International Journal of Biological Macromolecules 235 (2023) 123670

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Putative interactions between transthyretin and endosulfan II and its relevance in breast cancer

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ARTICLE INFO

Keywords:
Pesticides
Environmental toxicants
Good health and well-being
Sustainable cities and communities
Endocrine disruptor

ABSTRACT

The unregulated use of organochlorine pesticides (OCPs) has been linked to spread of breast cancer (BC), but the underlying biomolecular interactions are unknown. Using a case-control study, we compared OCP blood levels and protein signatures among BC patients. Five pesticides were found in significantly higher concentrations in breast cancer patients than in healthy controls: p′,p′ dichloro diphenyl trichloroethane (DDT), p′p′ dichloro diphenyl dichloroethane (DDD), endosulfan II, delta-hexachlorocyclohexane (dHCH), and heptachlor epoxide A (HTEA). According to the odds ratio analysis, these OCPs, which have been banned for decades, continue to raise the risk of cancer in Indian women. Proteomic analysis of plasma from estrogen receptor-positive breast cancer patients revealed 17 dysregulated proteins, but transthyretin (TTR) was three times higher than in healthy controls, which is further validated by enzyme-linked immunosorbent assays (ELISA). Molecular docking and molecular dynamics studies revealed a competitive affinity between endosulfan II and the thyroxine-binding site of TTR, pointing towards the significance of the competition between thyroxin and endosulfan, resulting in endocrine disruption leading to breast cancer. Our study sheds light on the putative role of TTR in OCP-mediated BC, but more research is needed to decipher the underlying mechanisms that can be used to prevent the carcinogenic effects of these pesticides on women's health.

1. Introduction

From 1990 to 2016, the incidence of cancer in Indian women declined; nevertheless, the incidence of age-standardized breast cancer increased by 40.7 %. Environmental factors (toxicants, applied chemicals, pharmaceuticals) and lifestyle changes (high smoking, alcohol consumption, low physical activity, and high stress) have been proposed as the major drivers of the prevalence of BC in developing countries, whereas genetic factors explain only 5–27 % of it [1–3]. Although there is a discrepancy between the levels of OCPs in the plasma of breast cancer patients and healthy controls [4], pesticide exposure is still considered an emerging determinant of chronic reproductive disorders, including breast cancer risk, especially in developing countries [5,6].

Proteins in blood have emerged as promising biomarkers in cancer

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https://doi.org/10.1016/j.ijbiomac.2023.123670
Received 24 November 2022; Received in revised form 4 February 2023; Accepted 10 February 2023
Available online 14 February 2023

treatment, with significant diagnostic, prognostic, therapeutic, and predictive values [7,8]. Some of the widely used serum-based markers for BC include carcinoembryonic antigen (CEA), antigen Ki 67, the soluble form of MUC-1 protein (CA15-3), Mammaglobin (MAM), circulating cytokeratins such as tissue polypeptide specific antigen (TPS), tissue polypeptide antigen (TPA), cytokeratin 19 fragment (CIFRA-21-1), and the proteolytically cleaved ectodomain of the human epidermal growth factor receptor 2 (s-HER2). Although these biomarkers are in clinical use, applying them to Indian populations remains questionable because most biomarkers were developed during studies on the Western population. Notably, not only does environmental quality differ between Western countries and India, but so do people's genetic make-up, lifestyle, and food habits [3].

Breast cancer is the leading cause of cancer-related disability-adjusted life years (DALYs) in Indian women (16.8 %). Environmental quality is considered one of the major causal factors of BC emergence in India [9]. However, the use of OCPs was prohibited in India in 1989, while the production and use of chlorinated pesticides such as endosulfan, p,*p*-dichlorodiphenyltrichloroethane (DDT), and hexachlorocyclohexane (HCH) are still permitted in the health, agricultural, and residential sectors [10]. Increased pesticide exposure from contaminated soil, water, food, and air has been linked to cancer in the Indian population [11,12]. Pesticides like o, p′dichlorodiphenyltrichloroethane (o,p′-DDT) and HCH isomers (α-HCH, β-HCH, γ-HCH, and δ-HCH) reduce gene expression and progesterone synthesis in ovarian granulosa cells even at low concentrations (4 ppm) [13–15]. However, determining the level of banned pesticides in women's blood and identifying the biomolecules that interact with pesticides and cause endocrine disruption, ultimately leading to the development of breast cancer, remains critical [16,17].

Links between pesticides and breast cancer remain ambiguous in European and American populations; however, Indian population show an association between pesticide exposure and breast cancer risks in younger women [16]. Given the disparity in pesticide exposure between developing and developed countries, the contradiction is understandable. Epidemiological and toxicological evidence suggest pesticide exposure is linked to changes in thyroid hormone biosynthesis, function, transport, and binding [18,19] as well as the development of hormone-related cancers such as breast [20], testicular, and prostate [21]. Despite mounting evidence linking pesticide exposure to hormone-related cancers, the mechanisms underlying this link remain unknown. As a result, we conducted a hospital-based case-control study on Indian females to look into the possible link between pesticide blood levels, breast cancer, and differential plasma protein expression. During endocrine disruption, possible biomolecular targets were identified using proteomics and bioinformatics. Our findings provide critical information about the biomolecular pathways induced by OCPs that contribute to BC in Indian women.

2. Materials and methods

2.1. Statement of ethics

Institutional Ethics Committee – Human Research (IEC-HR), University College of Medical Sciences, University of Delhi, Delhi-110,095, India, granted ethical clearance to the present study (10 February 2017). All participants provided pre-informed written consent to participate in this study. All the experiments were performed following relevant guidelines and regulations.

2.2. Participants and plasma sample preparation

Breast cancer patients and healthy controls (n = 38 for each group) from the Indian population were enrolled in the study. Blood samples were collected in EDTA anticoagulant tubes by venous puncture. Clinical parameters, such as age, menopause status, node status, estrogen receptor (ER), progesterone receptor (PR), and stage or histological grade, were recorded (Table S1). An age-matched healthy woman, free of any breast or gynecological pathology, was recruited as a control for each case. Patients with a history of treatment with chemo- or radiotherapy following any surgery were excluded.

Plasma was separated by centrifuging at 2000 ×*g* and 4 °C for 15 min. Plasma was stored in aliquots at −80 °C till further use. Plasma samples of fifteen individuals were selected randomly from each case group, and plasma from five individuals was pooled in equal volumes (50 μl) and used as triplicates for the proteomic analyses. We used only estrogen receptor-positive breast cancer patients for the proteomic analysis. Protein content in each pooled sample was estimated by Bradford's dye-binding assay by comparing it with a standard curve prepared using bovine serum albumin.

2.3. Estimation of organochlorine pesticides (OCPs) residue in the blood

Pesticide residues in the blood of both cases, BC patients, and healthy controls were estimated using gas chromatography [22]. OCPs were extracted in organic solvents (hexane and acetone; 2:1) using a standard method. The clear supernatant was collected in a fresh test tube, and the residue was reextracted with hexane (6 ml). Clean-up was done using Florisil (84 % silicon dioxide +16 % magnesium oxide) columns following the USEPA method. The column was packed with glass wool (1–2 cm) as a support matrix and a layer of activated Florisil (10 cm) followed by a layer of anhydrous sodium sulfate (1 cm). Prewetting of the column was done with 20 ml hexane (HPLC grade). Samples were allowed to pass through the column, and the sample was eluted with 15–20 ml of hexane. This process was repeated three times with the remaining residues. Finally, all the eluted samples were pooled and concentrated in a rotary evaporator. The concentrated residue was suspended in hexane (1 ml) and used for analysis.

