and ROC curve values considering BC x BBD and BC x CO groups.

<table>
<thead>
<tr>
<th>Phage A5</th>
<th>Phage A7</th>
<th>Phage C4</th>
<th>Phage D6</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCxBBD D</td>
<td>BCxC O</td>
<td>BCxBBD D</td>
<td>BCxC O</td>
</tr>
<tr>
<td>AUC</td>
<td>p-value</td>
<td>Cut-off</td>
<td>Sensibility</td>
</tr>
<tr>
<td>0.702</td>
<td>0.00052</td>
<td>&lt;0.043</td>
<td>82%</td>
</tr>
<tr>
<td>&lt;0.000</td>
<td>&gt;0.0001</td>
<td>&lt;0.028</td>
<td>92%</td>
</tr>
<tr>
<td>0.767</td>
<td>0.805</td>
<td>&lt;0.078</td>
<td>88%</td>
</tr>
<tr>
<td>&lt;0.000</td>
<td>&lt;0.000</td>
<td>&lt;0.07</td>
<td>92%</td>
</tr>
<tr>
<td>0.706</td>
<td>0.683</td>
<td>&lt;0.07</td>
<td>94%</td>
</tr>
<tr>
<td>0.0004</td>
<td>0.0016</td>
<td>86%</td>
<td>80%</td>
</tr>
<tr>
<td>0.000030</td>
<td>&lt;0.000</td>
<td>&lt;0.0295</td>
<td>86%</td>
</tr>
<tr>
<td>0.71</td>
<td>0.74</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

AUC: area under the curve; BC: breast cancer; BBD: benign breast disease; CO: healthy women.

The A7 phage presented prominent values: AUC = 0.767 (p = 0.01), sensitivity of 88% and specificity of 50%, comparing BC with BBD; and AUC = 0.805 (p = 0.01), sensitivity of 92% and specificity of 50%, comparing BC and CO. The A5 clone showed the lowest specificity to differentiate malignant patients from those diagnosed with benign disease. Odds ratio (OR) analysis supports the predictive value of our phages A5 (OR= 4.32 (95% CI
1.8 to 9.8), \( p = 0.0004 \), A7 (OR= 7.78 (95% CI 3.36 to 17.23), \( p = 0.0001 \), C4 (OR= 6.52 (95% CI 2.78 to 14.95), \( p = 0.0001 \) and D6 (OR= 4.66 (95% CI 2.22 to 9.59), \( p = 0.0001 \).

3.3 Synthetic peptides specificity

Circular ssDNA from the four validated clones were extracted, sequenced and translated by the ExPASy program. Different sequences were obtained: A5-LTPLTSPGTLLR, A7-RYLPTFDMVSRT, C4-VMPAAKYSRLVR and D6-VTPCSSFSSFLP, indicating that those motifs were positively selected during the biopanning process. The analyses in the SAROTUP database confirmed the non-redundancy of these sequences.

The four peptides were then tested for their diagnostic value against 150 patients’ serum (50 of each study group). Only the pD6 peptide showed differential reactivity between groups (Fig.3a). The ROC curve was significant for BC x BBD (Fig. 3b) (AUC = 0.68) and for BC x CO (AUC = 0.78) (Fig. 3c) with a sensitivity of 88.1% and 42.2% and a specificity of 94% and 44.4%, respectively (Cut-off = 0.059).

![Fig. 3. Reactivity of pD6 peptide to sera from patients with breast cancer (BC), benign breast disease (BBD) and healthy women (CO). The peptide discriminated the three study groups (a). ROC curve was constructed to predict the diagnostic power of pD6 in differentiating BC from BBD (b) and BC from CO (c). * p <0.05; ** p <0.01; *** p <0.0001.](image)

Since we aimed to map the FabC4 epitopes, the peptides were then chemically synthesized (GenScript) and evaluated for their ability to bind to this antibody fragment by ELISA (Fig. S2). All reacted to FabC4. The pC4 peptide showed the highest absorbance when compared to the others (p <0.05 for all comparisons), followed by pD6 (also p <0.05...
for all comparisons). By performing this immunoassay, we confirmed the selection of FabC4 epitopes through Phage Display technology.

3.4 Molecular docking

Molecular docking was performed in order to assess the best conformation of FabC4-epitopes (protein-ligand) complexes. The peptides pA5, pA7, pC4 and pD6 were modeled individually and in combination (Fig. 4).

Peptides were modeled by PEPFOLD 2.0 software and FabC4 was modeled by RAPTORX. PEPFOLD 2.0 to provide the 100 most likely models for each of the four peptides and their combinations. The best individual result was submitted to HPEPDOCK online tool as input in .pdb format with the three-dimensional (3D) structure of FabC4. This software provided the docking of FabC4-peptide complexes generating 100 different models, which were evaluated by the Fast Interaction REfinement in molecular DOCKing software (FIREDOCK). All dockings simulations are shown in Fig. 4a. Root Mean Square Deviation of atomic positions (RMSD) was calculated and the best scores for physical-spatial stability are presented in Fig. 4b. Docking of FabC4 and pD6 is demonstrated in Fig. 4c, showing the FabC4’s interaction residues Arg-91, Trp-96, Ser-256 and Arg-287.
Fig. 4. Docking of FabC4-peptide complexes. The selected peptides pA5, pA7, pC4 and pD6 were analyzed individually and in combination. (a) The most likely dockings of individual and combined peptides with FabC4 are presented. (b) Docking score for FabC4-peptide complexes, refined by FIREDOCK. Root Mean Square Deviation (RMSD). (c) FabC4-pD6 docking and interactions residues.

