Article

Novel Noninvasive Serum Biomarkers for Prompt Diagnosis of Breast Carcinoma

Muhammad Afzal, Razia Noreen,* Nosheen Aslam, Mohammad Mahtab Alam, and Maha Abdullah Momenah
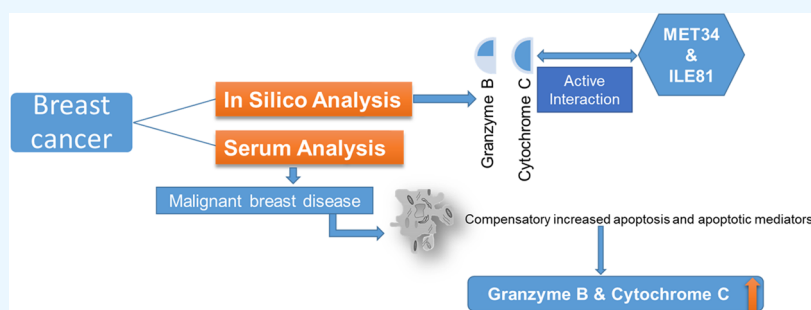
Cite This: ACS Omega 2024, 9, 1174−1182

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Immune cell infiltration is associated with improved prognosis in the microenvironment of breast cancer. The incidence of breast cancer in Pakistan is 2.5 times higher than that in neighboring countries of Asia, accounting for 34.6% of female cancers. The objectives of this study were to compare and determine apoptotic mediators and biomarkers for breast carcinoma, such as serum granzyme B, cytochrome C, and vitamin D by ELIZA and calcium spectrophotometrically. Study groups were differentiated into malignant breast disease G-I, benign proliferative breast disease G-II, and healthy control group G-III. The immune-related prognostic markers and therapeutic targets were determined through the interaction of proteins by molecular docking and AutoDock Vina software. The level of granzyme B and cyt C was higher in Group-I, -II, and -III. Likewise, the mean vitamin D level was greater in Group-I than those in other groups. Through SwissDock, the proteins granzyme B and cyt C with vitamin D, single amino acid residue MET34 (H-bond 2.75 Å), and ILE81(H-bond 2.092 Å) were revealed to actively participate in interactions. This study reveals granzyme B and cyt C as biomarkers for malignant breast disease and benign proliferative breast disease, while hypovitaminosis D and hypocalcemia are complications or comorbidities of breast cancer.

1. INTRODUCTION

Breast carcinoma is a major invasive carcinoma among females and a global health problem among women in both developing and industrialized countries.[1,2] It has engaged 22.9% of women as compared to other cancers. Among females belonging to the age group of 30−40 years, breast cancer (BC) covers 5 % of all breast carcinomas.[3] In Pakistan, the breast carcinoma incidence is 2.5 times more than that of neighboring countries.[4]

Classification of BC has been a focus for researchers after numerous recent worldwide integrative efforts outlined for assessing BC subtypes were published.[5] The main types of breast cancer are classified as in situ/noninvasive (benign) and invasive or infiltrative carcinoma (malignant).[6] Mammary carcinogenesis is believed as a multistep procedure involving a transition from standard breast to a benign proliferative breast disease to a ductal cancer in situ to penetrating ductal cancer.[7,8] Granzyme B (GrB) belongs to serine protease and cleaves nuclear membrane protein Lamin B, finally disrupting the integrity of the nuclear membrane.[9] GrB is secreted by activated natural killer cells (NK)/cytotoxic T lymphocytes (CTL). On effector−target interaction, vesicles of GrB are exocytosed and transported via perforin to the target sites in the cytoplasm.[10] GrB persuades apoptosis to form the apoptosome, consisting of a caspase cascade involving procaspase-3, -7, and -8 and cytochrome C released from mitochondria.[11] Additionally, GrB can induce the direct cleavage of a variety of death substrates and induce the production of mitochondrial ROS to induce apoptosis. GrB and cytochrome C are the mediators of apoptosis, which are compensatory increased in malignant breast disease.[12]
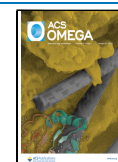
Apoptosis markers' serum concentrations, for example, soluble Fas ligand (FasL), cyt C, and GrB, are enhanced after the first cycle administration of the chemotherapeutic drugs. Measurement of such circulating apoptosis indicators can assist clinicians in assessing the treatment effectiveness in breast carcinoma.[11,13]

The antiapoptotic Bcl-2 protein confines toward the membrane of mitochondria and permeates the mitochondrial membrane to release cytochrome C (a component of the electron transport chain).[14−16] Cytochrome C triggers the execution of apoptosis, as it translocates into the cytosol.[17] Cytosolic cytochrome C binds to the apoptosis regulator Apaf-1 along with dATP and leads to the further initiation of the apoptosome formation to finalize the death cascade.[18,19]

Vitamin D active form that starts at 36 to 40 ng/mL is required for optimal neuromuscular and musculoskeletal functioning and proper immune response.[20] Vitamin D and its binding protein vit D binding protein (VDBP), a multifunctional plasma protein, form intermediary action in metabolite transport and angiogenesis that has important roles, including transport of vitamin D metabolites and fatty acids, control of bone development, actin sequestration, and inhibition of angiogenesis, as well as regulation of immune and inflammatory responses.[21] The vitamin D therapy evidenced no benefit in prostate cancer but rather worsened the scenario in some cancers.[22] In breast carcinoma, the insufficiency of vitamin D incidence differs among different populaces. The insufficiency of vitamin D was observed among 95.6% of women with breast carcinoma at Shaukat Khanum Hospital, Lahore.[23]

The vit D-derived hormone $1,25(OH)_2D3$ activates apoptosis in epithelial carcinoma cells and adult adipocytes through the initiation of apoptotic Ca^{2+} signal—a constant protracted boost in intracellular calcium concentration (Ca^{2+}).[24] The Ca^{2+} signal functions like an apoptotic marker that directly recruits apoptotic effectors and Ca^{2+}-dependent proteases in adipocytes and carcinoma cells. The cellular Ca^{2+} and $1,25(OH)_2D3$—apoptosis linkage in obesity and carcinoma provide insight into Ca^{2+}-dependent apoptotic proteases as hopeful targets regarding the invention of novel preventive and therapeutic agents for cancers. The notion of preserving an improved level of vit D to protect carcinoma and to decrease adiposity also demands further assessment.[25] Calcium signaling is one of the important regulators of the processes significant in carcinoma, for example, proliferation, apoptosis, invasion, and migration.[26,27]

The use of molecular signatures to add value to standard clinical and pathological parameters has impacted clinical practice in many cancer types, but perhaps most notably in the breast cancer field.[28−30] So far, GrB interactions with vit D and cyt C have not been focused on simultaneously in a single study; therefore, the present study is designed to address the research question "Can granzyme B, cytochrome C, vit D, and calcium act as apoptotic mediators and blood biomarkers for benign and malignant breast carcinoma in young females visiting a cancer hospital?" Furthermore, the variables will be assessed through bioinformatic tools that are important due to the considerable complexity of the disease at the clinical and molecular levels. The objectives of the present preliminary study are to evaluate and compare the apoptotic mediators such as GrB, cyt C, vitamin D, and calcium in patients of malignant breast cancer, benign proliferative breast disease, and a control group as a biomarker for breast carcinoma. In

silico analysis involved the docking of proteins cytochrome C and GrB, protein interactions, and their physiochemical properties with vit D.