OCPs were quantified by gas chromatography (Perkin Elmer Clarus 500) equipped with a ^{63}Ni electron capture detector. OCPs suspension (2 μl) was injected in GC at 170 °C with a 1 min hold time. The temperature was raised from 170 °C to 225 °C at 5 °C min^{-1} with 5 min hold time and finally from 225 °C to 275 °C at 6 °C min^{-1} with of 15 min hold time. Each sample was run for 40 min. OCPs were quantified by comparing with the peak area of a chromatogram of a mixed OCPs standard (Supelco, Sigma–Aldrich), and analytes were confirmed by spiking with pesticide standards. The detection limit of the detector was 0.05 pg perchloroethylene with nitrogen as a carrier gas, whereas the detection limit of the method was 4 pg mL^{-1} for each OCP. For quality control, five blood samples in triplicate were spiked with a mixed standard of OCPs at 5 and 25 ng ml^{-1}. The average recoveries of fortified samples exceeded 95 %. The case and control samples were run in the same analytical batches [23].

2.4. 2-Dimensional gel electrophoresis and analysis of gel images

Two-dimensional gel electrophoresis was performed to examine the protein differential expression [24]. Protein samples (50 μg) were mixed with rehydration buffer (7 M-urea, 2 M-thiourea, 2 % CHAPS, Sigma-Aldrich, USA) containing 40 mM dithiothreitol (DTT) and 0.2 % ampholyte (Sigma-Aldrich, USA). Isoelectric focusing (IEF) was carried out using Protean IEF cell (Bio-Rad, USA) as per the manufacturer's protocol. Proteins were separated in the first dimension on IPG strips (7 cm, pH-gradient strips, pH 4–7, Bio-Rad, USA) with IEF conditions as 200 V for 1 h followed by 500 V for 1 h and 1000 V for the next 1 h and rest of the focusing at 8000 V for 5 h for a total of 13,000 VH (Bio-Rad, USA). After completing the IEF, strips were equilibrated for 20 min each in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 4 % SDS, and 20 % glycerol) containing DTT (10 mg/ml) and iodoacetamide (40 mg/ml) (Sigma-Aldrich, USA) respectively.

Proteins were further separated in the second dimension using 12 %–4 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE). The gels were silver stained and scanned at 600 dpi for analysis. The relative intensities of protein spots were calculated by using Alpha Digidoc 1201 software, and densitometric data were subjected to cluster analyses.

2.5. Extraction of peptides and matrix-assisted laser desorption ionization-MS/MS

To extract proteins from gels, silver-stained protein spots were trypsinized using trypsin gold as per the manufacturer's protocol (Promega, USA). Gel blocks containing the desired protein spots were excised and washed with deionized water and kept in destaining solution (0.2 % K_3FeCN_6 and 0.04 % $Na_2S_2O_3$) (Sigma-Aldrich, USA) till the stain disappeared. The washing was continued with 100 mM NH_4HCO_3 and 100 % CH_3CN in a 1:1 ratio. The gel blocks were dehydrated with 40 μl of 1:1 acetonitrile (ACN) and water for 15 min, followed by washing with 40 μl of ACN. The gel pieces were treated with NH_4HCO_3 (50 μl of 100 mM) followed by ACN (50 μl) (Sigma-Aldrich, USA). The gel blocks were digested with a 10 ml digestion buffer containing 0.1 mg trypsin/ml (Promega, USA) for 45 min on ice. The 60 ml digestion buffer without trypsin (1 M $CaCl_2$, 1 M NH_4CO_3) was added again and incubated at 37 °C overnight. Supernatants were collected and dried in a vacuum centrifuge. The peptides were extracted by varying the concentration of ACN and trifluoroacetic acid (TFA) [24].

To identify the differentially expressed proteins, the trypsinized peptides were analyzed by MALDI-TOF MS/MS (Applied Biosystems, Life Technologies, USA) system. We mixed 1 μl of peptide solution with an equal volume of α-cyano-hydroxycinnamic acid (CHCA) matrix prepared in 70 % CH_3CN and 30 % 0.1 % TFA. The minimum S/N (signal/noise) filter was set at 25 μm with an exclusion list for α-cyano-4-hydroxycinnamic acid (CHCA) matrix peaks for the MS/MS precursor selection. A statistically significant probability of the protein's acceptance was based on Mowse score ($p \leq 0.05$).

2.6. ELISA-based quantification of TTR in human plasma

ELISA was used to quantify the expression of TTR in the plasma of BC patients and healthy controls. Each well of a 96-well microtiter plate was coated with anti-TTR antibody (ProSci Antibody, USA) in 1:2000 dilution with coating buffer (0.01 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6), and the plate was incubated overnight at 4 °C. After incubation, the plate was washed thrice with 100 μl PBST (phosphate-buffered saline with tween 20) and incubated with 200 μl blocking buffer (2 % BSA, Sigma-Aldrich, USA) for 1 h at RT. After incubation, the blocking buffer was removed, and the well was washed before incubating with primary antibodies for 3 h at RT. Again, the plate was washed and incubated with an anti-mouse HRP conjugated secondary antibody for 1 h and developed with ortho-phenylenediamine (1 mg/ml in 0.05 M citrate buffer and 5 μl/ml H_2O_2; Sigma-Aldrich, USA) for 30 min. The reaction was stopped by adding 2 N H_2SO_4 to each well, and OD_{492nm} was measured using an ELISA plate reader [25].

2.7. Molecular docking and dynamics of pesticides with TTR

To understand the molecular interactions of pesticides with TTR that result in its overexpression in BC patients, we performed molecular docking of TTR with identified pesticides using AutoDock 4.2.6. The crystal structure of the human transthyretin complex with thyroxine (T4) (PDB ID: 1ICT) was used as a template for docking with endosulfan II, p′,p′DDT, dHCH, HTEA, and p′p′-DDD. Thyroxine was used as a control for this study. Before docking, the solvent was removed, and the structure was energy minimized using Swiss PDB Viewer. The docking grid for the binding pocket was generated at the dimer interface of TTR based on the thyroxine structure. The residues Lys15, Leu17, Glu54, Leu110, Ser117, Thr119, and Val121 from both monomers form the docking site. Flexible docking was performed using the default parameters of the Lamarckian genetic algorithm. The free energy of ligand binding with TTR was calculated using the semi-empirical scoring function. Among the 10 docked poses, the best-docked pose was selected for all five pesticides and thyroxine on the basis of binding energy and inhibition constant (ki). Out of all these, endosulfan II showed binding energy close to thyroxine. Hence, endosulfan II and thyroxine TTR complexes were subjected to MD simulation analysis. All the interactions between the ligand and protein were analyzed using Pymol software [53].

