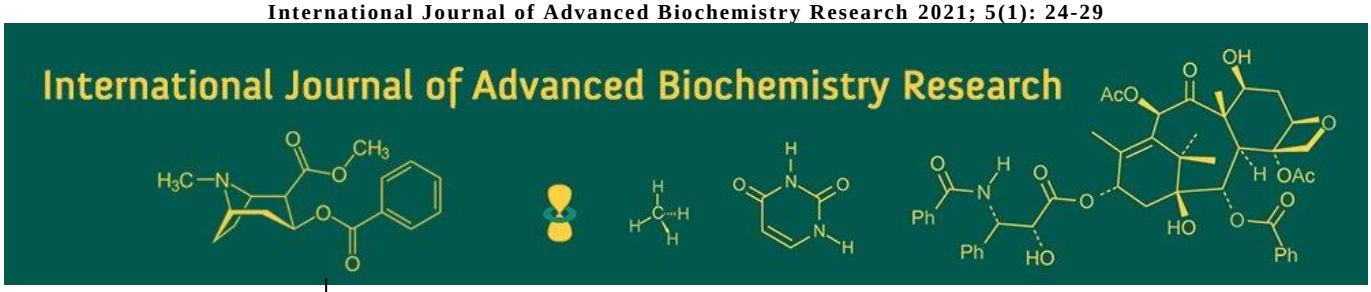

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A prospective study of biochemical markers in diagnosing carcinoma breast

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Abstract
Introduction: Various biochemical tumour markers have been studied singly or in combination, in breast malignancies which help in diagnosing carcinoma of breast cancer.
Aim: To assess the levels of enzymes Lactate dehydrogenase (LDH), Gamma glutamyl trans peptidase (GGT), and superoxide dismutase (SOD) in patients with newly diagnosed carcinoma breast and study if there is any clinical correlation between the levels of these enzymes with the presence of early untreated breast carcinoma.
Materials and Method: This is a prospective study done during the period February 2018 to Jan 2019 at the Government Pudukkottai Medical College. It was carried out in two groups, namely apparently healthy 27 females controls and 50 females with newly diagnosed carcinoma breast.
Results: The imbalance between free radicals and the anti-oxidants in the causation of breast cancer is being researched. Free radicals have been implicated in the initiation and promotion of carcinogenesis. This study was undertaken to evaluate if the levels of biochemical markers LDH and GGT, and the level of antioxidant enzyme SOD are altered in cases with carcinoma breast when compared to the levels in apparently normal controls.
In humans, three forms of superoxide dismutase are present. SOD1 is located in the cytoplasm, SOD2 in mitochondria and SOD3 in the extracellular matrix. SOD causes detoxifies superoxide to hydrogen peroxide. LDH is a glycolytic enzyme with five isoenzymes. The level of LDH is increased in various malignancies. GGT helps in the transportation of amino acids in the cells.
Conclusion: The levels of LDH and GGT were found to be significantly elevated in the cases, whereas the levels of SOD were found to be significantly lower in the cases. The SOD levels were lower in cases with stage-3 carcinoma breast than in cases with stage-2 carcinoma breast.

Keywords: Carcinoma breast, tumor markers, biochemical markers, LDH, SOD, GGT

Introduction
Tumour markers are biochemical indicators of the presence of tumour. Various tumour markers have been studied singly or in combination, in breast malignancies. In this study, the level of three biochemical parameters, namely SOD, LDH and GGT was assessed and analyzed for possible correlation to breast cancer.

Aim of the study
On reviewing the role of reactive oxygen species in the causation of carcinogenesis and the changes in biochemical parameters in serum of patients with carcinoma breast, this study has been taken up with keen interest to establish the following aims.

- To determine the reference ranges for the following biochemical parameters in female individuals of age between 35 and 75- serum lactate dehydrogenase, gamma glutamyl transpeptidase, total protein, albumin, albumin-globulin ratio, and blood superoxide dismutase.
- To determine the levels of these parameters in individuals with carcinoma breast
- To determine whether the levels of these biochemical parameters differ significantly in individuals with carcinoma breast when compared to apparently healthy individuals.

Materials and Methods
The study was done during the period February 2018 to Jan 2019. It was carried out in two groups, namely apparently healthy female controls and females with newly diagnosed carcinoma breast.

Control Group
The group comprised of 27 apparently healthy female subjects with no significant medical illness, and they were selected from the patients attending the general outpatient department of the Pudukkottai Govt Medical College.

Test Group
This group comprised of 50 females recently diagnosed with carcinoma breast through tissue diagnosis, being managed under the general surgery wards of the Pudukkottai Govt Medical College.