2. MATERIALS AND METHODS

2.1. Ethical Approval. The current study was carried out at the Department of Biochemistry Government College University Faisalabad, Pakistan, in collaboration with Faisalabad Medical University Faisalabad/Punjab Medical College Faisalabad and Allied Hospital Faisalabad, Pakistan. The research work was performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki 1964) and ethical principles of the WHO (2008) for experiments involving humans. The study was approved by the ethical review committee of Government College University, Faisalabad, for human and animal studies (ERC/GCUF/1967-IRB-588). Written Informed consent was obtained from all individual participants included in the study. The privacy rights of all participants were also observed.

2.2. Study Design and Sample Size. It was a cross-sectional study following the STROBE parameters in which 225 breast cancer patients were included, consisting of 75 newly diagnosed women with malignant breast disease (Group-I), 75 diagnosed women with benign proliferative breast disease (Group-II), and 75 healthy women completely free clinically as healthy/normal (Group-III). The sample size was calculated using the WHO Sample Size 2.0 formula. The subjects visiting the hospital were recruited voluntarily, and the sample was collected randomly and was completely unbiased. Clinical staging of breast cancer patients was based on the American Joint Committee on Cancer (AJCC) classification.

2.3. Sample Selection. The volunteers were recruited (15th April 2019 to 31st March 2020) and processed at the breast screening unit of Faisalabad Medical University Faisalabad/Allied Hospital Faisalabad, Pakistan, based on the following criteria.

2.3.1. Inclusion Criteria. Female subjects in the age range between 30 and 48 years and patients with malignant and benign proliferative breast tumors with confirmed histopathology were selected. The control group contained only healthy women without any disease.

2.3.2. Exclusion Criteria. The presence of immunological disorders and abnormal laboratory findings (LFT's, RFT's, etc.) were investigated and considered for the exclusion criteria, and patients suffering from diabetes, hypertension, and any other ailments were also excluded from the study.

2.4. Biochemical Evaluation. After all of the ethical and research aspects were completed, blood samples of patients and healthy subjects as control were taken under aseptic measure through venipuncture for detection of biochemical parameters. The data obtained by volunteers were further accessed for research purposes (biochemical analysis, data processing, and in silico studies) from 1st April 2020 to 15th July 2022. A monoclonal antibody is precoated onto well plates. Calibrators and samples of patients were placed in the wells. Specific substrates of granzyme B, cytochrome C, and vitamin D were used to visualize the reaction to give yellow color. The density of the yellow color is directly proportional to their concentrations present in serum. Serum GrB, cyt C, and vit D levels were based on standard sandwich ELISA, while the serum calcium level was measured by spectrophotometry.[21]

2.5. In Silico Analysis. SwissDock auto was used for docking cyt C and GrB with vit D, and the results were

visualized in UCSF chimera.[31] The general steps were performed, which included retrieval of proteins from the Protein Data Bank (PDB) and the ligand from the ZINC database,[32] Preparation of Coordinates files and removed extra protein parts, docking with SwissDock,[33] visualization of interactions compared to active sites of proteins. and analysis of results by PyMol[34] of the structure at the center of the most populated cluster in docking cytochrome C with B.

2.6. Statistical Analysis. Data were collected through a questionnaire, which was entered and statistically analyzed using SPSS 20.0. All qualitative variables were reported by using frequencies and percentages. All quantitative variables were reported by using mean $\pm$ SD. All quantitative variables were calculated and compared with controls using one-way ANOVA. The normality of the data was checked using a histogram with a normality curve. P values of ≤ 0.05 were considered statistically significant.[35]

3. RESULTS

Detailed record with reference to age is presented in Table 1, which describes that among patients of Group-I (malignant

Table 1. Frequency Distribution of Women According to Age

age	Group-I malignant breast disease		Group-II benign proliferative breast disease		Group-III normal healthy control	
	freq	%age	freq	%age	freq	%age
≤40 yrs	48	64.0	64	85.3	63	84.0
>40 yrs	27	36.0	11	14.7	12	16.0
total	75	100.0	75	100.0	75	100.0
mean ± SD	39.33 ± 7.271		35.85 ± 5.513		35.20 ± 6.020	

breast disease), 48 (64.0%) were up to 40 years old and 27 (36.0%) were more than 40 years old. The mean age of the women was 39.33 ± 7.271 years. Similarly, among women of Group-II (benign proliferative breast disease), 64 (85.3%) were up to 40 years old and 11 (14.7%) were more than 40

years old (Figure 1). The mean age of the women was 35.85 ± 5.513 years. In Group-III (normal healthy control), 63 cases (84.0%) were up to 40 years old and 12 (16.0%) were more than 40 years old, with a mean age of 35.20 ± 6.020 years.

Granzyme B levels are demonstrated in Table 2. Group-I possesses 18 cases (24.0%) with GrB levels <110 in 2 cases

Table 2. Frequency Distribution of Women According to the GrB Level[a]

GrB level	Group-I malignant breast disease		Group-II benign proliferative breast disease		Group-III normal healthy control	
	freq	%age	freq	%age	freq	%age
<110	18	24.0	15	20.0	48	64.0
110−500	2	2.7	7	9.3	3	4.0
>500	55	73.3	53	70.7	24	32.0
total	75	100.0	75	100.0	75	100.0
mean ± SD	605.173 ± 44.839		542.99 ± 287.932		346.64 ± 379.625	

[a]GrB < 110 pg/mL = normal concentration, 110−500 pg/mL = high concentration, >500 = very high concentration.

(2.7%), while 55 (73.3%) women had >500, and the mean GrB level was 605.17 ± 344.839. Among women of Group-II, 15 (20.0%) had GrB levels <110 in 7 cases (9.3%), while 53 (70.7%) women had >500. The mean GrB level was 542.99 ± 287.932. Among 75 women of Group-III, 39 (52.0%) had GrB levels <110 and 48(64.0%), while 24 (32.0%) women had >500.