The co-ordinates of the ligands with the best docked pose were used for generating the topology using the PRODRG server [26,27] without performing the energy minimization step. The .gro and .itp topologies files generated by the PRODRG server for both thyroxine and endosulfan II were further used for running the simulations with TTR dimer. Both TTR-endosulfan and TTR-thyroxine complexes were energy minimized and refined via molecular dynamics simulations (MDS) using GRMACS-5.0.7 with GROMOS 54a7 forcefield [28]. Each complex was solvated in a cubic box extending to 10 Å from protein-alone atoms in all directions using the SPC216 water model. The neutralization of solvated complex was performed by counter ions and energy minimization through the steepest descent algorithm with convergent criteria of <1000.0 kJ/mol/nm. The TTR-endosulfan and TTR-thyroxine complexes were further minimized and equilibrated at 300 K using NVT and NPT ensemble using the Leapfrog dynamics integrator and the Verlet scheme for 100 ps and 1 fs step-size. The Harmonic constraints using the LINCS algorithm, the Particle Mesh Ewald (PME) algorithm for long-range electrostatic interactions, the Berendsen coupling algorithm for temperature coupling, and the Parrinello-Rahman algorithm. A production MD run of 50 ns was performed. A similar protocol was used to perform MD simulations of native TTR dimers without a ligand [29,30].

2.8. Statistical analysis

Mann Whitney-*U* test was used to compare the pesticide levels in the blood of BC patients with healthy individuals (SPSS ver.16.0 for Windows). Odds ratio, associated p-value, and 95 % confidence interval were calculated using MEDCALC statistical software. We analyzed the significance of the difference in pesticide levels at $p < 0.05$. We used STRING 10.5 and Cytoscape to unravel the possible interaction between dysregulated proteins and analyzed using this molecular interaction tool and clustered using K-MEANS clustering algorithms.

3. Results

3.1. Quantification of pesticides in the blood of BC patients (case group) and healthy control group

We compared BC patients and healthy controls for blood levels of 12 OCPs i.e. endosulfan I, opDDT, heptachlor, methoxychlor, dieldrin, HTEB, ppDDE, endosulfan II, p′,p′ DDT, dHCH, HTEA, and p′p′-DDD. As compared with the healthy controls, BC patients showed significantly high levels of endosulfan II ($p < 0.001$), p′,p′ dichlorodiphenyltrichloroethane (DDT) ($p \leq 0.001$), delta-hexachlorocyclohexane (dHCH) ($p = 0.005$), and heptachlor epoxide (Fig. 1; Table 1). Odds ratio (OR) analysis also revealed that high levels of OCPs like p′,p′ DDT (OR: 49.86; $p = 0.001$) and p′p′-DDD (OR: 6.75: $p = 0.016$) pose a significant risk of BC in Indian population (Table S2).

3.2. 2-D gel electrophoresis analysis

Densitometry analyses of 2-DE protein profiles confirmed a differential expression of 17 proteins in the plasma of BC patients compared with the healthy volunteers and revealed a protein signature specific to disease conditions (Fig. 2A).

BC patients showed upregulation in 70 % of 17 differentially expressed proteins compared to the control group. Table 2 provides

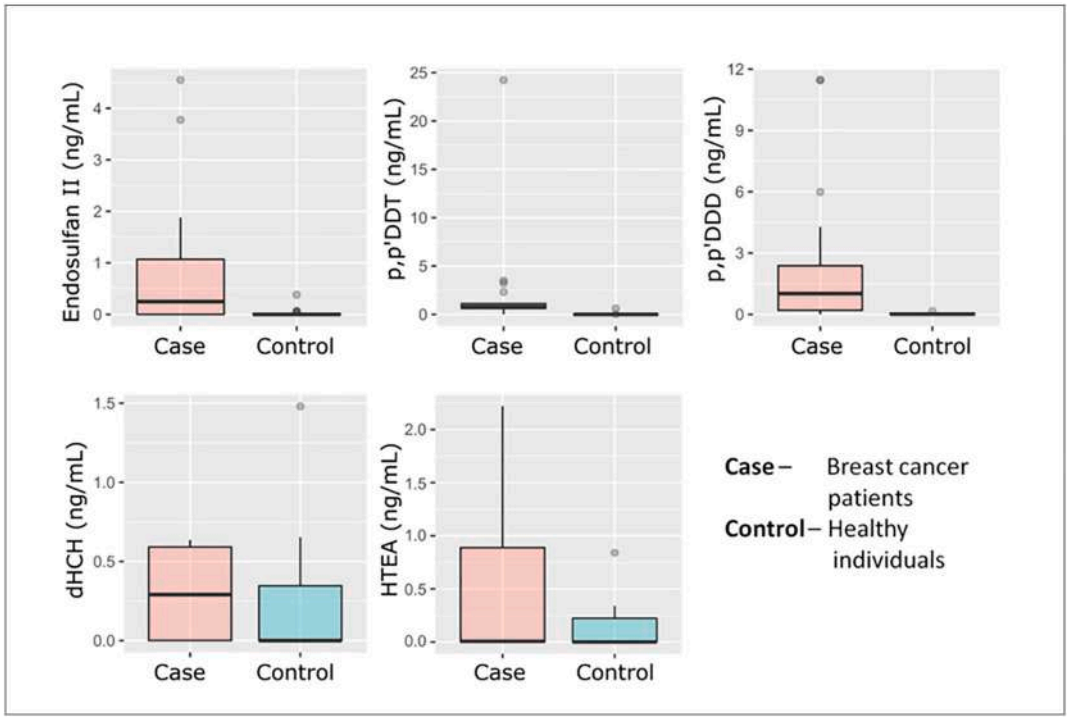

Fig. 1. Pesticides levels in blood of BC patients and healthy control. Median levels of endosulfan II ($p \leq 0.001$), p′,p′ dichlorodiphenyltrichloroethane (DDT) ($p \leq 0.001$), delta-hexachlorocyclohexane (dHCH) ($p = 0.005$), heptachlor epoxide A (HTEA) ($p = 0.002$), and p′p′-dichlorodiphenyldichloroethane (DDD) ($p \leq 0.001$) were found to be significantly high in breast cancer patients in comparison to healthy control.

Table 1
Blood levels of organochlorine pesticides in breast cancer patients and healthy controls.

Identified pesticides (ng/ml)	Breast carcinoma (mean ± sd)	Control median (mean ± sd)	Significance 'p'-value (Mann-Whitney)
dHCH	0.299 ± 0.31	0.21 ± 0.39	0.005
Endosulfan II	0.80 ± 0.95	0.03 ± 0.02	<0.001
HTEA	0.42 ± 0.64	0.12 ± 0.21	0.002
p,p′DDD	2.36 ± 1.61	0.03 ± 0.03	<0.001
p,p′ DDT	2.18 ± 0.54	0.03 ± 0.001	<0.001

p-Value≤0.05 is considered as significant.

details of differentially expressed proteins identified by MALDI-TOF/MS-MS analysis. Out of 17 differentially expressed proteins, transthyretin (TTR) protein showed the highest fold change (2.92) in BC patients. After TTR, ApoA1 was found to highly dysregulated. Five proteins (around 30 %) showed decreased expression in BC patients. The identified proteins were subjected to cluster analyses based on the densitometry values indicating expression patterns. Proteins were categorized into two different clusters by the k-means clustering algorithm. Cluster-1 comprises of fibrinogen gamma chain, isoform CRA_o, phosphatidylinositol transfer protein, beta, isoform CRA_c, chaperonin containing TCP1, subunit 8 (theta), isoform CRA_a, haptoglobin, interleukin 7 receptor, isoform CRA_b, HP protein, adenylate cyclase type 8, apolipoprotein A-I preproprotein, proapolipoprotein, phosphatidylinositol transfer protein, beta, isoform CRA_b, chain A, transthyretin, human transthyretin. However, cluster-2 comprises transferrin, MAP3K12-binding inhibitory protein 1 isoform 2, chain C, human fibrinogen, hypothetical protein FLJ40365, and interferon regulatory factor 4 (Fig. 2B).

