Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

Thesis submitted to Faculty of Medicine

BLDE (Deemed to be University) Vijayapura, Karnataka, India.

For the award of the degree of

DOCTOR OF PHILOSOPHY
In
MEDICAL PHYSIOLOGY

By

Dr Vitthalsa Khode
Reg. No-15PhD008

Department of Physiology
Shri B. M. Patil Medical College, Hospital & Research Centre, BLDE (Deemed to be University), Vijayapur-586103.

2022

BLDE (Deemed to be University)
Vijayapura, Karnataka, India.

DECLARATION BY THE CANDIDATE

I hereby declare that this thesis entitled –**Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer** is a bonafide and genuine research work carried out by me under the guidance of Prof **Dr. Sumangala Patil**, Professor of Physiology, BLDE (Deemed to be University)'s Shri B M Patil Medical College, Hospital & Research Centre, Vijayapura, and Prof **Dr. Praveenkumar Shetty**, Professor of Biochemistry, K.S. Hegde Medical Academy, Nitte (Deemed to be University), Mangalore, Karnataka. No part of this thesis has been formed the bases for the award of any degree or fellowship previously. Shall have the rights to preserve, use and disseminate this dissertation/thesis in print or electronic format for academic/research purpose.

Signature of the candidate

Dr Vitthalsa Khode,
Registration no: 15PhD008
Department of
Physiology, BLDE
(Deemed to be
University),
Shri B. M. Patil Medical
College, Hospital and
Research Centre
Vijayapura, Karnataka.

Date:
Place: Vijayapura

BLDE (Deemed to be University) Vijayapura, Karnataka, India.

CERTIFICATE BY THE GUIDE

This is to certify that this thesis entitled **"Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer."** is a bonafide research work carried out by Dr Vitthalsa Khode under my supervision and guidance in the Department of Physiology, BLDE (Deemed to be University)'s Shri. B.M.Patil Medical College, Hospital and Research Center, Vijayapura, Karnataka, India. In the fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology.

Signature of the Guide

Dr. Sumangala Patil
Professor of Physiology, BLDE (Deemed to be University), Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka.

Date:
Place: Vijayapura

BLDE (Deemed to be University) Vijayapura, Karnataka, India.

CERTIFICATE BY THE CO-GUIDE

This is to certify that this thesis entitled **"Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer."** is a bonafide research work carried out by Dr Vitthalsa Khode under my supervision and guidance for the award of the Degree of the Doctor of Philosophy.

Signature of the Co-Guide

Dr. Praveen Kumar Shetty
Professor of Biochemistry,
Department of Biochemistry,
K.S.Hegde Medical Academy,
NITTE (Deemed to be University),
Deralakatte, Mangalore,
Karnataka.

Date:
Place: Vijayapura

BLDE (Deemed to be University) Vijayapura, Karnataka, India

DECLARATION BY THE CANDIDATE

I hereby declare that the BLDE, Deemed to be University's Shri B. M. Patil Medical College, Hospital and Research Center, Vijayapur Karnataka, shall have the rights to preserve, use and disseminate this declaration/thesis in print or electronic format for academic/research purpose.

Signature of the candidate

Dr Vitthalsa Khode,
Registration no: 15PhD008
Department of Physiology,
BLDE (Deemed to be University),
Shri B. M. Patil Medical College,
Hospital and Research Centre,
Vijayapura, Karnataka.

Date:
Place: Vijayapura

BLDE (Deemed to be University)
Vijayapura, Karnataka, India.

ENDORSEMENT BY THE HOD AND PRINCIPAL

This is to certify that this thesis entitled **"Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer**.**"** is a bonafide research work carried out by Mrs. Roshni Sadashiv under the supervision and guidance of Dr. Sumangala Patil (Guide), Professor of Physiology, BLDE (Deemed to be University)'s Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, Dr. Praveen Kumar Shetty (Co-Guide), Professor of Biochemistry, K.S. Hegde Medical Academy, Nitte (Deemed to be University), Mangalore, Karnataka.

Signature of the HOD

Dr. Sumangala Patil
Professor & Head,
Department of Physiology,
BLDE (Deemed to be University),
Shri B.M.Patil Medical College, Research Centre,
Vijayapura, Karnataka.

Date:
Place: Vijayapura

Signature of the Principal

Dr Arvind Patil
Principal,
BLDE (Deemed to be University),
Shri B. M. Patil Medical College, Hospital and Research Centre,
Vijayapura, Karnataka. Hospital and

Date:
Place: Vijayapura

With the blessings of Lord Almighty, I dedicate this work to My Teachers & My Beloved Family..

ACKNOWLEDGEMENT

First and foremost, I shall always be grateful to **BLDE (Deemed to be University)‟s** Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, and **SDM University‟s** Shri Dharmasthala Manjunatheshwara College of Medical Sciences and Hospital for giving me a platform to conduct my research work.

I would like to express my sincere gratitude to my mentors, **Prof Dr. Sumangala Patil** (Guide), Department of Physiology, BLDE (Deemed to be University)‘s Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka and **Dr. Praveen Kumar Shetty** (Co-Guide), Professor of Biochemistry, K.S. Hegde Medical Academy, Nitte University, Mangalore, Karnataka for taking me under their scientific supervision.

I would like to thank my guide **Prof Dr. Sumangala Patil**, for the encouragement, timely advice, suggestions and feedback throughout this research project. Without your constant support and guidance this would not be possible. Thank you for your valuable time and precious patience and willing to help in anyway throughout the project. I consider myself lucky to have been under your able guidance and learn from your research expertise.

I express my deepest gratitude to my patient and supportive Co-guide **Dr. Praveen Kumar Shetty**. Thank you for carefully monitoring my research work at every stage with great enthusiasm and dedication. Your guidance throughout the research project inspired me to think beyond my capabilities. Your overall insights and knowledge in this field have made a remarkable impact. You are my inspiration for life.

I am grateful to **Dr. R. S. Mudhol,** Vice Chancellor and former late **Dr. M. S. Biradar**, Vice Chancellor, BLDE (Deemed to be University), **Dr. J. G. Ambekar,** Registrar, BLDE (Deemed to be University), **Mr. Satish Patil,** Deputy Registrar, BLDE (Deemed to be University)‘s Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka for their valuable support and timely help.

I thank immensely **Dr. Arvind Patil**, Principal, BLDE (Deemed to be University), **Dr. Tejaswini Vallaba, Vice Principal**, Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, for providing an excellent research environment and facilities in the institute.

I am grateful to **Dr. Niranjan Kumar**, Vice Chancellor, SDM University, **Col. U.S. Dinesh**, Registrar, SDM University, **Dr. Rathnamala Desai,** Principal, SDM University, **Dr. Chidanand shettar**, Vice principal, SDM University for permitting and encouraging me to conduct the research work amidst the departmental activities. Thank you for your guidance throughout the tenure.

It was a great honor for me to have the privilege of associating and exchanging knowledge with **Prof Kusal K Das**, Department of Physiology/Vascular Physiology, BLDE (Deemed to be University)'s Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka. Thank you sir, for being my constant source of inspiration.

I thank **Dr. Nilima Dongre**, Professor, Department of Biochemistry, BLDE (Deemed to be University)'s Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, for her timely advice and keeping a constant check on our PhD six monthly report submissions.

I thank the **PhD committee faculty**, BLDE University for their valuable suggestions and inputs during the tenure of eight PhD reports which has helped me enhance the quality of my research work.

The work would not have progressed without the immunohistochemistry investigations. My heartfelt thank you to **Col. U. S. Dinesh**, Registrar, SDM University, and his team of pathologists for doing immunohistochemistry of our cases. I would be ever grateful to the participants accepting to be part of this academic and research endeavor.

I would like to express my gratitude to the staff of Dept. of Physiology (BLDE and SDM University) whose cooperation was always available when I needed it.

I thank **Dr. Anil Bargale PhD**, **Dr Vishwas Kaveeshwar PhD, Dr. Satish G.**

Patil PhD SDM University, for their timely help and expertise and **Mr. Mohd Shannawaz,** Statistician, BLDE (Deemed to be University)'s Shri B.M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, BLDE University for carefully assessing the statistical analysis of the research work.

I would really like to thank my fellow PhD students. Special thanks to **Mrs. Komal Ruikar, Mr. Anil Bargale, Dr. Roshni Sadashiv, Mr. Sharath** for helping me throughout in the accomplishment of my thesis.

Special thanks to Chief librarian, **Dr. Prasanna Kumar B. M, Dr. Manjula**, former Chief librarian, and **Mr Shivkumar Acharya** asst. librarian and all the library faculty of BLDE University, for their much needed support and help during thesis preparation and plagiarism checks.

I immensely thank all the laboratory staff and my dear students.

If ever I could turn this science in to a scientific art it is only because of the wide information and knowledge that I could gather by reading the enormous literature written by various **authors** in the field of cell and cancer biology, pathology, biochemistry & embryology. The various journals and reviewers who helped me strengthened my work. Thank you for being my mentors throughout my PhD.

I was given an opportunity to present my research papers in national conferences held in **JNMC, Belgaum**, and **JSS Musuru**, Karnataka. I thank the organizers of all state and national conferences for providing me a platform, exchange and share my knowledge.

Finally, but not at all the least, I would like to express my immense gratitude to my friends who have supported me through the bad times as well as the good, thank you for putting up with me for the last five years. I extend my gratitude to my family members and dear wife Komal for togetherness in this venture, Anvi and Sharvi my wonderful daughters who believes their parents are the best. Thank you Komal, Anvi and Sharvi to make me feel proud of myself every single day. Here I lay another beautiful feather of knowledge on my cap!!!!