The mean GrB level was 346.64 ± 379.625. Table 3 demonstrates that among 75 women of Group-I, 44 (58.7%) had cytochrome C levels < 1000, 14 (18.6%) had between 1000 and 5000, and 17 (22.7%) women had >5000. The mean cytochrome C level in Group-I was 2231.16 ± 2387.808. In Group-II, 31 cases (41.3%) had cytochrome C levels <1000, 24 (32.0%) had between 1000 and 5000, and 20 (26.7%) women had >5000. The mean cytochrome C level was 2954.03 ± 2283.755. Group-III showed a majority of 72 (96.0%) cases with cytochrome C levels <1000 and only 3 (4.0%) had
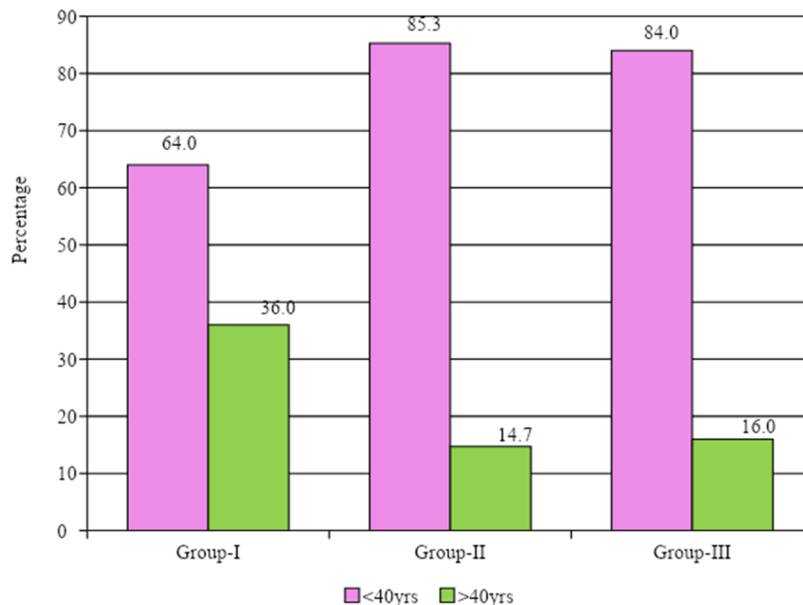

Figure 1. Frequency distribution of three groups of breast cancer according to age.

Table 3. Frequency Distribution of the Three Groups of Breast Cancer According to the Cytochrome C Level[a]

cytochrome C level	Group-I malignant breast disease		Group-II benign proliferative breast disease		Group-III normal healthy control	
	freq	%age	freq	%age	freq	%age
<1000	44	58.7	31	41.3	72	96.0
1000−5000	14	18.6	24	32.0	3	4.0
>5000	17	22.7	20	26.7	0	0.0
total	75	100.0	75	100.0	75	100.0
mean ± SD	2231.16 ± 2387.808		2954.03 ± 2283.755		366.07 ± 180.880	

[a]Cyt C < 1000 ng/mL = normal concentration, 1000−5000 ng/mL = high concentration, > 5000 ng/mL = very high concentration

between 1000 and 5000. The mean cytochrome C level in Group-III was 366.07 ± 180.880.

In Table 4, among the women of Group-I, 6 (8.0%) had vitamin D levels <10 ng/mL, 50 cases (66.7%) had 10−30 ng/

Table 4. Frequency Distribution of the Three Groups of Breast Cancer According to the Vitamin D Level[a]

vitamin D level (ng/mL)	Group-I malignant breast disease		Group-II benign proliferative breast disease		Group-III normal healthy control	
	freq	%age	freq	%age	freq	%age
<10	6	8.0	9	12.0	6	8.0
10−30	50	66.7	57	76.0	51	68.0
>30	19	25.3	9	12.0	18	24.0
total	75	100.0	75	100.0	75	100.0
mean ± SD	23.97 ± 14.013		21.11 ± 12.089		23.48 ± 11.073	

[a]Vit D < 10 (ng/mL) = deficient, (10−30 ng/mL) = insufficient, >30 (ng/mL) = sufficient.

mL, and 19 (25.3%) had >30 ng/mL. The mean vitamin D level was 23.97 ± 14.013 ng/mL. Vitamin D levels among the women of Group-II were <10 ng/mL in 9 cases (12.0%), 10−30 ng/mL in 57 cases (76.0%), and >30 ng/mL in 9 cases (12.0%). The mean vit D level was 21.11 ± 12.089 ng/mL. Likewise, Group-III had 6 cases (8.0%) with vit D levels <10 ng/mL, 51 cases (68.0%) had 10−30 ng/mL, and 18 (24.0%) women had >30 ng/mL. The mean vitamin D level was 23.48 ± 11.073 ng/mL.

The calcium study in Table 5 exhibits that among the women of Group-I, 22 (29.3%) had calcium levels <8.5 mg/dL, 17 (22.7%) had in the range of 8.5−10.5 mg/dL, and 36 (48.0%) women had >10.5 mg/dL. The mean calcium level was 10.641 ± 2.874 mg/dL. Among the women of Group-II, 21 (28.0%) had calcium levels <8.5 mg/dL, 20 (26.7%) had in the range of 8.5−10.5 mg/dL, and 34 (45.3%) women had >10.5 mg/dL. The mean calcium level was 10.492 ± 2.744 mg/dL. Among 75 women of Group-III, 9 (12.0%) had calcium levels <8.5 mg/dL, 18 (24.0%) had in the range of 8.5−10.5 mg/dL, and 48 (64.0%) women had >10.5 mg/dL. The mean calcium level was 11.872 ± 2.673 mg/dL.

The possible role of vitamin D was evaluated through in silico molecular docking with GrB and cytochrome C. This method is one of the most common assessments to determine the possible type of interactions responsible for the binding of ligands (vitamin D in our case) with proteins (Tables 6 and 7),

Table 5. Frequency Distribution of the Three Groups of Breast Cancer According to the Calcium Level[a]

calcium level (mg/dL)	Group-I malignant breast disease		Group-II benign proliferative breast disease		Group-III normal healthy control	
	freq	%age	freq	%age	freq	%age
<8.5	22	29.3	21	28.0	9	12.0
8.5−10.5	17	22.7	20	26.7	18	24.0
>10.5	36	48.0	34	45.3	48	64.0
total	75	100.0	75	100.0	75	100.0
mean ± SD	10.641 ± 2.874		10.492 ± 2.744		11.872 ± 2.673	

[a]Ca < 8.5 (mg/dL) = insufficient/hypocalcemia, Ca 8.5−11.5 (mg/dL) = normal, 11.5 (mg/dL) > hypercalcemia.

the affinity of their binding, and the distance between bonds and different orientations of the docked ligand at different sites of the target (Figure 2), specifically the active site (Figure 3). Cytochrome C is a major protein in energy chain pathways. Among all of the results generated with SwissDock, those were chosen that had at least one hydrogen bond of the ligand (vitamin D) with the receptor protein (Tables 6 and 7).

3.1. Protein Interactions by ClusPro. The protein interactions were studied via the online tool ClusPro, and the best-fitted model is shown in Figure 4. Visualization of the structure was performed by PyMol at the center of the most populated cluster in docking. For comparison, the X-ray structure of GrB in PyMol, aligned with the structure of the receptor (cytochrome C), is shown in Figures 5 and 6.