3.3. Functional classification and interactions of differentially expressed proteins

Fig. 3a–c shows the functional classification of seventeen differentially expressed proteins and their roles in the different pathophysiological states of BC. Gene ontology search revealed that differentially expressed proteins play significant roles in binding, catalytic and regulatory processes.

Cytoscape analysis involving 17 dysregulated proteins showed that TTR, ApoA, HP, FGG, and TF show strong interaction. These proteins are involved in angiogenesis pathways and the signaling by endothelin, GABA-B receptor II, G-protein, and gonadotropin-releasing hormone (Fig. 3d–e).

3.4. Validation of increased expression of TTR in absolute cases

To confirm the distinct upregulation of TTR expression across all the BC patients, ELISA was performed. In BC patients ($n = 38$), TTR showed 1.49-fold elevated expression ($p < 0.0001$) as compared to healthy volunteers ($n = 38$) (Fig. 4). Since TTR showed the highest upregulation in the BC patients, the molecular interaction of TTR with the OCPs found to be abundant in the blood of these patients were ascertained.

3.5. Molecular docking

The molecular docking showed that the organochlorine pesticides bind with human serum TTR protein with binding free energy varying from −5 kcal/mol to −9 kcal/mol. The detailed binding energy of identified pesticides and thyroxine are shown in Table 3. Of these five compounds, endosulfan II showed similar binding energy (−9.36 kcal/mol) as thyroxine (natural substrate) (−9.74 kcal/mol). The thyroxine is stabilized by two hydrogen-bonded interactions with Ser117 and Lys15. The compound is mostly stabilized by hydrophobic interactions. Similarly, endosulfan II formed two hydrogen bonded interactions with Lys15 and one interaction with back bone oxygen atom of Val16. Apart

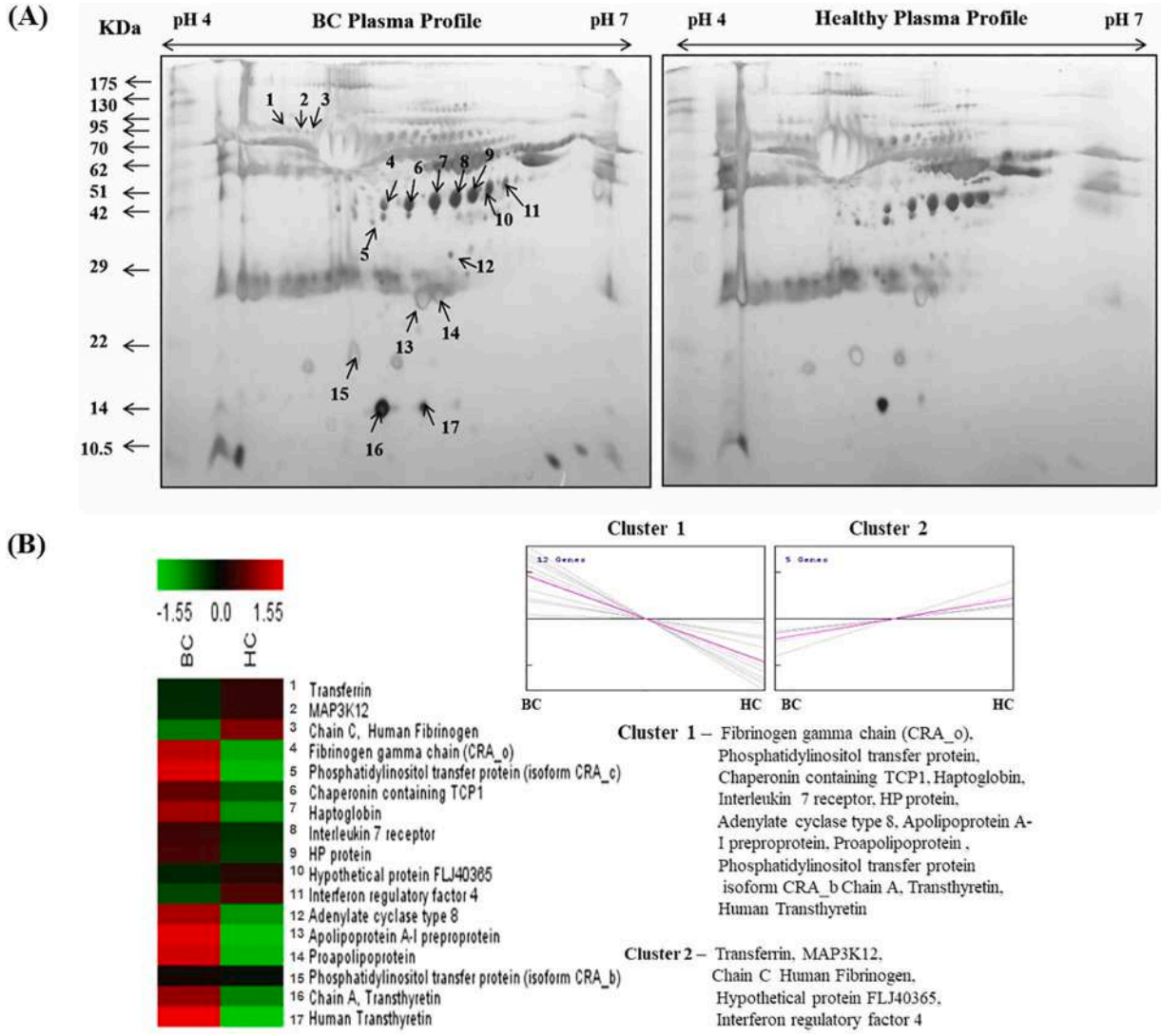

Fig. 2. (A) Silver-stained 2-D electrophoretic profiles of plasma proteins of BC patients and healthy controls. Differentially expressed spots were marked with numbers. (B) Heat map showing expression pattern analysis of dysregulated proteins in plasma of BC patients. The seventeen dysregulated proteins were clustered into two groups based on the difference in expression using MeV software. Cluster 1 contains 12 proteins, upregulated in the plasma of BC patients, whereas cluster 2 contains 5 proteins having down expression in BC patients. The mean of expression is marked in pink for each cluster, whereas grey lines represent the profile of each protein in the cluster. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Details of differentially expressed proteins identified by MALDI-TOF/MS-MS.