INDEX

Contents	Page No
List of tables	v
List of figures	vi
List of abbreviations	viii
Abstract	xii
Chapter 1: Introduction	
Introduction	3
References	6
Chapter 2: Aims and Objectives	
2.1: Aims and objectives	10
2.2: Hypothesis	10
Chapter 3: Review of literature	
3.1 Breast cancer	12
3.1.1 Breast cancer overview	12
3.1.2 Facts & Figures	12
3.1.3 Differences in Population of Breast Cancer	17
3.1.4 Pathological Breast Cancer Types	19
3.1.5 Stages of Breast Cancer	19
3.1.6 Genes related to breast cancer	21
3.1.7 Risk factors for breast cancer	24
3.1.8 Molecular classification of breast cancer	28

3.1.9Available treatment options for breast cancer	29
3.1.10 Distribution of molecular subtypes of breast cancer	33
3.2.1Oral contraceptives and breast cancer	35
3.2.2 Changing profile of oral contraceptive use	36
3.2.3 Biological relations with oral contrceptives	37
3.2.4 Epidemiological Studies of Breast Cancer and Oral Contraceptives	38
3.2.5 "Ever" Use of Oral Contraceptives	39
3.2.6 Duration of Oral Contraceptive Use	40
3.2.7 Oral Contraceptive use before first full-term pregnancy or before age 25	42
3.2.8 Duration since first use of oral contraceptives (latency)	43
3.2.9 Steroidal Potency of Various Formulations	44
3.2.10 Issues to Consider in Reviewing the Epidemiological Evidence	45
3.3.1 Oncogenic intracellular signaling in breast cancer	47
3.3.2 EGFR signalling	49
3.3.3 Oncogenic activation of the epidermal growth factor receptor	50
3.4 Cycloheximide chase for evaluation of protein degradation	52
3.5.1 Ubiquitination a one of the pathway for degradation of proteins.	56
3.5.2 Rapid Removal of Proteins	58
3.5.3 Quality Control Mechanism	59
3.5.4 Ub Is Linked to Substrates through an Enzymatic Cascade	60
3.5.5 E3s Recognize the Cellular Proteins That Undergo Ub Conjugation	61
3.5.6 Most Cell Proteins Are Degraded by the 26S Proteasome	64

3.6.1 Proteasome Inhibitors and Cancer Therapy	67
3.6.2 Protease inhibitor- MG-132	68
3.6.3 Peptide aldehydes	69
References	72
Chapter 4. Methods	
I. Human study	101
4.1Type of study	101
4.2 Study design	101
4.3Duration of collection of data:	101
4.4Place of conduct of research:	101
4.5Study population:	101
4.6 Sample size calculation	102
4.7 Selection criteria	102
4.8 Statistical analysis	104
II. In-vitro study	105
4.1Cell culture	105
4.2 Effect of 17 β-estrdiol on MDA-MB-231 and MCF-7 cell lines on EGFR expression	105
4.3 Cycloheximide chase to assess the effect of 17 β-estradiol on MDA-MB-231 cells on EGFR expression	106
4.4 EGFR degradation is due to ubiquitination.	107
4.5 Total cell extraction and western blotting	108
4.6 Wound healing assay	109
4.7 Statistical analysis	110

Chapter 5. Results	112
5.1 Human study	112
5. 2 In vitro study- Results	120
5.2.1 Effect of 17β-estrdiol on MCF-7 and MDA-MB-231 cell lines on EGFR expression	120
5.2.2 Cycloheximide chase to assess the effect of β-estradiol on MDA-MB-231 cells on EGFR expression	121
5.2.3 Cycloheximide chase to assess Ubiquitin mediated EGFR degradation.	121
5.2.4 Wound healing assay	124
References	125
Chapter 6. Discussion	128
6.1 Human study	128
6.2 In-vitro study	132
References	137
Chapter 7: Summary and Conclusion	143
Chapter 8: Annexure	145
Informed consent form	
Proforma	
Plagiarism Certificate	
Ethical clearance certificate	
Paper presentation certificates	
Publications	

LIST OF TABLES

Sl. No	Particulars	Page No
1.	Prevalence of molecular subtypes of breast cancer in different parts of the world.	24
2.	Demographic characters, parity and Age of menarche (AOM) of breast cancer patients in OCP users and non-users	132
3.	Reproductive history, family history of breast cancer (FHBC) and stage of breast cancer patients in OCP users and non-users.	134
4.	Phenotype/ clinical sub- types status in OCP users and non-users in breast carcinoma	135
5.	Age in Phenotypic sub types/ clinical sub types of breast carcinoma in OCP users and non-users.	137
6.	Logistic regression analysis of adjusted effect of age on selected parameters in OCP users and non-users	138

List of figures

Sl. No	Particulars	Page No
1.	Top sites of cancer in world population in 2020	13
2.	Estimated number of New cases of cancers in India in	14
3.	Estimated number of cancer Deaths in India in Females.	14
4.	A schematic diagram of risk factors is depicted in a pyramid-style structure	24
5.	Estrogen activates nuclear ER (genomic pathway) and ER in or near the membrane (non-genomic pathway).	50
6.	EGFR downstream signalling in cancer.	51
7.	Example showing to how cycloheximide chase is executed.	55
8.	Ubiquitin Proteasome Pathway	57
9.	The diagram shows different pathways of degradation of EGFR.	59
10.	The diagram shows different pathways of cullin-RING ligases are the Skp1–Cul1–F-box (SCF) complexes in degradation of EGFR.	62
11.	Diagram showing the mechanism of action of MG-132 inhibiting ubiquitination.	71
12.	Study design	103

13. A	(A) Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+ in total number of cases.	114
13. B	Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2+ enriched and Basal like (BA)/TNBC in total number of cases.	114
14.	Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+) in OCP users, non-users and total number of cases.	116
15.	Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2 enriched and Basal like (BA)/TNBC in OCP users, nonusers and total number of cases.	116
16.	Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+) in OCP users, non-users and total number of cases. *($p<0.05$);	119
17.	Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2 enriched and Basal like (BA)/TNBC	119
18A1	A1. Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours.	120
18A2	Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours.	120
18B1	Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours.	120
18B2	Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours.	120

19 A1	Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours.	122
19 A2	Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours.	122
19 B1	Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours.	122
19 B2	Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours.	122
20A1	Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1,2, 3 and 4 hours.	123
20 A2	Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1, 2, 3 and 4 hours.	123
20 B1	Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide +MG-132at 0, 0.5, 1,2, 3 and 4	123
20 B2	Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide + MG-132at 0, 0.5, 1,2, 3 and 4	123
21.	Effect of 100 nM 17β-estradiol on MDA-MB-231cellline with 100 nM of E2 on wound healing.	124

List of abbreviations

AKT	Ak strain transforming murine thymoma viral oncogene
ANOVA	Analysis of variance
AOM	age of menarche
AP-2	Adaptor protein 2
AS	Allred score
ASCO	American Society of Clinical Oncology
BA	Basal
BCA	bicinchoninic acid assay
BMI	Body mass index
BMI1	B lymphoma moloney murine leukaemia virus insertion region-1
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
CAP	American Pathologists
CASH	Cancer and Steroid Hormone
Cbl-gene	Casitas B-lineage Lymphoma
c-Cbl,	C-cbl Proto-oncogene Product
c-erbB-1	HER 1
CFTR	transmembrane conductance regulator protein
CHEK2	checkpoint kinase 2
CHIP	C-terminal of Hsp70-interacting protein
CHX	Cyclohexamide

CI	Confidence interval
DCIS	ductal carcinoma in situ
DMC1	DNA Meiotic Recombinase 1
DMEM	Dulbecco's modified Eagle's medium
E2	17β-estradiol
E3	Ub-protein ligase
E6-AP	E6-associated protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	epithelial mesenchymal transition
ER+	Estrogen Receptor
Erb2	Erythroblastic oncogene B, also Her2
Erα	Estrogen receptorα
ERβ	Estrogen receptor β
Esp15	Epidermal growth factor receptor substrate 15
FBS	Fetal Bovine Serum
FFTP	first full-term pregnancy
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Grb2	Growth factor receptor-bound protein 2
GTP	Gguanosine triphosphate
HECT	homologous to E6-AP carboxy-terminus)
HER2/neu	human epidermal growth factor receptor 2

HIF-1	hypoxia-inducible factor 1
HR	hazard ratio
H-ras,	Harvey Rat sarcoma
HRT	hormone replacement therapy
IARC	International Agency for Research on Cancer
IBC	inflammatory breast cancer
IHC	immune-histo-chemical
IS	intensity score
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JNK	c-Jun N-terminal kinase
K-ras	Kirsten Rat sarcoma
LA	Luminal A
LB	Luminal B
LCIS	lobular carcinoma in situ
LKB-1	liver kinase B1 (LKB1)
MAFbx	Muscle Atrophy F-box
MAPK	mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7,
MD-AMB-231	MD Anderson-Metastatic Breast-231
Mdm2	Mouse double minute 2 homolog
MDR	multi-drug resistance
MEK	kinase/MAP kinase-ERK

MG-132	Proteosome inhibitor
MHC	major histocompatibility complex
mTORC1	mammalian target of rapamycin complex 1
MuRF-1	muscle ring finger-1
MVB	multi-vesicular bodies
NCI	Program of the National Cancer Institute
NCRP	National Cancer Registry programme, India
NF-γB	nuclear factor kappa-light-chain-enhancer of activated B cells
NL	Non Luminal)/HER2+ enriched
NP-40	Nonidet P-40
N-ras,	neuroblastoma Rat sarcoma
OCP	Oral contraceptive pill
OR	odds ratio
p27Kip1	cyclin-dependent kinase inhibitor
PI3K	Phosphoinositide 3-kinases
PKA	protein kinase A
PR	Progesterone receptor
PR+	Progesterone receptor
PS	proportion score
Raf	Rapidly Accelerated Fibrosarcoma.
RGS4	regulator of G-protein signaling 4
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media