Interactive docking produced many models, but here we represented the top 10 models that showed energy. Model 0 is the best-fitted model (the lowest energy means the best fit) with a weighted score of −5527.7 (Table 8).

4. DISCUSSION

GrB was widely studied in terms of intracellular concentrations, especially in the context of apoptosis in cancer. The role of extracellular GrB has been reported previously in systemic lupus erythematosus, rheumatoid arthritis, and inflammatory disorders. GrB is particularly involved in the apoptosis of tumor cells. The released GrB is endocytosed in a receptor-mediated manner into the cytosol, where it mediates the recruitment of procaspases, mitochondria, and cytochrome C, which collectively contribute to apoptosis and DNA disintegration. The extracellular concentration of GrB increases in breast cancer with the increased levels of CTL and NK cells, which function in immune responses.[11]

To acquire appropriate outcomes, 225 women aged 30−48 years were included in the study. The breast carcinoma tendency is identified by the age of the patients and family history.[36,37] The higher incidence of breast carcinoma is associated with an increasing age. The findings of our study showed that the majority of the women in case groups (Group-I 64.0% and Group-II 85.3%) were ≤40 years old, while the remaining women were above 40 years old. Similarly, in the control group (Group-III), the majority of the women (84.0%) were ≤40 years old, and only 16.0% of women were above 40 years old. The mean ages of women in Group-I, -II, and -III were 39.33 ± 7.271, 35.85 ± 5.513, and 35.20 ± 6.020 years (Table 1), respectively. However, the results of the study demonstrated that only 21.4% of cases and 46.2% of controls were up to 40 years old and 78.6% of cases and 53.8% of

Table 6. Binding Energy and Bonds of Vitamin D toward Different Target Sites of Granzyme B[a]

ligand	receptor	fitness (kcal/mol)	score (kcal/mol)	H-bond	length	amino acid residue	binding site
vitamin D	granzyme B	−3047.11	−7.39	1	2.354	LEU171	not active
vitamin D	granzyme B	−3045.24	−7.38	1	2.75	MET34	active (Krivák and Hoksza[47])
vitamin D	granzyme B	−3043.62	−7.06	1	2.283	TYR245	not active
vitamin D	granzyme B	−3050.72	−6.83	1	2.481	PRO120	not active

[a]Note: ligand: vitamin D; fitness value: evaluation of clusters using the RMSD matrix, which is calculated by plotting RMSD against the crystal structure; score: energy of docking; H-bond: number of hydrogen bonds; length: bond length between the protein residue and the ligand.

Table 7. Binding Energy and Bonds of Vitamin D toward Different Target Sites of Cytochrome C

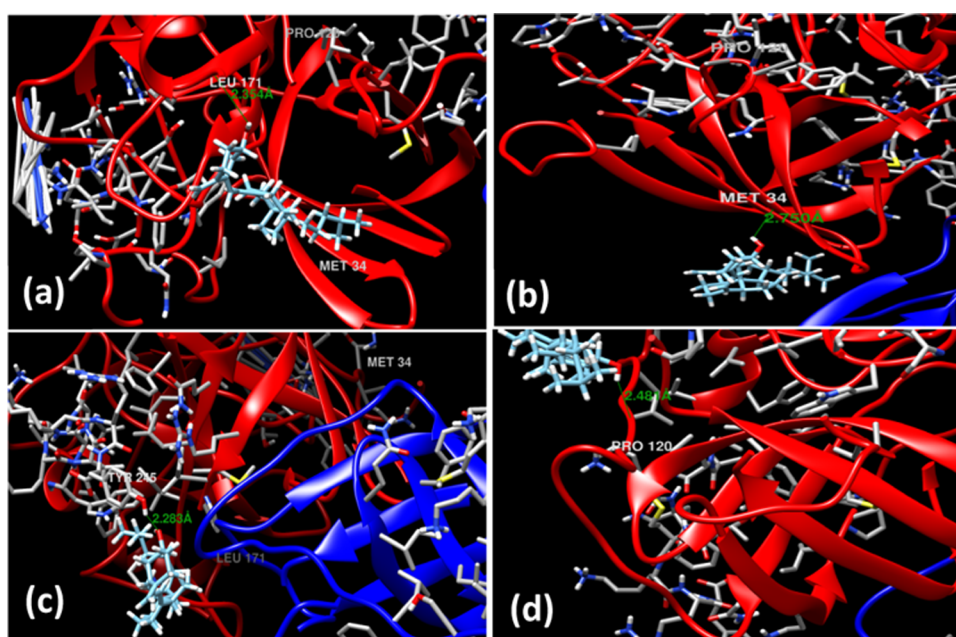| ligand	receptor	fitness (kcal/mol)	score	H-bond	length	amino acid residue	binding pocket
vitamin D	cyt C	−1431.17	−5.76	1	2.316	ASP62	not active
vitamin D	cyt C	−1424.9	−5.66	1	2.092	ILE81	active (Krivák and Hoksza[47])
vitamin D	cyt C	−1431.7	−5.56	1	2.525	GLY56	not active
vitamin D	cyt C	−1430.89	−5.45	1	2.486	HSE33	not active

Figure 2. Docking results of GrB with and vitamin D. (a) Docking of vitamin D at the receptor site LEU171 (1 H-bond with 2.354 Å bond length), (b) docking of vitamin D at the receptor site MET34 (1 H-bond with 2.750 Å bond length), (c) docking of vitamin D at the receptor site PRO120 (1 H-bond with 2.481 Å bond length), and (d) docking of vitamin D at the receptor site TYR245 (1 H-bond with 2.283 Å bond length).
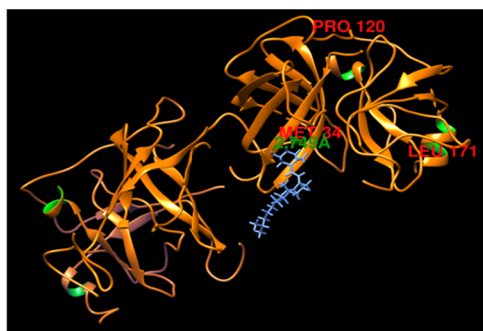

Figure 3. Docking results of GrB with vitamin D at its active site MET34 with one hydrogen bond and 2.750 Å bond length.
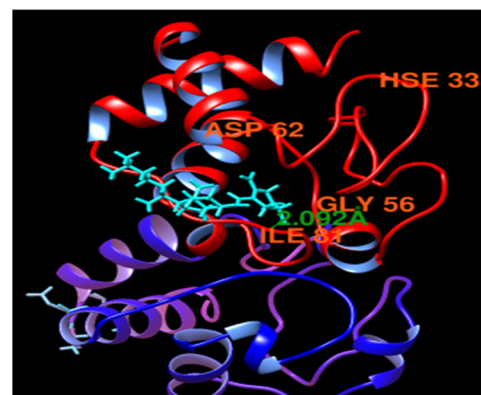