Inclusion criteria
Females with a confirmed diagnosis of carcinoma breast through tissue diagnosis, any stage of carcinoma, of any age group.

Exclusion Criteria
1. Individuals who have undergone any surgical intervention for the carcinoma breast other than tissue biopsy
2. Individuals who have received chemotherapy or radiotherapy for carcinoma breast.
3. Individuals diagnosed to have diabetes mellitus, liver disease, or pancreatic diseases
4. Individuals with a history of recent acute myocardial infarction.

Sample Collection
5ml of peripheral venous blood was withdrawn under sterile conditions with disposable syringes from all the 77 subjects of the study. 1.5 ml of blood was transferred into a test tube containing EDTA, for superoxide dismutase estimation. After thorough mixing, the contents were transferred into a 2 ml Eppendorf tube and labelled properly. These Eppendorf tubes were then stored at a temperature of -20 °C till the samples were analyzed.
The remaining blood of 3.5 ml from each subject was transferred to another test tube without any anti-coagulant. Serum separated from this tube was pipetted into a centrifuge tube and was centrifuged at 2000 rpm for 5 minutes to obtain clear fluid without cells. The clear serum was then analyzed for the estimation of serum lactate dehydrogenase, serum gamma glutamyl trans peptidase, serum total proteins, and serum albumin.
The biochemical parameters undertaken for the study were determined by semi-auto analyzer ERBA CHEM- 5 plus V2 using the following methodology:

Estimation of Superoxide Dismutase in Whole Blood
Principle
The role of superoxide dismutase (SOD) is to accelerate the dismutation of the toxic superoxide radical (O2●), produced during oxidative energy processes to hydrogen peroxide and molecular oxygen.
This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2- (4-iodophenyl)-3-(4-nitrophenol)-5 phenyl tetrazolium chloride (I.N.T) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of I.N.T. under the conditions of the assay.

$$\text{Xanthine} \xrightarrow{\text{XOD}} \text{Uric acid} + O2\bullet$$

$$\text{INT} \xrightarrow{O2\bullet} \text{Formazan dye}$$

Or,

$$O2\bullet + O\bullet + 2H+ \xrightarrow{\text{SOD}} O2 + H_2O$$

Sample Preparation
EDTA whole blood samples were used. 0.5 ml of whole blood was centrifuged for 10 minutes at 3000 rpm and then plasma discarded. The erythrocytes were washed with 3 ml of 0.9% NaCl solution and centrifuged for 10 minutes at 3000 rpm. The supernatant was discarded. This NaCl wash and centrifugation was repeated four times. The washed centrifuged erythrocytes was made up to 2 ml with cold redistilled water mixed and left to stand at 4 ℃ for 15 minutes. The lysate was diluted with 0.01 mol/L phosphate buffer pH 7 so that the percentage inhibition falls between 30% and 60%.

Reagent Composition
Contents initial concentration of solutions

R1a.	Mixed substrate	
	Xanthine	0.05 mmol/L
	I.N.T.	0.025 mmol/L
R1b.	Buffer	
	CAPS	40 mmol/L, pH 10.2
	EDTA	0.94 mmol/L
R2.	Xanthine Oxidase	80 U/L
	CAL Standard	4 U/mL

Stability and Preparation of Reagents
The contents of one vial of R1a. Was mixed with 20 ml of R1b buffer. This was stored at +2 ℃ to +8 ℃. R2 Xanthine oxidase one vial was reconstituted with 10 ml of redistilled water, and was stored at +2 ℃ to +8 ℃. One vial of the standard was reconstituted with 10 ml of redistilled water. Subsequent dilutions of this standard were prepared with Ransod sample diluents. The following dilutions were made of the Standard CAL (S6) to produce a standard curve:

Volume of	Volume of standard solution	Sample diluent
S6	Undiluted standard	---
S5	5ml of S6	5 ml
S4	5ml of S5	5 ml
S3	5ml of S4	5 ml
S2	3ml of S3	6 ml

S1 = sample diluent

Procedure
The semi auto analyzer was programmed to the following settings

Wavelength	505 nm
Cuvette	1 cm path length
Temperature	37 ℃
Measurement	against air

The following were pipette into separate cuvettes and mixed.

	Sample diluent	Standards S2-S6	Diluted sample
Diluted sample	---	---	0.05 ml
Standard	---	0.05 ml	---
Ransod sample diluent	0.05 ml	---	---
Mixed substrate (R1)	1.7 ml	1.7 ml	1.7 ml

Xanthine oxidase (R2) 0.25 ml was added to each of the above cuvettes.
The initial absorbance A1 was read after 30 seconds and the final absorbance A2 after 30 minutes.