RR	relative risk
RTK	Receptor tyrosine kinases
RT-PCR	Real time polymerase chain reaction
SCF	Skp1–Cul1–F-box
SEER	Surveillance, Epidemiology, and End Results
SPSS-20	Statistical Package for the Social Sciences
STAT	Statistical Package for the Social Sciences
TGF-α	Tumor growth factor alpha
TNBC	Triple negative breast cancer
TP53	tumor protein 53
TRCP	transducin repeat containing protein
Ub	Ubiquitin
UFD2	Ub fusion degradation protein 2
UICC	Union Internationale Contre le Cancer
UPP	ubiquitin–proteasome pathway
VBC	VHL-elongin BC
VHL	Von HippelLindau
WHO	World Health Organization

ABSTRACT

Background: Triple negative breast carcinoma (TNBC) is a breast cancer sub-type associated with high mortality rate and inadequate therapeutic options. Clinical data indirectly implicates where Oral Contraceptive Pill (OCP) usage is high, prevalence of Estrogen Receptor+ (ER+) breast cancer is high and prevalence of TNBC is low. This has lead to our hypothesis that OCP use may add to risk of ER+ breast cancer and OCP use may reduce the risk of TNBC. In in-vitro study we tried to differentiate the effect of estrogen on development of ER+ and triple negative breast cancer tumor affecting Epidermal growth factor receptor EGFR expression in respective cancer cell lines as TNBC commonly displays EGFR. It is known that effective EGFR degradation results in suppression of tumor in various models.

Aims and Objectives: We aimed at to comparing the prevalence and association of sub-types of breast cancer in OCP users and OCP non-users among woman 30 to 60 years of age, and in-vitro study we aimed at treating MDA-MB-231 cell lines with Cycloheximide with or without 17β-estrdiol to observe whether 17β-estradiol leads to EGFR degradation. We also aimed at whether degradation occurs through ubiquitination pathway.

Methods : This hospital-based observational human study of three year duration included 155 subjects of primary invasive breast cancer who got admitted at our institution. The data was obtained for ER, PR, HER2 condition, clinical classification and data related to demographic factors, reproductive history, and history of OCP use. They were divided into two groups. Group-1 included 48 patients with history of OCP use and group-2 included 107 patients who did not use OCP. In in-vitro study MDA-MB-231 cells were treated with 17β-estradiol (E2) and EGFR expression was evaluated by western blotting at different intervals by using Cycloheximide chase. To gauge ubiquitination pathway of degradation of EGFR in the MDA-MB-231 cell line, MG-132 was utilized. Data was analysed using SPSS-20.

Results: A significant increase in prevalence of molecular sub-types ER+, Progesterone Receptor+ (PR+) and Luminal B breast cancers in OCP users was

observed compared to non-users. There was considerable decrease in the age at the point of admission in ER+ cancer in OCP users (45.3 years) compared to non-users (52.2years). Whereas in OCP users age at the time of admission of Basal (TNBC) cancer patients (53.1 years) was higher when compared to non-users (45.4years). Logistic regression revealed the likelihood of ER+, PR+ and Luminal B in OCP users was 11%,10% and 13% less respectively with 1 year of higher age against the likelihood of TNBC among OCP users was 18% more and 8% less in non-users. In in-vitro study EGFR expression was reduced with β-estradiol treatment in MDA-MB-231 cell line with Cycloheximide chase. Upon Treatment with MG-132 and E2, EGFR expression did not reduce suggestive of that Estrogen degrades EGFR by ubiquitination pathway.

Conclusions: OCP use may be allied with increase in the prevalence of ER+, PR+ and Luminal B breast cancer. On the contrary OCP use is may be related with delay in the progression of the TNBC. In-vitro study conclusion was that estrogen degrades EGFR in MDA-MB-231 cells and this degradation occurs by ubiquitination.

Key words: oral contraceptive pill, triple negative breast cancer, MDA-MB-231,estrogen, epidermal growth factor receptor, MG-132

IMPLICATION OF ORAL CONTRACEPTIVE USE TO PHENOTYPIC EXPRESSION PATTERN OF RECEPTORS IN BREAST CANCER

Chapter 1

Introduction

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

1. Introduction

Breast cancer (BC) is the most common etiology for cancer death among women and the most frequently diagnosed cancer among women in 140 of 184 countries worldwide. It currently represents one in four of all cancers in women. BC is the most common cancer in women in India, in both males and females combined, in cities in India and in rural areas of India as well.

Breast cancer is a heterogeneous disease with different clinical, pathological, and molecular features. Expression patterns and immune-histo-chemical markers can differentiate BC subtypes and expected to reflect important differences in pathogenesis and aetiology [1-2]. Epidemiologic studies strongly suggest that Estrogen Receptor positive (ER+), Progesterone Receptor positive (PR+), Human Epidermal Growth Factor Receptor 2 positive **(**HER2+) and triple-negative breast cancers (TNBCs) are distinct entities that the etiologic factors, clinical characteristics, and therapeutic possibilities may vary by molecular subtypes [3-5]. Several investigations propose that reproductive factors and exogenous hormone especially estrogen use differently or even quite inversely affect the risk of ER+ and TNBC [6-8]. Estrogen was used in the treatment of breast cancers in the past. Meta-analysis has shown that obstetric history and Oral Contraceptive pill (OCP) intake increases risk of both ER+ and TNBC [9]. The role of some of the risk factors in the development of ER+ cancer is clear. Controversies concerning the precise role of risk factors in TNBC development and biological mechanisms behind the commencement of TNBCs are completely obscure. TNBC is clinically challenging type of breast cancer which occurs more frequently in younger women (<50 years) and Asian women and is related to significant aggressiveness as compared with other subtypes of BC.

Oral contraceptive use may promote or initiate tumours of the breast. The risk may be even greater for women due to family history of cancer or genetic mutation carrier status. However, overall results from studies are inconclusive. The association between OC use and breast cancer risk can be variable in different molecular subtype, specifically by joint estrogen receptor (ER), progesterone receptor (PR), HER2-neu (HER2) and TNBC (Triple Negative Breast Cancer) status. More than 90% of TNBC tumors are considered within the basallike subgroup, so called for its gene expression profile that imitates basal epithelial cells in other parts of the body and a characteristic morphology which includes high proliferative rate, a pushing border and central necrosis. Basal-like BC is associated with aggressive histology, poor prognosis, unresponsiveness to typical endocrine therapies, and is BRCA1-related BC. Even though triple-negative breast cancer is of growing attention in the clinical and research community, its etiology remains understudied. Western population which is considered socio-economically of higher status, OCP use is high. May be because of this they have more preponderance develop ER+ breast cancer and less preponderance to develop TNBC. African and Asian population have less preponderance to develop ER+ and more preponderance to develop TNBC. The reason could be here less use of OCP [10-12]. ER+ breast cancer development is influenced by estrogen via Estrogen Receptor α (ERα) receptors through independent mechanisms. Acting through ERα, it stimulates cell proliferation and initiates mutations that occur as a function of errors during DNA replication. But, estrogen has the contrary effect in TNBC when Estrogen Receptor β (ERβ) is there in excess. There are evidences estrogen decreases the proliferation of TNBC by non-genomic action [13]. These cumulative effects could be reason why western population has less number of TNBC. While the relationship between OCP use and BC risk has been extensively studied, the subject

remains an important research area as there are several key unanswered questions. There is scarceness in the studies whether OCP use associated with increased/reduced risk of especially with TNBC in Indian population particularly in younger age group. Addressing these issues is of public health importance given the high prevalence of use OC among Indian women and the greater aggressiveness of breast cancer in younger women especially Triple negative breast cancer. Therefore, in order to characterize the association between OCP use, and risk of different breast cancer subtypes among Indian young women, since these biological subtypes of breast cancers have therapeutic implication, we hypothesized that and there will be preponderance of ER+ breast cancer in subjects exposed to OCPs prior and there will be decrease in the prevalence in TNBC subjects exposed to OCP. Hence we aimed at this hospital based prospective observational study among women of younger age group (30-60 years) having different molecular subtypes of breast cancer. We also tried here to differentiate the effect of estrogen on development of ER+ and TNBC tumor utililising respective cancer cell lines.

References

1. Sorlie T, Tibshirani R, Parker J, Trevor Hastie, J S Marron, Andrew Nobel, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proceedings of the National Academy of Sciences of the United States of America 2003;100:8418–23.

2. Sotiriou C, Neo SY, McShane LM, Edward L Korn, Philip M Long, Amir Jazaeri,et al. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proceedings of the National Academy of Sciences of the United States of America 2003;100:10393–8.

3. Trivers KF, Lund MJ, Porter PL, Jonathan M Liff, Elaine W Flagg, Ralph J Coates, et al. The epidemiology of triple-negative breast cancer, including race. Cancer Causes Control. 2009;20:1071–1082.

4. Gaudet MM, Press MF, Haile RW, Charles FL, Sally LGr, Joellen S, et al. Risk factors by molecular subtypes of breast cancer across a population-based study of women 56 years or younger. Breast Cancer Res Treat. 2011; 130:587–597.

5. Yang XR, Sherman ME, Rimm DL, Jolanta L, Louise AB, Beata P, et al. Differences in risk factors for breast cancer molecular subtypes in a population-based study. Cancer Epidemiol Biomarkers Prev. 2007; 16:439–443.