Figure 4. Docking results of cytochrome C with vitamin D at its active site ILE81 with one hydrogen bond and 2.092 Å bond length.

controls were above 40 years old, while the mean age for cases was 47.6 years and for controls 40.1 years.[35]

When the GrB levels among women of all groups were assessed, the study found that the mean GrB levels were 605.17

± 344.839, 542.99 ± 287.932, and 346.64 ± 379.625 pg/mL in Group-I, -II, and -III, respectively (Table 2). The findings of
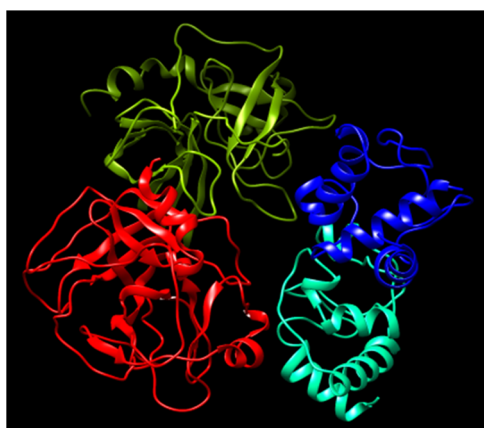
Figure 5. Visualization by PyMol of the structure at the center of the most populated cluster in docking. The docked ligand structure (lig.000.00.pdb) of cytochrome C with GrB is shown as red and green cartoons, whereas the receptor is shown as blue and cyan cartoons.
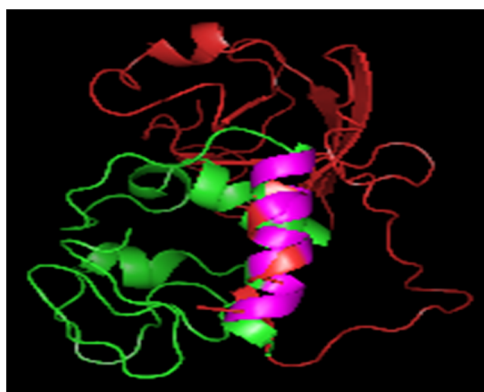

Figure 6. Visualization by PyMol of the structure at the center of the most populated cluster in docking. X-ray structure of the native pose of the aligned region of GrB and cyt C is shown as a magenta pink cartoon.

Table 8. Best-Fitted Models with Their Weighted Scores to Establish the Interaction between Cytochrome C and Granzyme B

cluster	members	representative	weighted score
0	249	center	−463.6
		lowest energy	−527.7
1	246	center	−579.4
		lowest energy	−579.4
2	72	center	−405.9
		lowest energy	−456.3
3	58	center	−441.2
		lowest energy	−481.1
4	54	center	−431.2
		lowest energy	−527.5
5	54	center	−422.6
		lowest energy	−482.3
6	40	center	−490.1
		lowest energy	−490.1
7	32	center	−416.6
		lowest energy	−438.5
8	32	center	−460.9
		lowest energy	−460.9
9	30	center	−434.1
		lowest energy	−456.0

helpful prognostic indicator among women with breast carcinoma.

Despite several experimental research studies repeatedly demonstrating vitamin D antineoplastic activities on breast carcinoma, results from randomized trials and epidemiologic research studies are not definitive.[42] The findings of our study showed that the majority of women in Group-I (74.7%), Group-II (88.0%), and Group-III (76.0%) had vitamin D deficiency/insufficiency (Table 4 and Figure 7a). The findings of a study[35] demonstrated that vitamin D deficiency was more prevalent in G1 cases (85.7%) than in controls (55.8%), and the results were found statistically significant. It also concluded that a deficiency of vitamin D is associated with breast carcinoma risk.

Numerous connections are found between breast carcinoma and calcium homeostasis alterations in the literature; however, no consensus links the mechanism to the disease prognosis. The study revealed that a few patients in Group-I (22.7%), Group-II (26.7%), and Group-III (24.0%) had normal calcium levels, while the remaining significant proportion in all three groups had hypocalcemia (Table 5 and Figure 7d). A study performed by Thaw and collaborators discussed contradictory results, showing a significantly positive association between calcium levels and tumor size, which is attributed to high bone resorption and increasing serum calcium levels,[43] while hypocalcemia is also referred to another study.[44]

In silico molecular docking studies were done using PyMol and the ZINC database, where four general steps were performed, including retrieval of required metabolites and enzymes, preparation of grid parameters and coordinate files, docking and visualization of interactions, and the analysis of results.[45] Zhao also reported a caspase-9/cytochrome C-mediated apoptosis in vitro and in vivo study of TNBC breast cancer, where expressions of caspase-3, caspase-9, Bcl-2, and Bax were detected.[46]

The vit D and GrB possible interactions at four active sites LEU171, PRO120, MET34, and TYR245 are shown in Figure

our study showed GrB higher concentration than a study undertaken[38] that ascertained that the mean GrB levels were 287.08 ± 118.43 in cases and 88.65 ± 59.68 in controls. However, a study carried out[39] on 228 females with TNBC who experienced resection with no neoadjuvant chemotherapy found that GrB expression was high among 46.5% while low among 53.5% of females.