Fold change	Protein name	M.W. (Da)	Score	Peptide matched	Accession no.	Spot no.
2.92	Human transthyretin	13,810	112	12	gi|443295	17
2.68	Apolipoprotein A-I preproprotein	30,759	80	27	gi|4557321	13
2.59	Phosphatidylinositol transfer protein, beta, isoform CRA_c	22,303	44	16	gi|119580149	5
2.45	Proapolipoprotein	28,944	206	36	gi|178775	14
2.20	Fibrinogen gamma chain, isoform CRA_o	47,971	45	18	gi|119625326	4
2.04	Adenylate cyclase type 8	76,945	48	16	gi|7687910	12
1.95	Haptoglobin	38,722	66	15	gi|3337390	7
1.88	Chain A, transthyretin	13,780	116	12	gi|2098275	16
1.54	Chaperonin containing TCP1, subunit 8 (theta), isoform CRA_a	60,011	45	17	gi|119630329	6
1.36	HP protein	38,868	94	18	gi|78174390	9
1.29	Interleukin 7 receptor, isoform CRA_b	9213	48	11	gi|119576329	8
1.05	Phosphatidylinositol transfer protein, beta, isoform CRA_b	22,401	53	15	gi|119580148	15
−0.57	Chain C, human fibrinogen	47,009	145	29	gi|237823916	3
−0.70	Interferon regulatory factor 4	13,938	41	32	gi|159164194	11
−0.8	MAP3K12-binding inhibitory protein 1 isoform 2	39,414	62	16	gi|222080055	2
−0.81	Transferrin	79,310	59	15	gi|37747855	1
−0.83	Hypothetical protein FLJ40365	57,400	51	12	gi|119604864	10

'−': denotes down-regulation of proteins, M.W.: molecular weight.

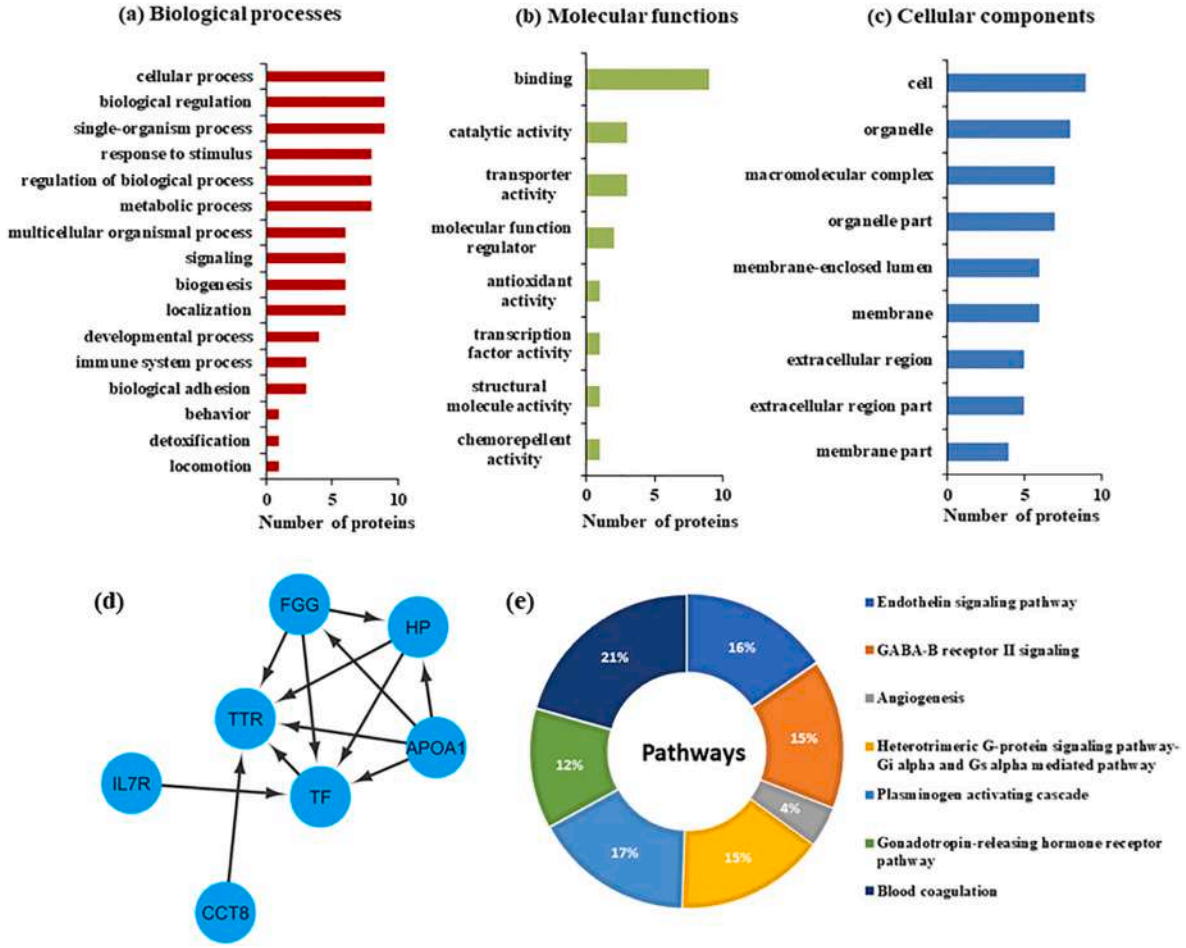

Fig. 3. Gene ontology-based functional classification of dysregulated proteins. Differentially expressed proteins play roles in (a) biological processes, (b) molecular functions and (c) cellular components. (d) The interaction network involving dysregulated proteins was developed using Cytoscape 3.8.2 and STRING analysis. The interactions with a confidence score ≥ 0.700 were considered. These proteins were clustered using MCODE clustering algorithms. The node is represented in blue circles, whereas the arrow indicates the interaction of a node with the other. (e) Doughnut diagram developed using PANTHER showing the role of dysregulated proteins in different metabolic pathways. The area of the colours in the doughnut diagram is proportional to the number of proteins in that specific pathway among all identified proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

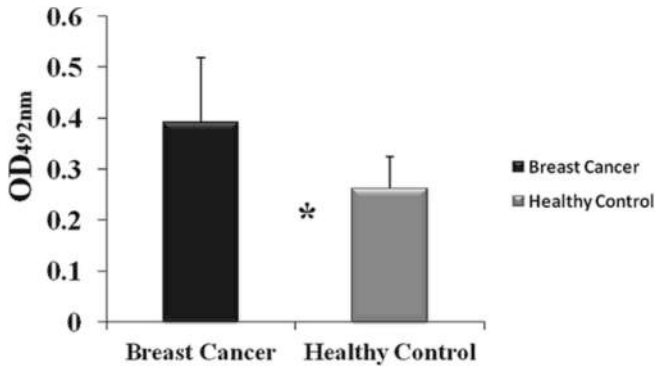

Fig. 4. Validation of TTR expression in BC patients and healthy controls using ELISA. ELISA analysis of plasma showed 1.49-fold increased TTR expression ($p < 0.001$) in BC patients compared with healthy controls ("*" denotes a significant difference in cases as compared to controls).

from hydrogen bonding of the compound is also stabilized by hydrophobic interactions with residues in the dimer interface (Table 3). The endosulfan occupies similar space like thyroxine (Fig. 5), indicating its potential to act as a thyroid disruptor. The molecule docking showed that the other compounds dHCH, HTEA, p,p′-DDD and p,p′-DDT forms only hydrophobic interactions with TTR residues. Hence, endosulfan II was selected for molecular dynamic simulation studies.

3.6. Molecular dynamic simulations

Molecular dynamics simulations of TTR-thyroxine and TTR-Endosulfan complexes were performed for 50 ns using GROMACS. The TTR-thyroxine complex showed a higher RMSD value than the TTR-endosulfan complex, indicating the high stability observed for endosulfan II. Upon comparing the TTR dimer, TTR-thyroxine and TTR-endosulfan complexes showed a decrease in the radius of gyration upon ligand binding, suggesting the compactness of the TTR dimer. The presence or absence of ligands does not significantly affect the solvent-accessible surface area. The TTR-thyroxine complex showed a higher RMSF than the native TTR and TTR-endosulfan complex (Fig. 6). Higher RMSF values indicated that the TTR dimer was stabilized in the presence of endosulfan II.