6. Ma H, Wang Y, Sullivan-Halley J, Linda W, Polly AM, Robert S, et al. Use of four biomarkers to evaluate the risk of breast cancer subtypes in the women's contraceptive and reproductive experiences study. Cancer Res. 2010; 70:575–587.

7. Yang XR, Chang-Claude J, Goode EL, Fergus JC, Heli N, Roger LM, et al. Associations of breast cancer risk factors with tumor subtypes: a pooled analysis from the Breast Cancer Association Consortium studies. J Natl Cancer Inst. 2011; 103:250–263.

8. Palmer JR, Boggs DA, Wise LA, Ambrosone CB, Adams-Campbell LL, Rosenberg L. Parity and lactation in relation to estrogen receptor negative breast cancer in African American women. Cancer Epidemiol Biomarkers Prev. 2011; 20:1883–1891.

9. Li li, YahuaZhong, Hongyan Zhang, Haijun Yu, Yong huang,zheng Li, Gaili Chen. et al. Association between oral contraceptive use as a risk factorand triple-negative breast cancer: A systematic review and meta-analysis. molecular and clinical oncology.2017; 7: 76-80.

10. Warner ET, Tamimi RM, Boggs DA, Rosner B, Rosenberg L, Colditz GA, et.al. Estrogen receptor positive tumors: do reproductive factors explain differences in incidence between black and white women? Cancer Causes Control. 2013; 24(4):731–9.

11. Rosato V, Bosetti C, Negri E, Talamini R, Dal Maso L, Malvezzi M, et.al, Reproductive and hormonal factors, family history, and breast cancer according to the hormonal receptor status. 2013. Eur J Cancer Prev.

12. Phipps AI, Buist DS, Malone KE, Barlow WE, Porter PL, Kerlikowske K, et.al, Reproductive history and risk of three breast cancer subtypes defined by three biomarkers. Cancer Cause Control. 2011; 22(3):399–405.

13. Y. Yan, X. Li, A. Blanchard H, C. Bramwell, K. I. Pritchard, , et al., Expression of both estrogen receptor-beta 1 (ER-β1) and its co-regulator steroid receptor RNA activator protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with estrogen receptor-alpha (ER-α)-negative early breast cancer (EBC). Annals of Oncology, 2013; 24(8): 1986–93.

Chapter 2

Aims and objectives

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

2.1 Aims and objectives

1. To evaluate whether oral contraceptive pill (OCP) use in woman associated with prevalence specific molecular subtypes of breast cancer.

2. To demonstrate effect β-estradiol on MCF-7 (Estrogen Receptor +) and MDA-MB-231 (Triple Negative Breast Cancer) cell lines on Epidermal Growth Factor Receptor (EGFR) expression.

3. To demonstrate whether degradation of EGFR by β-estradiol in MDA-MB-231 cell line is mediated through ubiquitination pathway.

2.2 Research Hypothesis

We hypothesized that and there will be preponderance of ER+ breast cancer in subjects exposed to OCPs prior and there will be decrease in the prevalence in TNBC subjects exposed to OCP.

We hypothesized estrogen is involved in this mechanism of upgradation of EGFR in MCF-7 cell line and degradation of EGFR in MD-AMB-231 cell nine

We hypothesized that β-estradiol facilitates the degradation of EGFR in MD-AMB-231 cell line by facilitating ubiquitination process.

Chapter 3

Review literature

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

3. Review of literature

3.1 Breast cancer

3.1.1 Breast cancer overview

Breast cancer (BC) represents 1 in 4 cancers diagnosed among women globally. Colorectal, lung, cervical, and thyroid cancers are also common among women. Lung cancer and prostate cancer are the most frequent among men, accounting for nearly one-third of all male cancers. For the first time, female breast cancer has turn out to be the most commonly diagnosed cancer, surpassing lung cancer, in particular due to high prevalence in low and middle income countries. Lung cancer remains the leading cause of cancer deaths, not only in many low and middle income countries but also in most higher-income regions like North America, Europe and Australia.

3.1.2 Facts & Figures

3.1.2a Humans are comprised of millions of cells approximately 3.72×10^{13}. Some cells are specific to certain tissues, for instance, epithelial cells are found throughout the gastrointestinal tract, bladder, lungs, vagina, breast and skin. This group of cells accounts for approximately 70% of cancers, one of these epithelial cancers is breast cancer that shares 43.3% of total age standardized rate in females in the world.

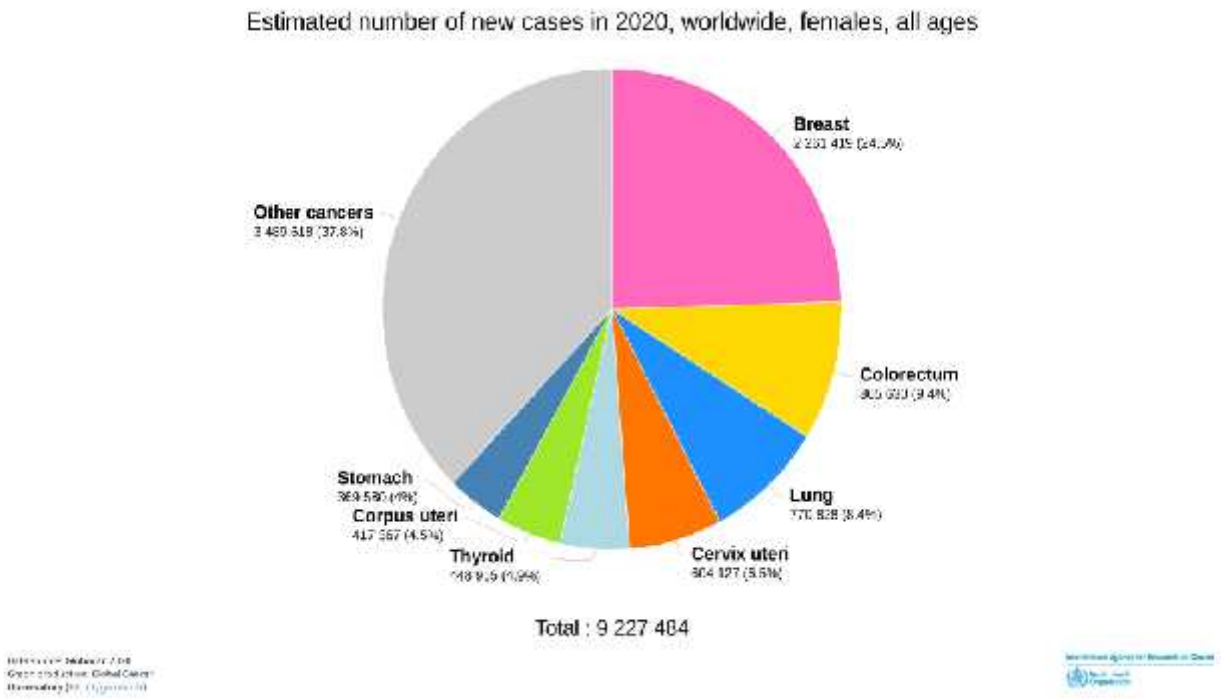


Figure-1: Top sites of cancer in world population in 2020 (Source GLOBOCAN 2020, IARC)

IARC released on 14th December the updated version Globocan 2020 with new estimates on the global cancer burden, representing that it has risen to 19.3 million cases and 10 million cancer deaths in 2020. The International Agency for Research on Cancer (IARC) approximates that globally, 1 in 5 people develop cancer at some stage in their lifetime. 1 in 8 men and 1 in 11 women breathe their last breath from the disease. These new estimates imply that more than 50 million people are living within five years of a past cancer diagnosis. Ageing populations globally and socio-economic risk factors remain among the primary factors driving this increase [1] (Fig 1).

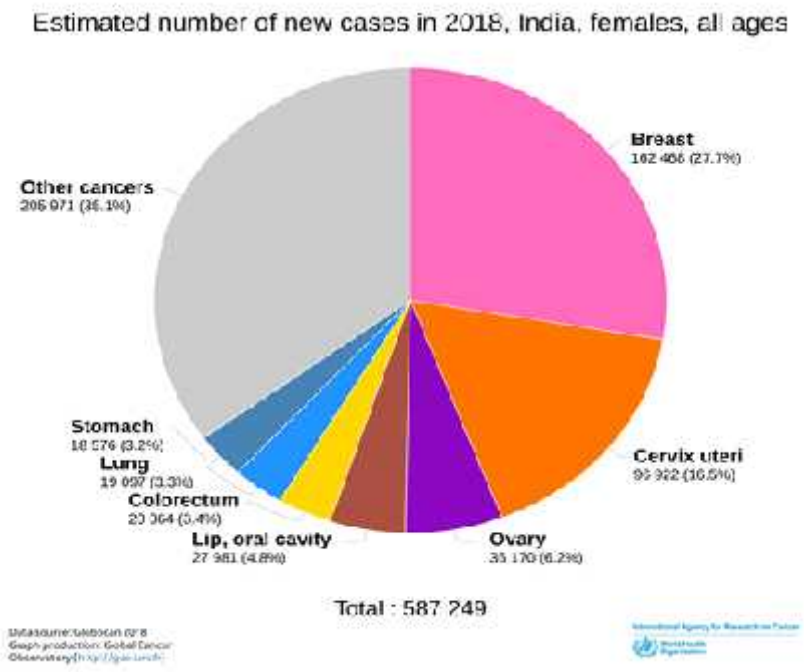


Figure-2: Estimated number of new cancer cases in India in Females.