It is anticipated that through the immune surveillance process, malignant and precancerous cells are identified through the immune system and are targeted for eradication.[40] As far as cyt C levels are concerned, the study disclosed that the mean level of cytochrome C was higher among women of Group-I (2231.16 ± 2387.808) and Group-II (2954.03 ± 2283.755) than Group-III women (366.07 ± 180.880; Table 3 and Figure 7c). The apoptotic cascade requiring high serum cyt C as revealed from the significant levels (p = 0.05) in benign patients. Another study assessed the levels of cyt C as an innovative role of tumor markers among operable malignant breast carcinoma patients.[41] Results showed that cyt C levels were found to be elevated among females with malignant tumors compared to benign breast disease and healthy controls. Another puplication[42] included 90 women with breast carcinoma and 35 with benign proliferative breast disease and found that release from cyt C can be measured as a
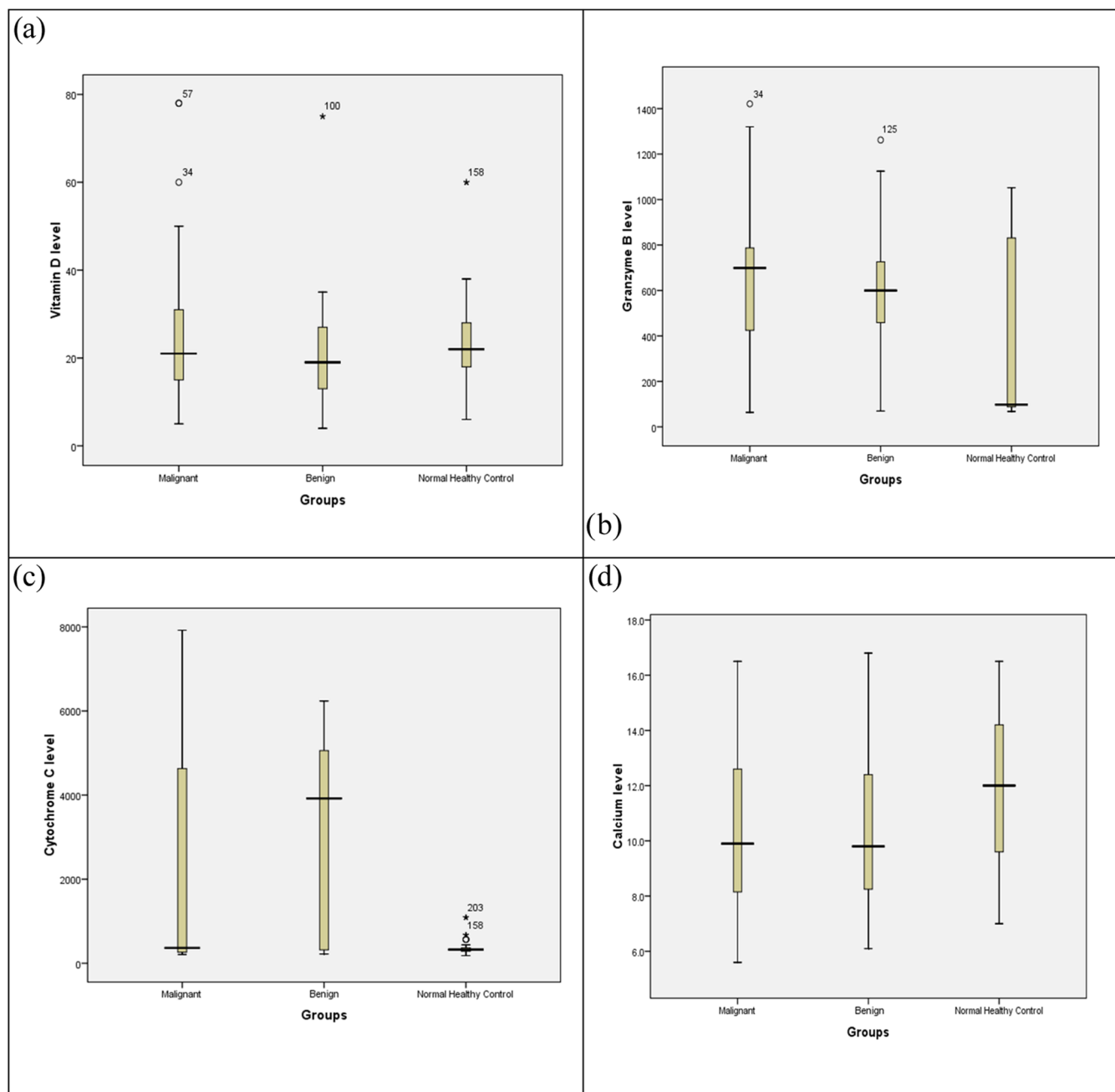
Figure 7. Breast cancer (BC) biomarkers and their comparison among malignant and benign cases versus normal $p = 0.05$. (a) Vit D level is significant with respect to normal, while benign is nonsignificant. (b) GrB levels show nonsignificant results. (c) Cytochrome C levels are highly significant in malignant patients of BC. (d) Calcium levels are significant in both malignant and benign cases of BC as compared to normal.

2. As revealed in docking results, for the single active binding site at MET34 with one hydrogen bond and a fitness energy of −3045.24 kcal/mol (Table 7 and Figure 3), a bond length of 2.75 Å is ideal for interaction, as demonstrated by Krival and co-workers.[47]

Regarding proliferation, vit D at pharmacological doses usually causes growth arrest, but the situation here is different, in which extremely low doses of vit D (picomolar range in tissue) stimulate cell proliferation and effectively enhance photodynamic therapy, as shown previously. Vit D stimulates differentiation and proliferation in MDA-MB-231-luc tumors, as detected by intratumor collection of protoporphyrin IX.[22] Similarly, the SwissDock interaction between vit D and cytochrome C resulted in one active binding site at ILE8 (1

H-bond with 2.092 Å bond length; Figure 4), with a 5.66 score and a −1424.9 kcal/mol fitness (Table 8), being the only active site attributing the proliferative activity of GrB underlying the breast cancer and conferring the cytochrome C activity in an apoptotic cascade. For comparison, the X-ray structure of GrB into PyMol, aligned with the structure of the receptor (cytochrome C), is shown in Figure 5. Abdel-Razeq explained in his study that the vitamin D level compared to those with normal, deficient patients had a larger tumor size (46.7% vs 2.9%), presented at an advanced stage of the disease.[37] A study conducted by Yao et al.[48] highlighted that vitamin D deficiency was more prevalent in cases (80.0%) than in controls (63.0%), and the results were found statistically significant. A study performed by Karkeni and associates

(2019) highlighted that vitamin D played a role in decreasing the tumor growth of breast cancer. Ismail[35] indicated that vitamin D deficiency had a negative impact on overall and disease-free survival among breast carcinoma cases. In malignant breast disease and benign proliferative breast disease, the levels of GrB are significantly higher (50−110 pg/mL) than normal (20−40 pg/mL). High serum concentrations of cyt C (>1000 ng/mL) than normal and benign cases are also observed. Further studies are required to be conducted on a large scale in the clinical setting, with a combination of the vit D approach, to detect the apoptotic mediators as biological markers for breast carcinoma.

5. CONCLUSIONS

The higher levels of GrB and cyt C can be considered biomarkers for malignant breast disease and benign proliferative breast disease. Vit D deficiency and hypocalcemia seem to be aggravating factors for the development of malignant breast disease and benign proliferative breast disease. In silico molecular docking studies of vit D and cytochrome C with GrB revealed the possible types of interactions responsible for binding and confirmation of their apoptotic and proliferative activity. As there are no commercially available serum biomarkers for breast carcinoma, these apoptotic mediators may open a new window for further researchers to establish the work on serum biomarkers as well as their molecular studies.

■ ASSOCIATED CONTENT

Data Availability Statement

All of the data used to support the findings of this study have been included in the manuscript.