Calculation

$\frac{A2-A1}{3}$ = ΔA per minute of standard or sample

Sample diluent rate (S1 rate) = rate of uninhibited reaction = 100%

All standard rates and diluted sample rates were converted into percentages of the sample diluent rate and subtracted from 100% to give a percentage inhibition.

$$100 - \frac{(\Delta AStd/min \times 100)}{=(\Delta AS1/min)} \text{ percentage inhibition}$$

$$100 - \frac{(\Delta ASample/min \times 100)}{=(\Delta AS1/min)} \text{ percentage inhibition}$$

Percentage inhibition for each standard against log10 was plotted. The percentage inhibition of the sample was obtained from the curve

Lactate Dehydrogenase
Method: UV Kinetic (IFCC and SFBC) Method Kit Used: Autopak of Bayer Diagnostics

Principle
Lactate Dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate and NADH to NAD. LDH activity in serum/plasma is directly to proportional to the rate of decrease in absorbance of NADH at 340 nm.

Pyruvate + NADH + H+ ↔ Lactate + NAD+

Sample collection, storage & stability
Serum was used for estimation of LDH, and was stored at 2 ℃ to 8 ℃ for one week. Reagents

Reagent 1 (co enzyme)	
NADH	240 µmol/L
Reagent 1A (Buffer)	
Tris buffer, pH 7.2	80 mmol/L
Sodium Chloride	200 mmol/L
Pyruvate	1.6 mmol/L

Reagent reconstitution
The reagents were allowed to attain room temperature. 3 ml of reagent 1A was added into one bottle of reagent 1, mixed by gentle swirling till complete dissolution. The reconstituted reagent mixture was used after 5 minutes. The reconstituted reagent was stored at 2 ℃ to 8 ℃.

Procedure
The reconstituted reagent was brought to room temperature prior to use. Assay parameters

General System Parameters

Reaction Type	:	Kinetics
Reaction slope	:	Increasing
Wave length	:	405 nm
Flow cell Temp	:	30 ℃
Delay time	:	60 secs
No. of readings	:	4
Interval	:	60 secs
Sample volume	:	100 µ L
Reagent volume	:	1 ml
Path length	:	1 cm
Factor	:	1111
Zero setting with	:	distilled water

The semi-auto analyzer was calibrated to the above mentioned system parameters. 1 ml of the reconstituted reagent and 100 µL of the sample were dispensed into a test tube, mixed and analyzed in the semi-auto analyzer immediately.

Total protein
Method: BIURET method
Kit used: Bayer AUTOPAK

Principle
Peptide bonds of protein form a blue violet colored complex with cupric ions in an alkaline medium. The intensity of colour is proportional to the amount of peptide bonds and the colour is read at 540 nm (530 to 570 nm). The final colour is stable for 8 hours. Sample used: Serum.

Reagents

Reagent 1 (biuret reagent)	
Sodium hydroxide	3.8 mol/l
Potassium sodium tartrate	0.1 mol/l
Cupric sulphate	33 mmol/l
Potassium iodide	30 mmol/l
Reagent 1A (surfactant)	
Surfactant	20 g/L
Standard (total protein 6 g/dl)	
BSA	60g/dl

Reagent reconstitution
The reagents were allowed to attain the room temperature. 41 ml of distilled water was added to one bottle of reagent 1 and then the contents of one bottle of reagent 1A were added, mixed gently to avoid foaming.

Procedure
The reconstituted reagent was brought to room temperature prior to use.

System parameters

Reaction type	:	Endpoint
Reaction Slope	:	Increasing
Wavelength	:	546 nm (530-570 nm)
Flow cell temp	:	30
Incubation	:	20 min at room temp
Reagent volume	:	1 ml
Std. Concentration	:	6 g/dl
Zero setting with	:	reagent blank

1 ml of reagent was taken in all test tubes. For standard, 10 µL of the standard was added. For the test, 10 µL of samples were added and incubated for 20 min at room temperature and the readings were taken at 546 nm.

Albumin

Method: Bromocresol green method

Principle

Albumin in a buffered solution reacts with the anionic Bromocresol green (BCG) with a dye binding reaction to give a proportionate green colour which is measured at 628 nm (600-650 nm). The final colour is stable for 10 minutes.

Sample: Serum.

Reagents

Reagent 1 (Bromocresol green)

Succinic acid 94 mmol/L

Sodium hydroxide 10.2 mmol/L BCG 0.149 mmol/L

Standard (albumin 5 g/dL)

BSA 50 g/L

Reagent reconstitution: Albumin reagent is ready to use.