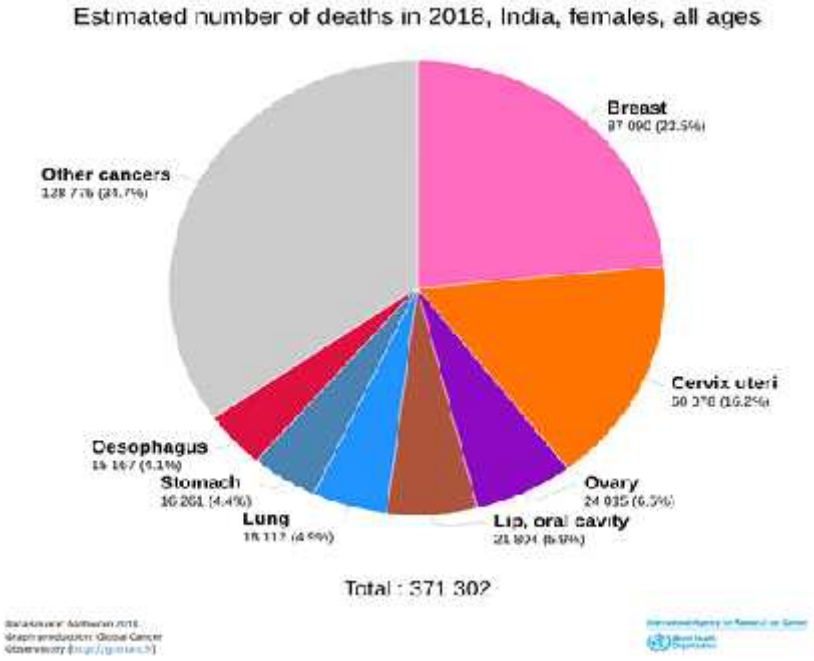


Figure-3: Estimated number of cancer Deaths in India in Females.

BC is the most familiar reason of cancer in women in India, in both males and females combined, in cities in India and in rural areas of India as well. There are a range of sources of statistics for BC in India. The statistics and images shown here are all from Globocan 2018 (Globocan belongs to IARC - International Agency for Research on Cancer). They contain data from NCRP (National Cancer Registry programme, India) as well, but as the last publication of NCRP data is 2014, they have represented data from Globocan, that is for the year 2018. The BC incidence has been rising especially in the lower age groups [2].

Incidence means the numbers of women diagnosed with BC in that particular year. The graphs represent data for the year 2018. Therefore the numbers in 'Incidence' correspond to the number of women who were newly detected with BC for the year 2018 (Fig-2).

Mortality (Death) defines the numbers of women who died of BC in that particular year, and is represented by the pink color in the graph. In the graph, the mortality numbers have been highlighted (Fig-3).

3.1.2b Breast Cancer in 25-49 years age group

In India, for the year 2018: 61,264 out of projected total 186,965 cases of cancer were due to BC, for the age group of 25-49 years in women. BC accounted for **32.8%** of all cancer cases in women in India for that year 2018, for that particular age group. Roughly, that translates into one third of all cases of cancer in the age group of 25-49 years, being due to BC. 21,892 women died of BC for this age group cancer of the cervix and that of the ovary are the second and third most common cancers in this particular age group. These three account for more than 60 percent cancers in women

in 25-49 age groups in India. Subsequently, the BCs in 25-49 years age group accounted for 37.70% of all BC in women in India, for the year 2018 [2].

3.1.2c Breast Cancer in 50-69 years age group

In women in India, for the year 2018: 75,574 out of projected total 271,076 cases of cancer, were due to BC, for the age group of 50-69 years. BC accounted for **27.9%** of all cancer cases in women in India for that year 2018, for that particular age group. Roughly, that translates into a little more than one fourth of all cases of cancer in the age group of 50-69 years, being due to BC. 44,051 women died of breast cancer for this particular age group. Cancer of the Cervix and Cancer of the Ovary are the second and third most frequent cancers in this age group. These three account for about 50 percent of cancers in women among 50-69 age groups [2].

3.1.2d Breast Cancer in the age group of 70 and above

In women in India, for the year 2018: 25,056 out of projected total 107,165 cases of cancer, were due to BC, for the age group of 70 and above. BC accounted for **23.4%** of all cancer cases in women in India for that year 2018 for that particular age group. Roughly, that translates into a little less than one fourth of all cases of cancer in the age group of 70 and above, being due to BC. 20,938 women died of BC for this age group. Cancer of the Cervix and Cancer of the Lip and Oral Cavity are the second and third most frequent cancers in this particular age group [2].

BC is fairly familiar in the younger age group (25-49 years), accounting for almost **37.7%** of all cases, that is a pretty high number. BC peaks in the age group of 50-69 which accounts for almost **46.5%** of all cases and then starts reducing in the age group of 70 years and above. It may not actually be reducing; however it has

something to do with life expectancy. In women, the maximum possibility of developing any cancer are in the 50-69 years age group, that has the highest number of cancer overall. In younger and middle age group, ovarian cancer is third most frequent, while in the age group of 70 and above, its frequency reduces. Combining the age group of 25-49 and 50-69, BC seems to be more common in the latter half of 25-49 and earlier half of 50-69 which means the 40-60 years age group. Accordingly we must target this age group for education and breast awareness as well as for learning on regular screening mammography.

It is important to understand that BC can occur in the younger population as well. We saw that 37.7% of all newly detected BC cases were in the 25-49 years age group. For most, BC is an illness of the elderly, nevertheless it is not so. Also, many young BCs tend to be very aggressive and have to be tackled in proper succession of surgery and other treatments, and needless to say, early detection will indefinitely give soaring cure rates.

3.1.3 Differences in Population of Breast Cancer

Breast cancer variation which exists among different population, or the regional differences in the types have been attributed to the following factors; Prevalence of major risk factors, availability and quality of treatment, availability and use of medical practices such as cancer screening, completeness of reporting, and age. However, geographic areas, and counties within countries also determine the occurrence of the most commonly diagnosed cases BC or deaths [3]. The highly penetrant but rare susceptibility genes, BRCA1 and BRCA2 [4] and more prevalent, but lower penetrance genes, CHEK2 and EGFR [5] have been sugested to be the key inter-individual and inter-group differences in the distribution of risk among breast

cancer patients. Countries with massive economic growth over the past 40-50 years, such as Japan, Singapore, and urban areas of China have experienced an increase in breast cancer incidence [6]. Age-standardized occurrence rates for BC 1998–2002 were 110 (non Hispanic Caucasians, California), 82.3 (Ontario, Canada), 41.3 (Hong Kong) and 14.7 (Jiashan, China) [7]. Reports on migration studies reveal that the incidence of breast cancer changes significantly over one to two generations to more closely reflect the breast cancer risk in the adopted country [8], which seems to occur in parallel with dynamics in diet and certain indicators of acculturation [9]. Notably, evaluation of differences in risk factors and natural history of all breast tumor types would permit for comparisons based on geographical regions, socioeconomic status and levels of industrialization [10]. Other differences in population of breast cancer are outlined in studies conducted: In a study by Li et al [11], it was publicized that the majority of breast tumours from Asian women are estrogen receptor (ER) negative. Also it has been indicated that both pre-and postmenopausal Asian women with breast cancer, are likely to have ER positive tumors as Caucasians [12]. ER positivity among premenopausal breast cancer cases was greater when compared with the comparison group of Caucasian women in Australia in a Vietnamese cohort. [13]. Variation in the gene profiles of tumors from populations of different genetic/ethnic backgrounds have also been reported which was considerable. About 15% of sporadic breast cancer, which are BRCA1 origin in Caucasian women, appears to have the basal phenotype. On the other hand, studies have also suggested that breast cancer in women of African descent may have a higher proportion of basal phenotype. In similar manner a study among Nigerians shown a high frequency of basal like tumors, where 87 of 148 (59%) breast cancer patients were both ER- and HER2-[14].

3.1.4 Pathological Breast Cancer Types

Each breast has 15-20 sections called lobes, which have numerous smaller sections called lobules. The lobes and lobules are connected by slim tubes, called ducts. On the basis of this anatomical difference, breast cancer can broadly be classified into two major types (a) ductal (b) lobular. The most common type of BC is ductal cancer. The disease can also be classified on the basis of extent of spread, as non-invasive and invasive. The term non-invasive refers to cancer that has not spread past the area where it initially developed, whereas invasive term refers to the spread (metastasis) of the disease to other tissues. Other uncommon types of breast cancer are inflammatory breast cancer, Phyllodes tumor and Paget disease of the nipple. Some special types of breast cancer are medullary carcinoma, mucinous carcinoma, tubular carcinoma, etc.

3.1.5 Stages of Breast Cancer

The staging systems currently in use for describing severity of breast cancer are based on the clinical size and extent of invasion of the primary tumor (T), the clinical absence or presence of palpable axillary lymph nodes and confirmation of their local invasion (N), together with the clinical and imaging evidence of distant metastases (M). This is then converted into the TNM classification. It has been subdivided into following five stages by UICC (Union Internationale Contre le Cancer).

Stage 0: Carcinoma in situ lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS)

Stage I: Early stage of breast cancer where the tumor is <2 cm across and has not spread beyond the breast.

Stage II: Early stage of breast cancer where the tumor is either < 2 cm across and has spread to the lymph nodes under the arm; or the tumor is between 2-5 cm (with or without spread to the lymph nodes under the arm); or the tumor is > 5 cm and has not spread outside the breast.

Stage III: Locally advanced breast cancer where the tumor size is >5 cm across and has spread to the lymph nodes under the arm; or the cancer is widespread in the underarm lymph nodes; or the cancer has spread to lymph nodes near the breast bone or to other tissues near the breast tissue.

Stage IV: Metastatic breast cancer where the cancer has spread outside the breast tissue to other organs in the body.