■ AUTHOR INFORMATION

Corresponding Author

Razia Noreen − *Department of Biochemistry, Government College University Faisalabad, Faisalabad 38000, Pakistan;* ⊙ orcid.org/0000-0001-9269-1117; Email: drrazianoreen@gcuf.edu.pk, itsrazia@yahoo.com

Authors

Muhammad Afzal − *Department of Biochemistry, Government College University Faisalabad, Faisalabad 38000, Pakistan*

Nosheen Aslam − *Department of Biochemistry, Government College University Faisalabad, Faisalabad 38000, Pakistan*

Mohammad Mahtab Alam − *Department of Basic Medical Sciences, College of Applied Medical Science, King Khalid University, Abha 61421, Saudi Arabia*

Maha Abdullah Momenah − *Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, Riyadh 11671, Saudi Arabia*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.3c07431

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Veiga, G. L. d.; Silva, R. D. M. d.; Pereira, E. C.; Azzalis, L. A.; Alves, B. d. C. A.; Gehrke, F. d. S.; Gascón, T. M.; Fonseca, F. L. A. The role of Survivin as a biomarker and potential prognostic factor for breast cancer. *Rev. Assoc. Med. Bras.* **2019**, *65*, 893−901.

(2) Tiwari, S.; Tiwari, S. P.; Yadav, R.; Vaishnav, V. A. D. Recent Advancement of Nano Formulations in Treatment of Breast Cancer. *J. Pharm. Negat. Results* **2023**, 3650−3653.

(3) Aghaei, M. The Breast Cancer, a Global Health Crisis. *J. New Find. Health Educ. Sci. (IJHES)* **2023**, *2* (1), 107−117.

(4) Shaukat, U.; Ismail, M.; Mehmood, N. Epidemiology, major risk factors and genetic predisposition for breast cancer in the Pakistani population. *Asian Pac. J. Cancer Prev.* **2013**, *14* (10), 5625−5629.

(5) Abhisheka, B.; Biswas, S. K.; Purkayastha, B. A comprehensive review on breast cancer detection, classification and segmentation using deep learning. *Arch. Comput. Methods Eng.* **2023**, *30*, 1−30.

(6) Neagu, A.-N.; Jayathirtha, M.; Whitham, D.; Mutsengi, P.; Sullivan, I.; Petre, B. A.; Darie, C. C. Proteomics-based identification of dysregulated proteins in breast cancer. *Proteomes* **2022**, *10* (4), 35.

(7) Arif, J. M.; Kunhi, M.; Bekhit, A. A.; Subramanian, M. P.; Al-Hussein, K.; Aboul-Enein, H. Y.; Al-Khodairy, F. M. Evaluation of apoptosis-induction by newly synthesized phthalazine derivatives in breast cancer cell lines. *Asian Pac. J. Cancer Prev.* **2006**, *7* (2), 249.

(8) Lakshmi, S. G.; Kamaraj, M.; Nithya, T.; Chidambaranathan, N.; Pushpalatha, G.; Santhosh, P.; Balavaishnavi, B.; Mahajan, M. Network pharmacology integrated with molecular docking reveals the anticancer mechanism of Jasminum sambac Linn. essential oil against human breast cancer and experimental validation by in vitro and in vivo studies. *Appl. Biochem. Biotechnol.* **2023**, 1−32.

(9) Kuppili, R. R.; Bose, K. Calpains and Granzymes: Non-caspase Proteases in Cell Death. *Proteases in Apoptosis: Pathways, Protocols Translational Adv.* **2015**, 53−94.

(10) Baigrie, D.; Nookala, V. Bullous Pemphigoid. In *StatPearls [Internet]*; StatPearls Publishing, 2022.

(11) Kadam, C. Y.; Abhang, S. A. Serum levels of soluble Fas ligand, granzyme B and cytochrome c during adjuvant chemotherapy of breast cancer. *Clin. Chim. Acta* **2015**, *438*, 98−102.

(12) Yuan, L.; Cai, Y.; Zhang, L.; Liu, S.; Li, P.; Li, X. Promoting apoptosis, a promising way to treat breast cancer with natural products: A comprehensive review. *Front. Pharmacol.* **2022**, *12*, No. 801662.

(13) Alemzadeh, E.; Allahqoli, L.; Dehghan, H.; Mazidimoradi, A.; Ghasempour, A.; Salehiniya, H. Circulating tumor cells and circulating tumor DNA in breast cancer diagnosis and monitoring. *Oncol. Res.* **2023**, *31* (5), 667.

(14) Anderson, A. J.; Jackson, T. D.; Stroud, D. A.; Stojanovski, D. Mitochondria—hubs for regulating cellular biochemistry: emerging concepts and networks. *Open Biol.* **2019**, *9* (8), No. 190126.

(15) Cho, M.-Y.; Park, S.-Y.; Park, S.; Lee, Y. R.; Han, G.-D.; Kim, J.-A. Geranyl derivative of phloroacetophenone induces cancer cell-specific apoptosis through Bax-mediated mitochondrial pathway in MCF-7 human breast cancer cells. *Biol. Pharm. Bull.* **2012**, *35* (1), 98−104.

(16) Vandenabeele, P.; Bultynck, G.; Savvides, S. N. Pore-forming proteins as drivers of membrane permeabilization in cell death pathways. *Nat. Rev. Mol. Cell Biol.* 2023, 24 (5), 312−333.

(17) Li, J.-y.; Sun, X.-a.; Wang, X.; Yang, N.-h.; Xie, H.-y.; Guo, H.-j.; Lu, L.; Xie, X.; Zhou, L.; Liu, J.; et al. PGAM5 exacerbates acute renal injury by initiating mitochondria-dependent apoptosis by facilitating mitochondrial cytochrome c release. *Acta Pharmacol. Sin.* 2023, 1−12.

(18) Barczyk, K.; Kreuter, M.; Pryjma, J.; Booy, E. P.; Maddika, S.; Ghavami, S.; Berdel, W. E.; Roth, J.; Los, M. Serum cytochrome c indicates in vivo apoptosis and can serve as a prognostic marker during cancer therapy. *Int. J. Cancer* 2005, 116 (2), 167−173.

(19) Borna, H.; Hosseini Qale Noe, S. H.; Harchegani, A. B.; Talatappe, N. R.; Ghatrehsamani, M.; Ghanei, M.; Shahriary, A. A review on proteomics analysis to reveal biological pathways and predictive proteins in sulfur mustard exposed patients: roles of inflammation and oxidative stress. *Inhal. Toxicol.* 2019, 31 (1), 3−11.

(20) Goodwin, P. J. Vitamin D in Cancer Patients: Above All, Do No Harm-2009. *J. Clin. Oncol.* 2009, 27 (13), 2117−2119.

(21) Schexnayder, C.; Broussard, K.; Onuaguluchi, D.; Poché, A.; Ismail, M.; McAtee, L.; Llopis, S.; Keizerweerd, A.; McFerrin, H.; Williams, C. Metformin inhibits migration and invasion by suppressing ROS production and COX2 expression in MDA-MB-231 breast cancer cells. *Int. J. Mol. Sci.* 2018, 19 (11), 3692.

(22) Rollakanti, K. R.; Anand, S.; Maytin, E. V. Vitamin D enhances the efficacy of photodynamic therapy in a murine model of breast cancer. *Cancer Med.* 2015, 4 (5), 633−642.