Procedure

The reagent was brought to room temperature before use.

General system parameters:

Reaction type	:	Endpoint
Reaction slope	:	Increasing
Wavelength	:	628 nm (600-650 nm)
Flowcell temperature	:	30
Incubation	:	1 minute, room temperature
Sample volume	:	10 µL
Reagent volume	:	1 mL
Standard concentration	:	5 g/dl
Zero setting with	:	reagent blank

The instrument was set using the above parameters

To 1 ml of reagent 10 µL of samples was added and incubated for 1 minute at room temperature and readings were taken.

Results

The mean age of the controls was 51.88±8.59 and the mean age of the cases was 52.28±10.93. The biochemical parameters, namely the antioxidant enzyme superoxide dismutase, gamma glutamyl transferase and lactate dehydrogenase, serum total protein, serum albumin, albumin-globulin ratio, obtained from the 77 subjects taken up in this study.

Chart-1 shows the compilation of biochemical parameters for the cases & controls. The mean and standard deviation of cases & Controls were as follows LDH-423±198; GGT-29±6; SOD 151.7±10.7; and LDH 213±112, GGT 20 ±6, SOD 189.8 ±14.8 respectively.

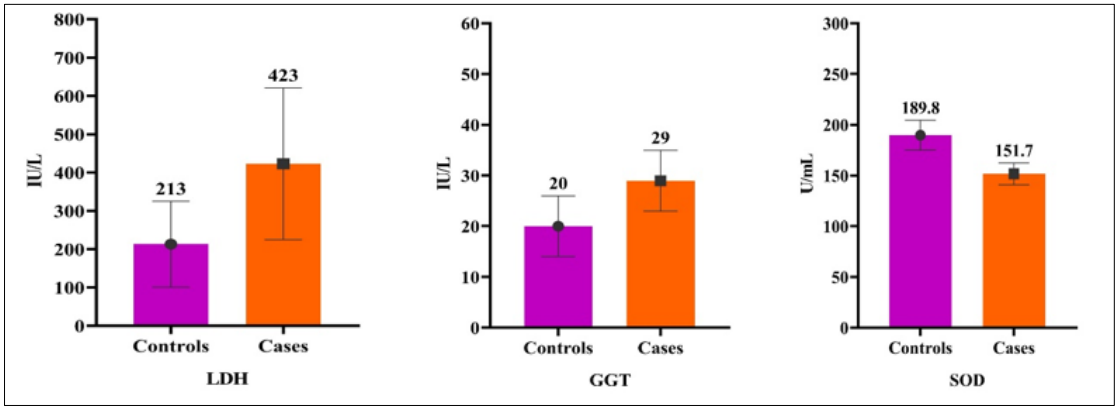
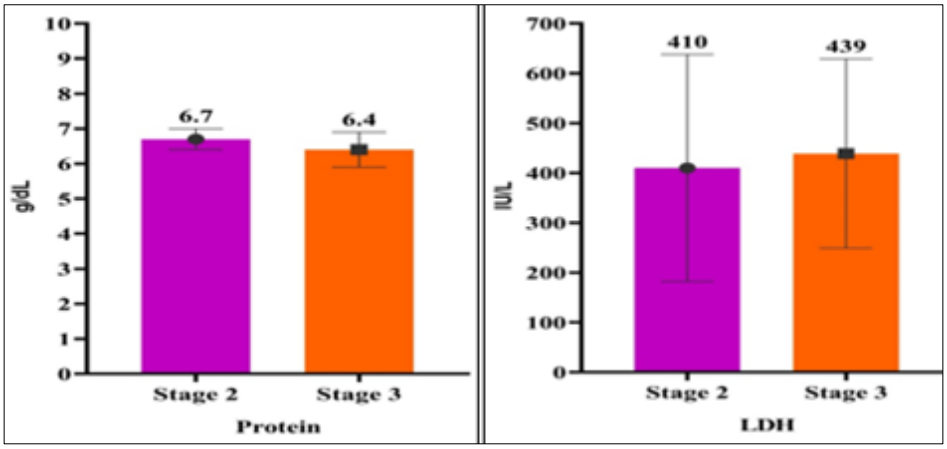


Chart 1: Biochemical Parameters of LDH, GGT and SOD

Chart-2 shows the compilation of biochemical parameters for the Stage 2 & Stage 3 of Breast cancer among the cases