The molecular interactions of a ligand's thyroxine and endosulfan II with TTR before and after MD simulation showed that thyroxine maintained the H-bond with Ser117 and formed new H-bond with Ala108. While it almost maintained other hydrophobic interactions throughout the MD simulation (Fig. 7a–c). In case of endosulfan II, it lost both hydrogen-bonded interactions with Lys15 and Val16 but remain stable inside the dimer interface by forming hydrophobic interactions

Table 3
Interactions and the Binding affinity formed by pesticides with TTR.

Organochlorine pesticides (OCPs)	BE (kcal/mol)	Ki (μM)	Hydrophobic interactions		H-bond interactions	
			Chain A	Chain B	Chain A	Chain B
Thyroxine (T4)	−9.74	0.072	M13, K15, E54, T106, A108	M13, K15, L17, H56, T106, A108	S117 (OG)	K15 (NZ)
dHCH	−5.99	40.440	L17, A108, A109, L110, S 117, T118, T119	L17, A109, L110, S117	Not found	Not found
Endosulfan II	−9.36	0.138	K15, V16, L17, P24, S52, G53, A108	K15, L17, T106, A108, T119, V121	K15 (NZ) V16 (O)	K15 (NZ)
HTEA	−7.35	4.130	K15, L17, A108, A109, L110, T118, T119	K15, L17, A108, L110, T119,	Not found	Not found
p,p′-DDD	−6.76	11.150	K15, L110	K15, L17, T106, A108, A109, L110, S117, T118, T119, V121	Not found	Not found
p,p′-DDT	−7.47	3.320	K15, V16, L17, A108, S117, T118, T119,	K15, L17, L110, V121	Not found	Not found

BE - binding energy, Ki - inhibition constant.

with Lys15, Leu17, Ala108, Leu110 and Thr123 (Table 4; Fig. 7d-e).

4. Discussion

Blood levels of Endosulfan II, p′,p′ DDT, dHCH, HTEA, and p′p′-DDD were significantly higher in estrogen receptor-positive breast cancer patients compared to healthy individuals, indicating an increased risk of breast cancer. DDT and DDE levels in breast cancer patients' adipose tissue were also significantly elevated as also reported by others [30]. A recent Indian study also found that cancer patients had much higher levels of DDT, DDE, and HCH than healthy controls [16]. The EPA classifies DDT, DDE, and HCH as group B2 carcinogens, and bioaccumulation of OCPs in blood or tissues in cancer patients has been documented in comparison to the control group [31,32]. OCP, such as methoxychlor, acts as an environmental estrogen with estrogenic and anti-androgenic activity during in vivo research, influencing fertility, uterine development in females, and early pregnancy [5,33]. The presence of HCH has also been linked to breast, prostate, and ovarian cancer [34].

A large-scale epidemiological study from Spain found a significant prevalence of BC (81 %) in pesticide-contaminated areas [35]. Pesticide exposure is known to harm the immune system, promoting cancer growth by inducing chronic inflammation and altering tumour immunogenicity [36]. However, little is known about the biomolecules and interactions that cause the effects of high pesticide blood levels. Our proteomic analysis revealed biomolecules that were dysregulated in BC patients with significantly elevated OCP levels.

TTR, ApoA1, HP, and FGG, all of which are involved in the acute phage response pathway, were found to be significantly overexpressed in breast cancer patients (Fig. 2). TTR, ApoA1, HP, and FGG have also been linked to prognosis in breast, liver, and colorectal cancers [37]. Altered levels of ApoA1 affect lipid metabolism, which has also been linked to breast cancer [38]. A 2.68-fold increase in ApoA1 in breast cancer patients' plasma (Table 2) demonstrates its role in determining breast cancer risk in women. Several studies have shown that ApoA1 levels change during cancer onset and progression; thus, ApoA1 may be useful for early cancer detection and prognostic classification. However, increased expression of haptoglobin or HP (spot 7, 9) reveals its specific role in BC. HP is an acute-phase inflammatory protein that is upregulated during angiogenesis [39]. Our results corroborate with findings of other workers [40], which found a link between elevated serum HP levels and tumour growth.

In addition to ApoA1 and HP, the current study discovered a 2.2-fold increase in fibrinogen beta chain (FBB) in cancer patients' plasma. In contrast, the expression of fibrinogen chain C was reduced (0.57-fold). Other researchers have discovered that the fibrinogen alpha, beta, and gamma chains are upregulated in advanced breast cancer [41]. A 2.92-fold increase in TTR expression, a homotetrameric protein released by liver hepatocytes, has predictive value in the diagnosis of cancer in BC patients (Table 2). Nasim et al. [52], on the other hand, discovered a predominance of TTR downregulation (68.75 %) in the sera of BC patients compared to healthy controls. The serum TTR level has also been described as a prognostic marker for lung and ovarian cancer [42,43].

OCPs, as endocrine disruptors, cause hormone-mediated cancers such as BC [13,44]. Proteomic analysis indicates disruption of many endocrine pathways due to elevated TTR levels in the plasma of BC patients. Because it transports the thyroxine (T4) hormone and the retinol-binding protein complex throughout the body, TTR is an essential endocrine protein. TTR has also been linked to the aetiology of a number of diseases [45].

Given our findings and previous data implicating TTR in the genesis and diagnosis of BC, further research into the pesticide-TTR interaction that causes endocrine disruption is required. Therefore, we docked pesticides with the crystal structure of human TTR to ascertain their competitive binding. TTR levels in serum are linked to smaller ovarian cancer [46]. Endosulfan II is one of the most persistent OCPs that pose a serious risk to human health for their chronic toxicity, persistence, and bioaccumulation [47]. Endosulfan is associated with reproductive and developmental damage in both animals and humans. It is also reported that endosulfan triggers epithelial-mesenchymal transition via the PTP4A3-mediated TGF-β signaling pathway in prostate cancer cells [48]. But there is no study that explains the mechanism of endosulfan as an endocrine disruptor in breast cancer. Docking results confirmed the interactions between endosulfan II and TTR. Endosulfan II had a higher binding energy with the thyroxine-binding site of TTR than thyroxine, indicating a possible role in endocrine disruption and subsequent risk for BC. As indicated by RMSD and RMSF values, endosulfan increases the stability of the TTR complex in MD analysis, whereas the presence of the natural substrate thyroxine decreases the stability of the TTR dimer complex. Endosulfan competitively inhibits the thyroxine transport pathway, as evidenced by its increased stability over the TTR dimer. A recent study demonstrated endosulfan-induced dopaminergic neurotoxicity in Parkinson's disease (PD), and TTR is regarded as a powerful diagnostic marker for PD [49]. Several organic pollutants, including bisphenol-A and triclosan, have been shown to bind to TTR, causing endocrine disruption [50]. Our findings suggest that pesticides' ability