TNM or UICC classification is less often used since it does not incorporate many biological variables that affect the progress of disease. Knowledge about such variables is increasingly widened now, so a pragmatic approach to classify patient according to the treatment they require is essential.

3.1.6 Genes related to breast cancer

Many genes have been identified in relation to breast cancer types. Mutations and abnormal amplification of both oncogenes and anti-oncogenes play vital roles in the processes of tumor initiation and development.

3.1.6a BRCA1/2

BC associated gene-1 and -2 (BRCA1 and BRCA2) are two most important anti-oncogenes for BC risk. BRCA1 and BRCA2 are situated on chromosome 17q21 and 13q12, respectively. Both of them encode tumor suppressor proteins. Dysregulation of cell cycle checkpoint, abnormal centrosome duplication, genetic instability and eventually apoptosis [15, 16] are caused by BRCA1 deficiency. BRCA1 expression is repressed by “pocket proteins” such as p130, p107 and the retinoblastoma protein in an E2F-dependent method. The BRCA1 gene has been linked to the formation of a loop between the promoter, introns, and terminator regions, which regulates this gene via interactions with its own promoter [17, 18]. BRCA2 protein by interacting with RAD51 and DMC1, regulates recombinational repair in DNA double-strand breaks [19,20]. BRCA2-associated BCs are likely to be high-grade invasive ductal carcinomas, but with a luminal phenotype [21].

BRCA1/2 mutations are inherited as an autosomal dominant manner even though the second allele is normal. The risk of breast cancer increased greatly if an individual inherited harmful mutations in either BRCA1 or BRCA2 genes. About 20-25% of hereditary breast cancers and 5-10% of all breast cancers are caused by BRCA1/2 mutations [22, 23]. Chen et al in their meta-analysis showed that the breast cancer risk ratio (RR) in women older than 70 years carrying BRCA1 or BRCA2 mutations was 57% and 49%, respectively [24].

3.1.6b HER2

Human epidermal growth factor receptor 2 (HER 2), also called as c-erbB-2, is an important oncogene in BC and is located on the long arm of human chromosome 17 (17q12). The homologene in mice is Neu, which was first to be identified in 3-methylcholanthrene induced rat neuroblastoma cells [25]. The expression of HER-2 gene is activated primarily through the gene amplification and re-arrangement. HER2 protein is an EGFR of tyrosine kinase family and form heterodimers with other ligand-bound EGFR family members such as HER-3 and HER-4, to activate downstream signaling pathways [26]. Knockout of HER-2 in mouse models shown it disrupts normal mammary duct formation. Overexpression of HER-2, which is detected in about 20% of primary breast cancers, amplifies the number of cancer stem cells by TEN/Akt/mTORC1 signaling, and indicates poor clinical outcomes [27, 28].

3.1.6c Epidermal Growth Factor Receptor (EGFR)

EGFR is also known as c-erbB-1 or HER-1 in humans. It is located on the short arm of chromosome 7 (7p12). The EGFR protein is a cell surface glycoprotein of tyrosine kinase family and it is activated by binding to EGF, amphiregulin, betacellulin, TGF-α and so on. The downstream signaling pathways of EGFR including PI3K, JNK and Ras-Raf-MAPK are activated or triggered to promote cell proliferation, cell invasion, angiogenesis and to protect cells that not in favor of apoptosis [29, 30]. Overexpression of EGFR is associated with in more than 30% of cases of the inflammatory breast cancer (IBC), a aggressive subtype of breast cancer. Cases with EGFR-positive IBC have a poorer prognosis than those with EGFR-negative tumors [31, 32]. More than half of triple-negative breast cancer (TNBC) patients, characterized by the absence of estrogen receptor (ER), progesterone receptor (PR)

expression and HER2 amplification, also have EGFR overexpression [33]. Therefore, targeting the EGFR pathway and their downstream signaling pathways might be a promising therapy for these malignant tumors.

3.1.6d Ras

There are three members exists in the Ras gene family: H-ras, K-ras and N-ras. They are located on the chromosome of 11 (11p15), 12 (12p12) and 1 (1p22) respectively. The proteins encoded by these genes are extremely homologous in nature, and they belong to the small guanosine triphosphate (GTP)-binding protein superfamily [34]. Point mutations are commonly linked with the over expression of these three human Ras genes, and most of these are mis-sense mutations located at the coding domain for GTP binding site. Though mutations of Ras proteins are infrequently in BC (less than 5%), the abnormality of Ras signal transduction pathway are observed in both the types of benign and malignant mammary tissues [35]. H-ras can be associated with B lymphoma moloney murine leukaemia virus insertion region-1 (BMI1) to facilitate proliferation, invasion, and to inhibit apoptosis in breast cancer cells [36]. H-ras overexpression is detected in both the primary and advanced breast cancer cases, signifying a poor prognosis [37, 38]

3.1.7 Risk factors for breast cancer

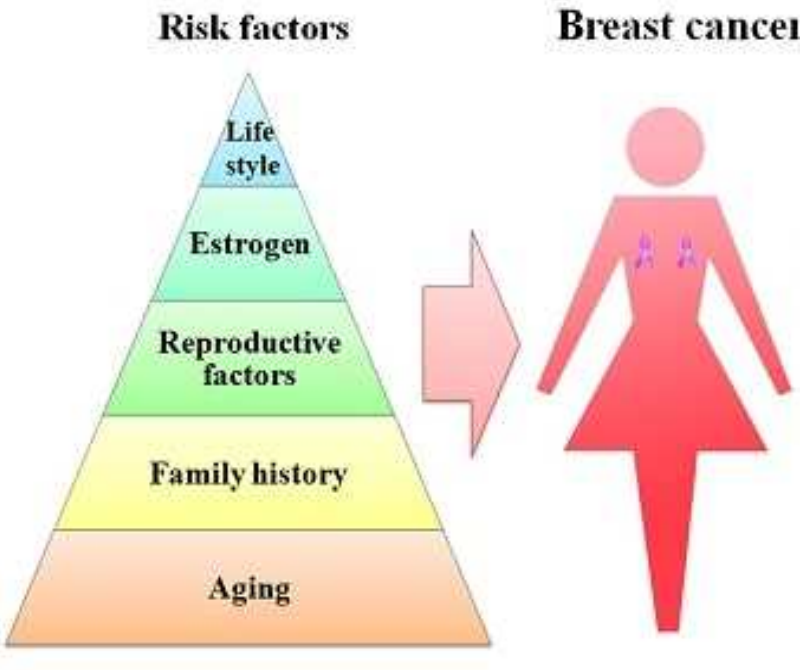


Figure 4: A schematic diagram of risk factors is depicted in a pyramid-style structure.

3.1.7a Aging

Aging is one of the most important risk factors of breast cancer beside gender, because the incidence of breast cancer is highly related to the increasing age. In 2016, deaths of all breast cancer in America reported in women over the age of 40 and 60, were approximately 99.3% and 71.2% respectively. Therefore, it is necessary to have a mammography screening in advance in women aged 40 or older.

3.1.7b Family history

Nearly a one fourth of all breast cancer cases are related to family history of BC [39]. Women, whose sister or mother has a breast cancer, are prone to this disease. A cohort study of over 113,000 women in UK has shown that women with one first-degree relative with BC will have a 1.75-fold higher risk of developing this disease than women without any diseased relatives. Moreover, the risk becomes high as 2.5-fold or higher in women with two or more first-degree relatives with BC [39]. The inherited susceptibility to breast cancer is attributed to the mutations of breast cancer related genes such as BRCA1 and BRCA2.

3.1.7c Reproductive factors

Reproductive factors such as early menarche, late age at first pregnancy, late menopause, and low parity can increase the breast cancer risk. Each one-year delay in menopause increases the risk of BC by 3 percent. Each one year delay in menarche or each additional birth decreases the risk of BC by 5 percent or 10 percent, respectively [40-42]. A recent Norwegian cohort study showed that a hazard ratio (HR) is around 1.5 between late (more than 35 years) and early (less than 20 years) age at first birth [43]. Reproductive factors are strongly related with the ER condition, with differences in the odds ratios (OR) between ER positive and ER negative breast cancer for parity (OR: 0.7 vs. 0.9 for more than 3 births vs. nulliparae) and age on the first birth (OR: 1.6 vs. 1.2 for age more than 30 vs. less than 25 years) [44].

3.1.7d Estrogen

BC is associated with both endogenous and exogenous estrogens. The endogenous estrogen is mainly produced by the ovary in premenopausal women and removal of ovary can reduce the risk of breast cancer [45]. The main sources of exogenous estrogen are the OCPs and the hormone replacement therapy (HRT). The OCPs have been widely used since 1960s and their have been many formulations upgraded to reduce side-effects. However, the odds ratio is still higher than 1.5 for African-American women and Iranian populations [46, 47]. Nevertheless, OCPs do not increase the risk of breast cancer in women who discontinue using them for more than 10 years [40]. HRT involves the administration of exogenous estrogen and other hormones for the menopausal and postmenopausal women. Plenty of studies have shown that the use of HRT can increase the breast cancer risk. A cohort study of 22,929 females in Asia revealed HRs of 1.48 and 1.95 after HRT use for 4 and 8 years, respectively [49]. The information of Million Women Study in UK stated that a relative risk (RR) of 1.66 between current users of HRT and those who never used HRT [48]. However, the risk of breast cancer has been shown to reduce significantly after two years of discontinuation of HRT [50]. The recurrence rate is also more in breast cancer survivors who take HRT, and the HR for a newly diagnosed breast tumor is 3.6 [51]. Since the adverse effects of HRT were published in early 2003 based on the Women's Health Initiative randomized controlled trial, the incidence rate of breast cancer in America has reduced by approximately 7% due to the decrease in the use of HRT [52].