4. Discussion

The complexity of BC relies on the accumulation of numerous molecular changes that lead to the progression towards a metastatic malignant phenotype [23,24]. More studies are needed in order to better elucidate the scenario in BC. FabC4 was previously bioengineered and has been already described as a tool for BC diagnosis and prediction of the clinical evolution of TNBC [10]. The targets were sequenced, and the depiction of the epitopes will certainly allow the discovery of new antigens associated with this tumor. In the present study, Phage Display platform was applied to select peptides binding to FabC4. Both
the recombinant peptide-displaying phages and the synthetic forms of these peptides presented diagnostic potential value since they are recognized by FabC4 and by BC patients’ sera.

Due to its versatility, the Phage Display technology remains widely used in the selection of specific ligands and is especially useful for epitope mapping [25–29]. In fact, in the present study, five specific peptides were selected as FabC4 ligands, either when presented by recombinant phages or in their chemically synthesized format. In Phage-ELISA assays, four of these phages reacted differentially to serum from patients with BC, BBD and CO. Since this is a simple, specific, reproducible and low-cost method [30], our results emphasize the importance of epitope mapping for future diagnostic purposes. Indeed, previous studies have also identified peptides expressed on bacteriophage surfaces with diagnostic potential [31,32]. ROC curve analysis validated our findings, since the AUC summarizes the diagnosis’ accuracy indicating the probability of the test assigning a higher score to BC patients [33]. Our data suggest that the identified antigens circulate in BC patients’ blood and may contribute to the diagnosis of the disease.

Although the reactivity of the clones was successfully validated by the ELISA assays, it was not possible to establish a consensus sequence among the displayed peptides. However, linear correlations between absorbances suggest that they are mimicking distinct regions of the same protein. Considering the chemically synthesized peptides, the ELISA results differed from those obtained by phage clones. Among all the selected peptides, the pD6 was the only one that discriminated the BC from the other groups although with less accuracy than the recombinant phage. The bacteriophage stabilizes the fused peptide conformation by optimizing its binding to the target while the synthetic peptide may not be able to acquire the same secondary or tertiary structure. Thus, synthetic peptides can present a slightly altered behavior. However, the sensitivity and specificity of pD6 demonstrate its potential in clinical screening as a complementary method to those already routinely used as mammography and breast ultrasonography.

It is noteworthy that FabC4 efficiently recognized all peptides, pA5, pA7 pC4 and pD6. To predict the FabC4-peptides complexes, we performed molecular docking analysis. Three-dimensional structures of FabC4 and the peptides were predicted. The ability to predict in silico epitopes by molecular docking is highly desired as it provides relevant information
about possible interactions that help experimental decision. Docking accurately simulates biological scenarios and provides valuable information on molecules binding sites characterization [34,35].

Considering that FabC4 is a predictor of TNBC survival [10], our results shed light on a new antigen related to TNBC, which needs to be extensively studied. Taken together, our study succeeds in mapping the epitopes of the FabC4 antibody fragment. Phage particles were selected against FabC4 and could differentiate serum immunoglobulins of BC patients from healthy controls. Among all the chemically synthesized peptides that are recognized by FabC4 however, only the pD6 presented diagnostic potential. Molecular docking analyzes validated the interactions between the selected peptides and FabC4. Our results are one of a kind in the elucidation of new molecular mechanisms associated with BC and possibly offer alternative tools for diagnostic and therapeutic strategies.

**Ethics approval and consent to participate**

This project was carried out in conjunction with the Obstetric Service of the University Hospital of UFU. The study protocol was approved under No. 176/2008 by the Local Research Ethics Committee in accordance with the Declaration of Helsinki of 1975, revised in 2008, and a written informed consent was obtained from all participants.

**Conflict of interest**

We confirm that there is no conflict of interest. The results presented in this manuscript are part of one patent application. Authors: Araújo, T. G.; Goulart, L. R.; Maia, Y. C. P.; Alves, D.A.; Mota, S. T. S.; Araujo, G. R.; Vaz, E. R.; Ribeiro, M. A.; Zoia, M. A. P.; Vecci, L. “Peptídeos recombinantes ligantes ao anticorpo tumoral específico câncer de mama e uso”, 2019. Registration number: BR1020190143029. Date of deposit: 07/10/2019.

**Acknowledgements**

The authors would like to thank the financial support by Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).
References


9. Nobel The Nobel Prize in Chemistry 2018 was divided, one half awarded to Frances H. Arnold “for the directed evolution of enzymes”, the other half jointly to George P. Smith and Sir Gregory P. Winter “for the phage display of peptides and antibodies.” Available online: https://www.nobelprize.org/prizes/chemistry/2018/summary/ (accessed on Feb 12, 2019).

10. Araújo, T.G.; Paiva, C.E.; Rocha, R.M.; Maia, Y.C.P.; Sena, A.A.S.; Ueira-Vieira, C.; Carneiro, A.P.; Almeida, J.F.; de Faria, P.R.; Santos, D.W.; et al. A novel highly reactive Fab antibody for breast cancer tissue diagnostics and staging also discriminates a subset of


**SUPPLEMENTARY FIGURES**

![Fig. S1. Schedule of the strategy used in the biopanning cycles of Phage Display for FabC4 epitope mapping.](image)

The elution in two stages is highlighted, aiming to identify proteins related to the tumor process. The cycle presented was repeated three times. BC: breast cancer; BBD: benign breast disease; CO: healthy women.
Fig. S2. Reactivity of synthetic peptides to FabC4. Peptides pC4 and pD6 presented higher absorbance values. * p <0.05; ** p <0.01; *** p <0.0001.