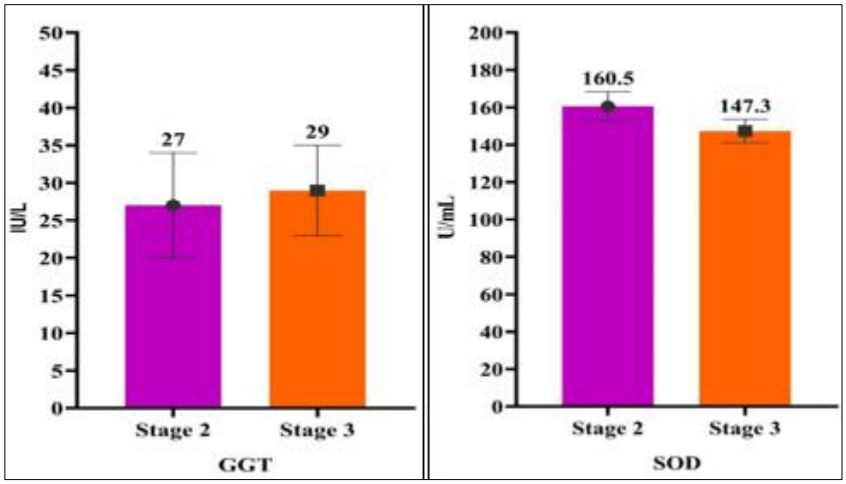


Chart 2: Biochemical Parameters in stage 2 and 3 of protein, LDH, GGT and SOD

Table 1: Comparison of Biochemical Parameters in Cases and Controls

	Study group	LDH Mean ± SD IU/L	GGT Mean ± SD IU/L	SOD Mean ± SD U/mL
1	Controls (n=27)	213±112	20±6	189.8±14.8
2	Cases (n=50)	423±198	29±6	151.7±10.7
p-value		0.01	0.01	0.01
Level of significance*		Significant	significant	significant

Table 2: Comparison of biochemical parameters in Stage-2 & Stage-3 CA Breast

Sl. No.	Study group	Protein Mean ± SD g/dL	LDH Mean ± SD IU/L	GGT Mean ± SD IU/L	SOD Mean ± SD U/mL
1	Stage 2	6.7±0.3	410±228	27±7	160.5±7.9
2	Stage 3	6.4±0.5	439±190	29±6	147.3±6.3
p-value		0.05	0.18	0.28	0.01
Level of significance*		Significant			Significant

The reference ranges for the analyzed biochemical parameters of the apparently healthy female controls with a mean age of 51.88±8.59 years are as follows:

Total protein	7.4±0.6 g/dL
Serum albumin	4.3±0.5 g/dL
A: Gratio	1.4±0.2
Serum LDH	213±112 IU/L
Serum GGT	20±6 IU/L
Blood SOD	189.8±14.8 U/mL

These mean values fall within the accepted reference ranges, and therefore could be accepted as valid for this study.

Comparison of the levels of various biochemical parameters under study, between the cases and controls is shown in Table-1 From this table, it is found that the levels of LDH have been found to be significantly elevated in the cases with a mean value of 423±198 compared to controls mean value 213±112 IU/L. (P-value <0.00) The levels of serum GGT have been found to be significantly elevated (p-value <0.00) in the cases. The levels of blood SOD have been found to be significantly decreased (p-value <0.00) in the cases.

In comparing the levels of the biochemical markers in Table 2 among the subjects with stage-2 and stage-3 breast cancers, only SOD levels were lowered which is significant (p-value 0.00)- mean blood level of 147.3 IU/mL in stage 3 compared to the mean blood level of 160.5IU/mL in stage-2. This could be indicative of further radical-induced inhibition of SOD activity in advanced stages of breast cancer.

Conclusion

The levels of biochemical markers LDH and GGT have been found to be significantly elevated. The levels of antioxidant enzyme SOD significantly lowered in cases with carcinoma breast.

The SOD levels were lower in cases with stage-3 carcinoma breast than in cases with stage-2carcinomabreast. Definite association of these markers with carcinoma breast might open up the scope for use of these biochemical markers as adjuvant in breast cancer screening. The association of the levels of these biochemical parameters with the clinical course of the carcinoma breast, is yet to be established. Further, the levels of these biochemical markers in nonmalignant breast tumours need to be studied. Definite association of these markers with carcinoma breast might open up the scope for use of these biochemical markers as adjuvant in breast cancer screening. This also would warrant further studies of these biochemical markers in patients post-surgery and/or chemotherapy and/or radiotherapy, which could open up possibilities of these biochemical markers to be used in estimation of these in the diagnosis, management and follow-up of individuals at risk of and those diagnosed to have carcinoma breast which will considerably help in reducing morbidity and mortality associated with cancer breast.

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