3.1.7e Lifestyle

Modern lifestyles such as too much dietary fat intake and excessive alcohol consumption can increase the risk of breast cancer. Alcohol consumption can increase the level of estrogen-related hormones in the blood and trigger the estrogen receptor related pathways. Modern western diet has too much of fat and excess intake such fat, especially the saturated fat, is associated with increased mortality (RR=1.3) and poor prognosis in breast cancer patients [55]. A meta-analysis based on 53 epidemiological studies stressed that an intake of 35-44 grams of alcohol per day can elevate the risk of breast cancer by 32%, with a 7.1% increament in the RR for each additional 10 grams of alcohol per day [53, 54]. Although the relationship between breast cancer risk and smoking remains contentious mutagens from cigarette smoke have been identified in the breast tissue from non-lactating women. The risk of breast cancer is also increased in women who both drink and smoke (RR=1.54) [56]. Untill now, accumulating evidences demonstrate that smoking, especially at an earlier age, has a increased risk on breast cancer occurrence [57-60].

A common apprehension among women is that hormonal contraception could add to cancer risk. While studies have time and again found that use of OCPs reduces risks for ovarian and endometrial cancers, findings as regards breast cancer have been uncertain and controversial. To assess associations between OCP use and risks for these cancers, researchers used data from the UK Biobank, which recruited a huge cohort of individuals in between 2006 and 2010, as well as from national databases.

Among over 250 thousand women born between 1939 and 1970, more than 80% had used or were currently are using OCPs. In analyses attuned for 10 parameters, ever users of OCPs had significantly lower risks for ovarian (OR= 0.72) and endometrial

cancers (OR= 0.68) than never users, but this correlation did not extend to breast cancer. In analyses of women at dissimilar follow-up ages, ever users at ages 35, 50, 55, and 60 had lower risks for the two gynecologic cancers but a minimally yet significantly elevated risk for breast cancer (age 55: OR= 1.10). Increasing duration of OCPs use was related with higher prevention of ovarian and, particularly, endometrial cancers than no OCP use (≥20 years of use: ORs, 0.60 and 0.36, respectively); however, risks for breast cancer were comparable between never and long-term users [61].

3.1.8 Molecular classification of breast cancer

BC is a heterogeneous disease, comprising of numerous distinct cell types having different biological features and clinical behaviour. Therefore, classification of BC cannot be restricted to the one based on localization and the extent of the tumor. To further classify on molecular basis, various investigators utilizing different molecular techniques and comprehensive gene profiling analysis have revealed following four major subtypes of BC.

- Basal like (ER-/HER2-)
- HER2 enriched (ER-/HER2+)
- Normal breast like, & luminal A (ER+)
- Luminal B (ER+/HER2 +)

Such molecular differences lead to discrete clinical outcomes and responses to treatment. Amongst the subtypes, luminal A-type tumors have best prognosis and are low-grade tumors. On the other hand, luminal B type tumors are aggressive high grade tumors. Besides, basal-like tumors are high grade with relatively poor

prognosis. These tumors may or may not be triple negative (ER-/PR- and HER2-). The better insight of the molecular subtypes of BC eases the choice of therapy regime for the patients and ensures more sensitivity to the treatment.

The overall pooled prevalence of luminal A, luminal B, HER2-enriched, and TNBC subtypes of breast cancer were 0.33 (95% CI 0.23–0.44), 0.17 (95% CI 0.12–0.23), 0.15 (95% CI 0.12–0.19), and 0.30 (95% CI 0.27–0.33), respectively. Amongst molecular subtypes of breast cancer, luminal A was the the majority prevalent subtype followed by TNBC, luminal B, and HER2-enriched subtypes. The overall occurrence of TNBC in India is high compared to other regions of the world. Additional research is warranted to recognize the determinants of high TNBC in India. Differentiating TNBC from other molecular subtypes is important to guide therapeutic management of BC.

3.1.9Available treatment options for breast cancer

In the recent years, life-saving treatment strategies for BC highly developed bringing new hope and excitement. Instead of just one or two, various treatment options are available for BC. Furthermore, a option of treatment based on the factors like age, physiological condition of patients and stage of cancer is possible now. Treatment options for the breast cancer could be characterized as follows

Local treatment options: This type of treatment specifically targets the tumor and rest of the body parts remains untouched. It constitutes the following procedures.

Surgery: Surgical removal is recommended to patients having a localized tumor below 4 cm. Lumpectomy is a type of surgery referring to surgical removal of the

tumor in breast along with negligible amount of surrounding tissue. Another edition, partial mastectomy is extensive and removes more amount of normal tissue surrounding the tumor. This is also referred to as quadrantectomy. These two surgical procedures make up the 'breast conserving surgery' as the removal of complete breast is avoided. Furthermore, patients with a more advanced stage of breast cancer may undergo a total mastectomy or total removal of breast with a sentinel lymph node biopsy. For a more advanced tumor radical mastectomy is recommonded that includes removal of breasts along with removal of lymph nodes in the armpits and chest. Surgeries are the preferred mode of treating BC when accompanied with radiotherapy or chemotherapy.

Radiation therapy: Surgical removal of tumor is frequently followed by radiation therapy to remove residual microscopic cells. Superiority of this combination can be proved by data demonstrating a recurrence rate of 14.3% for those patients undergoing breast conservation therapy followed by radiation as compared to 39.2% for those undergoing surgery alone.

Systemic treatment options: Molecular classification of BC has made it likely to administer specific enzymes, drugs, antibodies and hormones for treating cancer. When these compounds are administered either through blood vessels or orally, it is characterized as systemic therapy. [62]

Chemotherapy: Chemotherapy is usually suggested for all women with an invasive BC that is hormone receptor-negative. It is usually administered through blood steam or given orally. On the other hand, regional chemotherapy is the administration of drugs directly into the cerebrospinal fluid, an organ, or a body cavity such as the

abdomen, mainly affecting cancer cells in those areas. Several therapeutic drug options are available for the treatment through chemotherapy such as cisplatin is the most effective drug that is in the clinical use since 1972 to treat a variety of cancer types. Similarly, epirubicin, doxorubicin, antimetabolite as 5-fluorouracil, microtubule inhibitor paclitaxel are few examples of this category.

Hormone therapy: Hormone therapy removes hormones or inhibits their action and stops cancer cells from growing. For example, hormone therapy with tamoxifen is often given to patients with early stages of breast cancer and those with metastatic breast cancer. Moreover, hormone therapy with an aromatase inhibitor is given to some postmenopausal women who have hormone-dependent BC.

Targeted therapies: Conventionally, chemotherapeutics act on dividing cells thus cannot differentiate between normal or malignant cells. Targeted therapy blocks the proliferation of cancer cells by interfering with specific molecules essential for tumor development and growth. Some of these molecules may be present in normal tissues, but they are often mutated or over expressed in tumors. Therapies targeting a specific downstream protein or a metabolite come under this category and are being considered extensively. They have become potential candidates in modern pharmacotherapy for most of the cancers including that of breast (63). In adding up to these drugs, many other drugs have been developed or are being developed that target hallmarks of cancer specifically, e.g. avoiding immune destruction, sustaining proliferative signaling, activating invasion and metastasis, resisting cell death and so on. Various molecular modifications that generate and sustain these tumorigenic processes have been spelled out as druggable targets. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering

with specific molecules engaged in tumor growth and progression (64, 65). In the present in-vitro study is evaluating the effect of estrogen on EGFR expression which can control the growth and proliferation of cancer tissue in either direction.

Gene therapy: A number of gene therapy approaches for carcinoma of the breast have been developed. These approaches can be broadly divided into: (1) Transgene expression, (2) molecular chemotherapy or suicide gene therapy, (3) proapoptotic gene therapy, (4) antiangiogenic gene therapy, (5) genetic immunopotentiation, and (6) genetic modulation of resistance/sensitivity, (7) Ablation of oncogenes by RNA based methods. The field of cancer gene therapy embraces a range of ideas and technologies from direct attack on tumour cells to harnessing the immune response to tumour antigens. Clinical trials for breast cancer have been initiated to evaluate safety, toxicity, and efficacy. Clinical trials for cancer treatment shares 64% of total gene therapy clinical trials worldwide till 2012 which increased to 66% in 2014. It is expected that as new therapeutic targets and approaches are acknowledged and advances in vector design are realized, gene therapy will play a lead role in clinical breast cancer treatment.

By spotlighting on molecular and cellular changes that are specific to cancer, targeted cancer therapies may be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells than other types of treatments including chemotherapy and radiotherapy. Our study is one initiative in that direction.

3.1.10 Distribution of molecular subtypes of breast cancer

The study which was done in India with sample of 2062 patients ranging between 22–100 years with a mean age of 51.18 years, prevalence of subtypes of cancer was similar to those reported by Mane et al a another Indian study. 37% of patients were luminal A, 8% were luminal B, 11% were HER2 rich, and 26% were basal-like. In the case of Mane et al. 43.8% were luminal A, 14.8% were luminal B, 16.1% were Basal-like, and 16.1% were HER2 rich [66, 67]. Table -1 shows the prevalence of molecular subtypes of breast cancer in different parts of the world.

Table 1: Prevalence of molecular subtypes of breast cancer in different parts of the world.