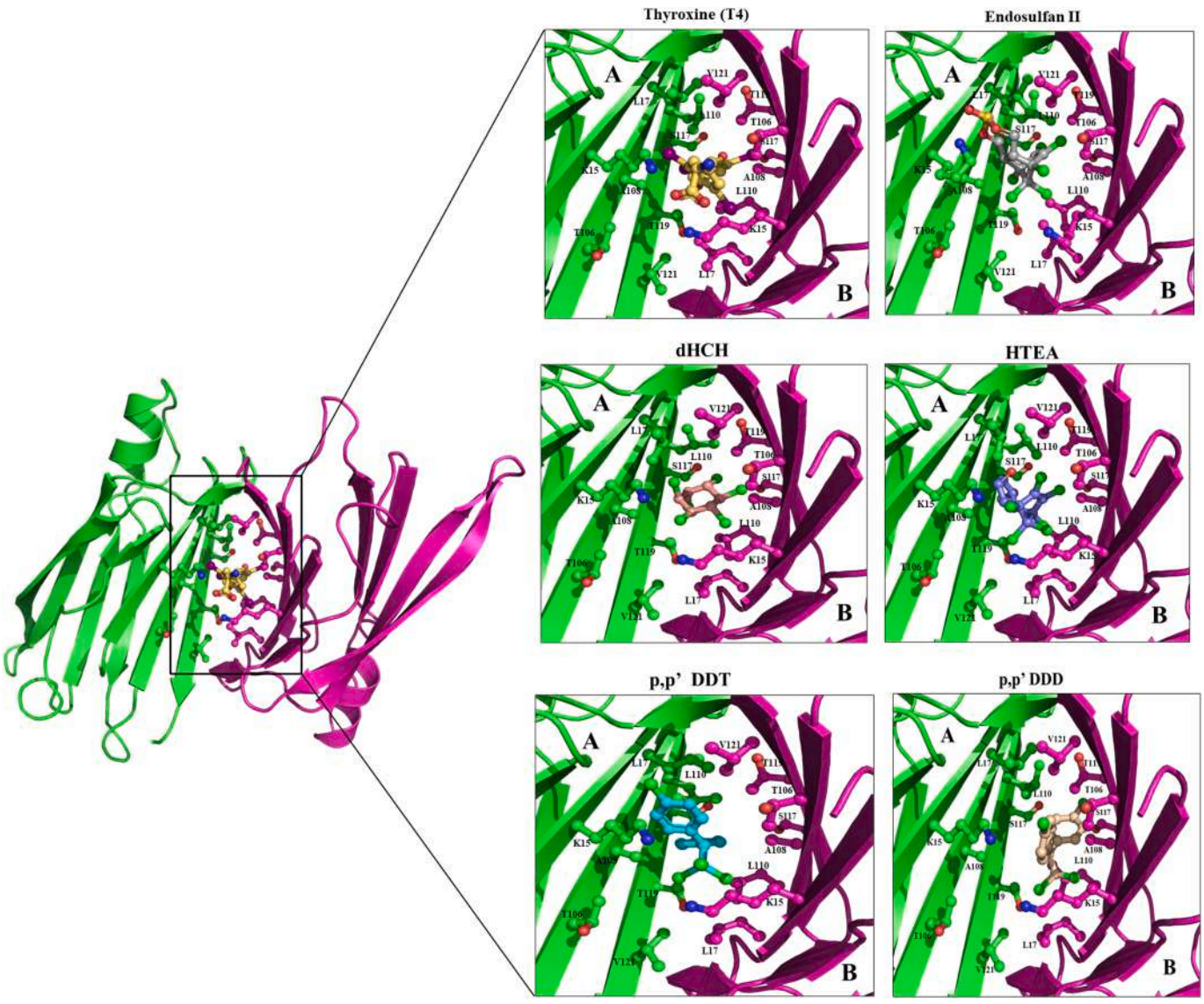

Fig. 5. Molecular docking of TTR with pesticides. TTR dimer was modelled and docked with identified pesticides. The magnified view of docking position (right-hand side) highlights the side-chain conformations of TTR dimer in the presence of thyroxine (−9.74 kcal/mol), endosulfan II (−9.36 kcal/mol), dHCH (−5.99 kcal/mol), HTEA (−7.35 kcal/mol), *p,p′*-DDT (−7.47 kcal/mol) and *p,p′*-DDD (−6.76 kcal/mol) detected in the blood of BC patients.

to bind with TTR is linked to their endocrine disrupting properties. Although the interactions between pesticides and TTR, as well as their effects on endocrine disruption, have not been thoroughly studied, their association with the onset of BC warrants further investigation.

Relaxed regulatory policies and poor execution continue to be a primary cause of widespread pesticide use in India, posing increased cancer risks, particularly for women. For example, in India, the maximum residue limits (MRL) for lindane and DDT are 40–100 times higher than European Union standards [12]. Because pesticides are widely used and have high residue limits, the amount of OCPs in the environment and food chains has increased [10,51]. The rising prevalence of OCPs in Indian breast cancer patients is cause for concern in the health and policy sectors. It would be critical to determine whether the contamination is due to legacy OCPs or current unregulated pest control use. Appropriate legislative measures are required to protect particularly women, from OCPs, exposure due to both legacy and currently used pesticides.

In conclusion, blood levels of ppDDD, ppDDT, Endo II, D-HCH, and HTEA in breast cancer patients were significantly higher than in healthy controls. Increased blood levels of pesticides accompanied by significant upregulation of TTR, ApoA1, HP, and FGG in BC patients indicate their potential to serve as BC biomarkers. Molecular docking confirmed the significant affinity between endosulfan II and TTR at the thyroxine-binding site. Increased TTR expression, a key protein of inflammatory and endocrine pathways with a competitive affinity for thyroxine-binding region of TTR, suggests pesticide-induced endocrine system disruption in BC patients. The emergence of BC in India may be an additive or synergistic effect of OCP misuse and its legacy nature, necessitating policymakers' and environmental managers' attention.

CRediT authorship contribution statement

SS: Conceptualization, Data curation, Methodology, Formal analysis, Writing – original draft, Visualization, Investigation. **LM:** Molecular dynamics and editing. **PM:** Methodology, Formal analysis. **NK:** Sample collection, Data curation. **TK:** Sample collection, Data curation. **CVS:** Proteomics analysis. **ASE:** Software, Writing – review & editing, Conceptualization, Supervision. **BDB:** Supervision, Writing – review & editing. **VM:** Supervision, Conceptualization, Writing – review & editing, Resources. **RSS:** Conceptualization, Writing – original draft,