(23) Buttigliero, C.; Monagheddu, C.; Petroni, P.; Saini, A.; Dogliotti, L.; Ciccone, G.; Berruti, A. Prognostic role of vitamin d status and efficacy of vitamin D supplementation in cancer patients: a systematic review. *Oncologist* 2011, 16 (9), 1215−1227.

(24) Aldeewan, A. B.; Abbas, B. A.; Jaber, N. N.; Khudor, M. H. Role of Apoptotic signaling pathways in the immune system. *Iran. J. Ichthyol.* 2023, 10, 209−218.

(25) Badar, F.; Mahmood, S.; Faraz, R.; Yousaf, A.; Quader, A. U.; Asif, H. Epidemiology of breast cancer at the Shaukat Khanum memorial cancer hospital and research center, Lahore, Pakistan. *J. Coll. Physicians Surg. Pak.* 2015, 25 (10), 738−742.

(26) Sergeev, I. N. Vitamin D-mediated apoptosis in cancer and obesity. *Horm. Mol. Biol. Clin. Investig.* 2014, 20 (2), 43−49.

(27) Prevarskaya, N.; Skryma, R.; Shuba, Y. Calcium in tumour metastasis: new roles for known actors. *Nat. Rev. Cancer* 2011, 11 (8), 609−618.

(28) Lee, J. M.; Davis, F. M.; Roberts-Thomson, S. J.; Monteith, G. R. Ion channels and transporters in cancer. 4. Remodeling of Ca2+ signaling in tumorigenesis: Role of Ca2+ transport. *Am. J. Physiol.: Cell Physiol.* 2011, 301 (5), C969−C976.

(29) Azimi, I.; Roberts-Thomson, S.; Monteith, G. Calcium influx pathways in breast cancer: opportunities for pharmacological intervention. *Br. J. Pharmacol.* 2014, 171 (4), 945−960.

(30) Abrao Nemeir, I.; Saab, J.; Hleihel, W.; Errachid, A.; Jafferzic-Renault, N.; Zine, N. The advent of salivary breast cancer biomarker detection using affinity sensors. *Sensors* 2019, 19 (10), 2373.

(31) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 2004, 25 (13), 1605−1612.

(32) Sterling, T.; Irwin, J. J. ZINC 15—ligand discovery for everyone. *J. Chem. Inf. Model.* 2015, 55 (11), 2324−2337.

(33) Grosdidier, A.; Zoete, V.; Michielin, O. SwissDock, a protein-small molecule docking web service based on EADock DSS. *Nucleic Acids Res.* 2011, 39 (suppl_2), W270−W277.

(34) DeLano, W. L. Pymol: An open-source molecular graphics tool. CCP4 Newsl. *Protein Crystallogr.* 2002, 40 (1), 82−92.

(35) Shaukat, N.; Jaleel, F.; Moosa, F. A.; Qureshi, N. A. Association between vitamin D deficiency and breast cancer. *Pakistan J. Med. Sci.* 2017, 33 (3), 645.

(36) Khazaee-Pool, M.; Montazeri, A.; Majlessi, F.; Rahimi Foroushani, A.; Nedjat, S.; Shojaeizadeh, D. Breast cancer-preventive behaviors: exploring Iranian women's experiences. *BMC Womens Health* 2014, 14, 1−9.

(37) Ismail, A.; El-Awady, R.; Mohamed, G.; Hussein, M.; Ramadan, S. S. Prognostic significance of serum vitamin D levels in Egyptian females with breast cancer. *Asian Pac. J. Cancer Prev.* 2018, 19 (2), 571.

(38) Yousef, A. A.; Alsalahy, M. M.; Almahdy, M. A.; Essawy, T. S.; Belal, K. M. Study of serum Granzyme B in heavy cigarette smokers with and without chronic obstructive pulmonary disease. *Egypt. J. Chest Dis. Tuberc.* 2014, 63 (4), 815−819.

(39) Kawaji, H.; Kubo, M.; Motoyama, Y.; Shimazaki, A.; Hayashi, S.; Kurata, K.; Yamada, M.; Kaneshiro, K.; Kai, M.; Nakamura, M. Functional analysis of tumour infiltrating lymphocytes in triple negative breast cancer focusing on granzyme B. *Ann. Oncol.* 2019, 30, v5.

(40) Cullen, S. P.; Brunet, M.; Martin, S. Granzymes in cancer and immunity. *Cell Death Differ.* 2010, 17 (4), 616−623.

(41) Osaka, A.; Hasegawa, H.; Yamada, Y.; Yanagihara, K.; Hayashi, T.; Mine, M.; Aoyama, M.; Sawada, T.; Kamihira, S. A novel role of serum cytochrome c as a tumor marker in patients with operable cancer. *J. Cancer Res. Clin. Oncol.* 2009, 135, 371−377.

(42) Mabrouk, G. M.; Zohny, S. F.; Shehata, H. H.; Ali, E. M.; Basyouni, M.; El-Shinawi, M. Prognostic significance of cytochrome C release and tissue lactate dehydrogenase in patients with breast cancer *Egypt. J. Biochem. Mol. Biol.* 2010, 282 DOI: 10.4314/ejbmb-.v28i2.60792.

(43) Thaw, S. S. H.; Sahmoun, A. E.; Schwartz, G. G. Serum calcium, tumor size, and hormone receptor status in women with untreated breast cancer. *Cancer Biol. Ther.* 2012, 13 (7), 467−471.

(44) Diniotis M D, B.; Sternberg, E.; Shakuntala, S.; Chiha, M.; Khosla, P. Hypocalcemia in malignancy-unexpected but common. *Cureus* 2015, 7 (12), No. e442.

(45) Ahmed, E. Antidepressants in Patients With Advanced Cancer: When They're Warranted and How to Choose Therapy *Oncology (08909091)* 2019, 332.

(46) Zhao, Y.; Jing, Z.; Lv, J.; Zhang, Z.; Lin, J.; Cao, X.; Zhao, Z.; Liu, P.; Mao, W. Berberine activates caspase-9/cytochrome c-mediated apoptosis to suppress triple-negative breast cancer cells in vitro and in vivo. *Biomed. Pharmacother.* 2017, 95, 18−24.

(47) Krivák, R.; Hoksza, D. P2Rank: machine learning based tool for rapid and accurate prediction of ligand binding sites from protein structure. *J. Cheminform.* 2018, 10, 1−12.

(48) Yao, S.; Sucheston, L. E.; Millen, A. E.; Johnson, C. S.; Trump, D. L.; Nesline, M. K.; Davis, W.; Hong, C.-C.; McCann, S. E.; Hwang, H.; et al. Pretreatment serum concentrations of 25-hydroxyvitamin D and breast cancer prognostic characteristics: a case-control and a case-series study. *PLoS One* 2011, 6 (2), No. e17251.