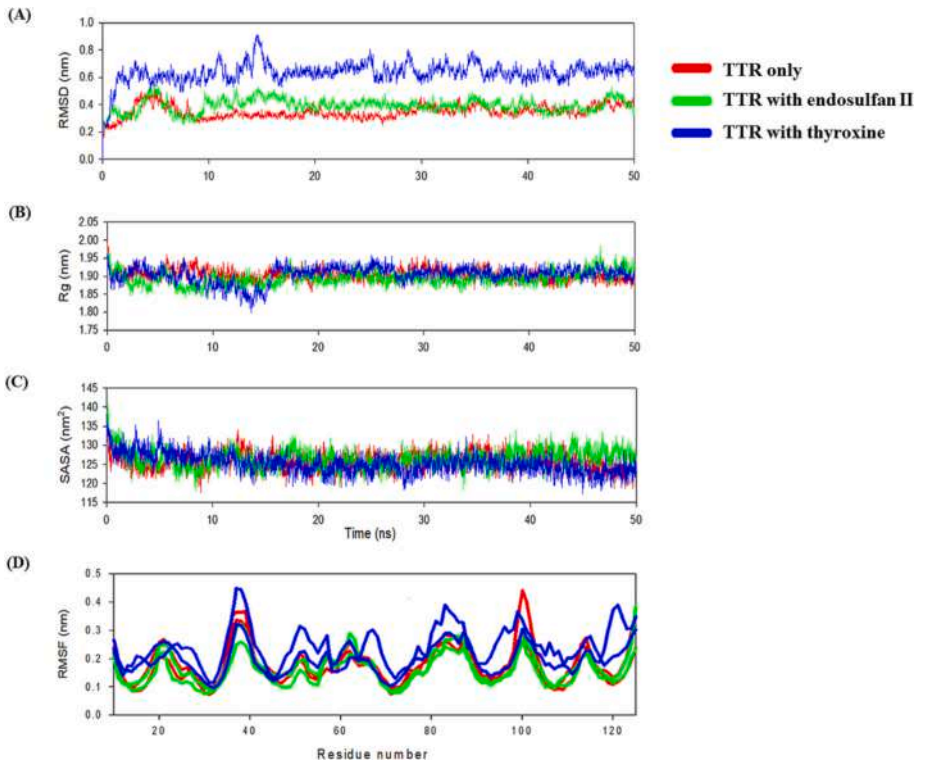

Fig. 6. RMSF, radius of gyration, SASA and RMSD graph of TTR with thyroxine and endosulfan II. Root Mean Square Fluctuation of each residue of the monomer. The RMSD value of the TTR-thyroxine complex was found to be more than RMSD of the TTR-Endosulfan complex (A). The Radius of gyration was found to be decreasing initially in both the TTR-endosulfan and TTR-thyroxine complexes compared to TTR alone, describing the compactness of TTR dimer upon ligand binding, then Rg peak becomes similar to native TTR dimer peak (B). The solvent-accessible surface area (SASA) shows the change in the solvent surface area upon ligand binding (C). The RMSF value of the TTR-thyroxine complex was found to be high as compared to TTR only, whereas the RMSF of TTR- endosulfan was found very similar to TTR only (D).

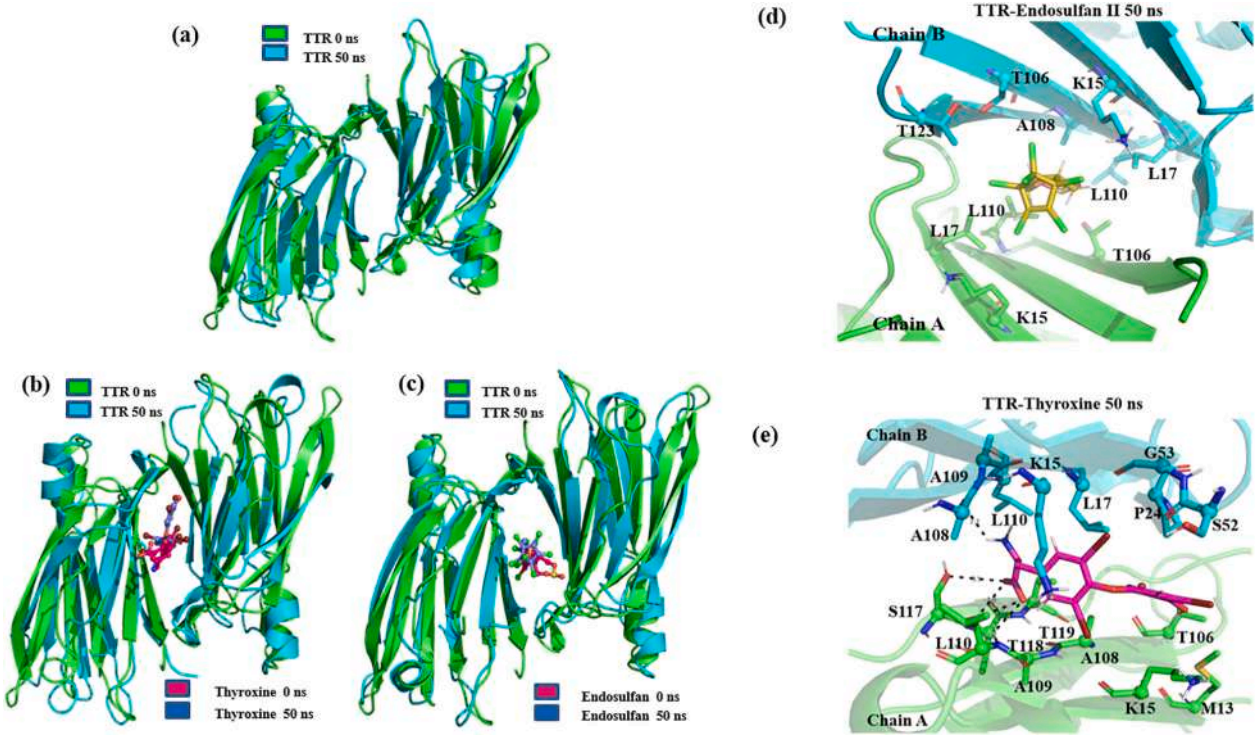

Fig. 7. An overlapped image of TTR dimer alone (a), with thyroxine (b), and with endosulfan II (c) at 0 and 50 ns of molecular dynamics simulations. Interaction between the Endosulfan-TTR (d) and Thyroxine-TTR (e) complex at 50 ns of MD simulations. The cartoon structure diagram represents the residues of both TTR monomers in stick orientation which are involved in the formation of either Hydrophobic interactions or H-bond with the Endosulfan/Thyroxine. The H-bond distances are shown in the black dashed line.

Table 4
Interactions formed between TTR-Thyroxine and TTR-Endosulfan II complex before and after 50 ns of MD simulations.

Organochlorine pesticides (OCPs)	Hydrophobic interactions		H-bond interactions	
	Chain A	Chain B	Chain A	Chain B
Before MD simulations				
Thyroxine (T4)	M13, K15, E54, T106, A108	M13, K15, L17, H56, T106, A108	S117 (OG) - OH4′ - 2.6 Å	K15 (NZ) - OH9–2.6 Å
Endosulfan II	K15, V16, L17, P24, S52, G53, A108	K15, L17, T106, A108, T119, V121	K15 (NZ) – Cl- 2.8 Å V16 (O) - OH – 2.9 Å	K15 (NZ) - Cl = 3.3 Å
After 50 ns MD simulations				
Thyroxine (T4)	M13, K15, T106, A108, A109, L110, S117, T118, T119	K15, L17, P24, S52, G53, A108, L110	S117 (O) - OH10–3.5 Å S117 (OG) - OH10–3.0 Å S117 (OG) - OH9–3.0 Å L110 (NH) - O9–2.7 Å	A108 (O) - NH8 = 2.7 Å
Endosulfan II	K15, L17, A108, T119	K15, L17, T106, A108, L110, T123	Not found	Not found

Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

VM and RSS thank the Institute of Eminence, University of Delhi, for Faculty Research Grant-Projects (Ref. No./IoE/2021/12/FRP dated 31.08.2022). RSS acknowledges special research grant to Delhi School of Climate Change & Sustainability by Insititute of Eminence, University of Delhi. Dr. Gayatree Mohapatra, RCB, Faridabad, is acknowledged for helping in proteomic analysis and Shipra, UCMS, New Delhi, for helping in GCMS. SS acknowledges the Research Associate fellowship of ICMR and SRF of CSIR.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.123670.

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