Study	Luminal A	Luminal B	HER2 enriched	Basal-like	Total no of patients
British Columbia Cancer Agency [68, 69]	71%	6%	7%	15%	3348
Mayo Clinic Breast Cancer study [70]	86%	9%	2%	4%	256
Vancouver General Hospital study [71]	78%	4%	6%	12%	246
University of British Columbia [72].	42%	15%	17%	26%	365
Carolina breast cancer study [73].	51.4%	15.5%	6.6%	26.4%	496
Dawood et al. [74].	65.8%	14.3%	4.9%	15%	1945
Mane et al. [67]	43.8%	14.8%	16.1%	25.3%	521
Tubtimhin et al.	31.6%	15.6%	9.9%	11.3%	523
ElidrissiErrahhali et al.	61.1%	16.1%	8.6%	14.2%	2260

3.2.1Oral contraceptives and breast cancer

BC appears to be prejudiced by hormonally mediated factors leads to the theory that the high rate of exposure to OCPs among women may also be associated with this increase. The possibility of increased BC risk related to OCP use is a major concern to the scientific community.

Evaluation of cancer incidence data from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute (NCI) proposes that there has been an overall increament in the incidence of breast cancer, with increase of the mainly affecting among women over age 50 as it is discussed earlier. Most of the early research on this topic studies conducted prior to 1980 found no association between OCP use and breast cancer, comforting many in the research and clinical communities that there was little or no increased risk [74-78]. Investigations reported in the early 1980s offered little to cause observers to change their minds. The majority of studies, counting the largest case-control study in the United States, found no consistent suggestion of elevated risk [79-86], although several studies reported elevated risk estimates for particular aspects of oral contraceptive use [87-91]. There was no constancy among these elevated risk estimates.

The image seems to have changed since 1986. There are a number of investigations during this period that do not support an increased risk related to OCP use, including more analyses of the Cancer and Steroid Hormone (CASH) Study data and the updated analysis of data from the United States nurses' cohort study [92-96]. But an increasing number of studies have appeared that suggest an elevated risk in relation to

some aspects of OCP use [97-100]. These studies, especially those with encouraging findings, have received much publicity and generated renewed concern over the safety of OCPs.

3.2.2 Changing profile of oral contraceptive use

OCPs were introduced in the early 1960s but did not find general acceptance until the late 1960s and early 1970s. There are four major types of OCPs. Combination pills or classical pills, which contain unchanging amounts of estrogen and progestin and act by suppressing ovulation, were the first OCPs approved in the United States and have always been the most popular type of pill used. Sequential pills, in which estrogen alone is given for the first two weeks followed by an estrogen-progestin mixture during the last six days, were also introduced early but were removed from the market around 1977 [101,102]. The, or progestin-only pills or minipills introduced in 1972 contain no estrogen and a lower amount of progestin. They do not affect ovulation but rather inhibit ovum transfer and implantation by thickening the cervical mucus. Phasic oral contraceptives, combination pills that contain estrogen along with a progestin dose that varies in amount throughout the month, were introduced in 1983 and have since become increasingly accepted. These oral combinedor classical oral contraceptives were used in our study in most of the cases.

Following the advent of OCPs, two major changes have occurred that must be well thought-out in evaluating the research on OCPs and breast cancer: (1) changes in the formulation of OCPs and (2) changes in the patterns of their use and in the women who use them. Concerning the first change, over the past three decades, the formulations of oral OCPs have undergone considerable modifications. Both the types

and doses of steroids have changed; the doses of both estrogen and progestins have been greatly reduced; several new progestins have entered the market whereas others have been withdrawn; and sequential pills are utilized no longer. High-estrogen-potency OCPs constituted 94 percent of the market in 1964, but by 1976 low-estrogen oral contraceptives less than 50 µg of estrogen, which were introduced in 1967, controlled the market [101]. The profiles of OCP users have also changed over time. OCPs were most commonly used first in the 1960s by married women to space pregnancies and only later by single women as a method to postpone first pregnancy [90]. Routine prescription to younger single women was not familiar until the early 1970s in Great Britain and the late 1970s in the United States [90]. In the United States, only women born since the 1940s have had the chance to be exposed to long-term use of the pill early in life.

At present the use of OCPs is an extremely prevalent exposure among young women. With the recent epidemic of teenage pregnancies in the United States, routine prescription to teenage girls as young as age 13-15 is not uncommon. Unpublished data from a case-control study conducted by the author and colleagues of breast cancer diagnosed between 1983 and 1988 in young women born after 1944 in Seattle, Washington, showed that 92 percent of both the cases and controls reported "ever" having used OCPs; among women under age 20 the proportion of "ever" use increased to 100%.

3.2.3 Biological relations with oral contrceptives

An essential criterion for establishing causality is the biological plausibility of the relationship. Estrogen causes proliferation of breast tissue and would be expected to

augment BC risk by stimulating growth of intermediate and cells stem cells. Progestin causes alveolar cell growth ans development in the estrogen-primed breast, however it also causes differentiation. It is unclear, therefore, whether the predicted net result would be to increase or decrease BC risk.

The influence of estrogen and progestin on breast epithelium proliferation and differentiation appears to differ with age. Cancer risk is thought to be a function of the number of cells at risk that varies with age. It is possible to posit that any carcinogenic risk of OCPs may be strongly mediated by age of exposure or by the timing of exposure in relation to other events that are thought to affect epithelial proliferation or differentiation e.g., menarche, first full-term pregnancy.

The etiology of BC has strong hormonal subject matter: oophorectomy decreases risk, late age at first full-term pregnancy increases risk, early menarche and late menopause increases the risk. These effects seem to last for decades. Accordingly, if use early in life affects risk, it may be many years before harmful outcomes are seen. It is possible that the studies conducted so far have not permitted an adequate interval between exposure to OCPs and the onset of BC, consequently that even if an association were present, it might not nevertheless be detectable. Thus, vigilance must be maintained and search of this issue should continue in the future even though we might conclude, based on current data, that findings are too conflicting to be alarming at present.

3.2.4 Epidemiological Studies of Breast Cancer and Oral Contraceptives

As discussed earlier, the studies conducted prior to 1980 carry little implication of an increased risk for BC in relation to OCP use. These studies focused on cases diagnosed before 1975; therefore, they included a large proportion of women who,

because of their birth years, had modest opportunity to have ever used OCPs or to have used OCPs for a long time, and virtually had no chance for exposure at an early age. For these grounds, as well as the briefness of the time between exposure and diagnosis, investigations conducted before 1980 cannot add any insight into the current controversy. Unfortunately, these same complicities plague some of the studies published in the early 1980s as well.

3.2.5 "Ever" Use of Oral Contraceptives

Twenty-five of the thirty studies for which a relative risk (RR) for "ever" use was reported had RR close to 1.0. Two of the five studies that report higher risk estimates for "ever" use of the OCP are ones for which questions with in view either to basic study design, low exposure prevalence low response rates, or have been raised [102, 103]. Taken as a whole, there is no evidence of increased risk of BC in women who meet the criterion of "ever" using OCPs. The finding of no relationship between "ever" use of OCPs and BC risk has been quite steady throughout the past 20 years of research."Ever" use of OCPs is probably too rough a measure to provide much insight into any association with BC risk because such use is so widespread that it typically includes more than 90 percent of all young women. Further, understanding is difficult because there are subgroups of women who try OCPs but discontinue because of side effects soon after they start it. Women who never use OCPs may be a unique subgroup. For instance, they may have a family history of BC, or an increased awareness or distrust of undiagnosed infertility or health problems that contraindicate the use of OCPs; these factors, in turn, may corelate to both their decision not to use OCPs and their risk for BC.

3.2.6 Duration of Oral Contraceptive Use

There was very little evidence of increased risk related to long-term use of OCPs in any of the studies published prior to 1986. Of the case-control investigations published since that time, seven reported an additional risk for use of long duration. The largest case-control study of breast cancer conducted to date, the CASH study, illustrated no evidence of an association of breast cancer risk and long-term use of OCPs among women aged 20 to 54 [104]. Despite its size the study comprised more than 4,700 cases and a similar number of controls in eight geographic regions of the United States, the majority of the women were over age 45. A Yugoslavian study of women under age of 55 years, found an RR of 2.4 for OCP use exceeding seven years, as well as a significant dose-response pattern. Of late, the World Health Organization (WHO) study, a multinational case-control study carried out in three developed and seven developing countries, stated a suggestive dose-response pattern of increasing risk with years of OCP use; this alliance, however, could well be attributable to a recency effect since risk was highest in present users and steadily reduced with time since last exposure. Paul and colleagues (1986) reported an RR of 4.6 for use of 10 or more years among women aged 25–34. The first ever report suggesting a dose-response relationship with duration of OCP use among young women: for use of 8 to 11 years, an RR of 1.4 was found; for 12 or more years of use, a 2.2-fold additional risk of BC was found [105]. McPherson and coworkers (1987) showed an increased RR of 1.8 for use above 11 years among a group of British women through age 45. A well-conducted study in the United Kingdom [100] among women under age 36 reported a significant dose-response pattern for duration of use.

OCP use of 49 to 96 months was associated with a 1.4-fold excess risk, and use exceeding 96 months was associated with a 1.7-fold excess breast cancer risk. This case-control study was one of the few that were able to validate the self-reported data on OCP use so as to rule out the often-raised criticism of recall bias. A hospital-based case-control study conducted in the northeastern United States (Miller et al., 1989) among women under age 45 observed a twofold excess risk for OCP use of five to nine years duration and a fourfold excess risk for use of 10 or more years.

Contradictory observations have been recorded among the three large prospective cohort studies. The largest, the Nurses Health study in the United States [105], found no increase in risk for any duration of use or for any other aspect of OCP use except current use. Current use of OCPs was associated with an overall adjusted RR of 1.5. This additional risk was confined to women between ages 40 and 50. However, the Royal College of General Practitioners cohort in the United Kingdom reported excess BC risk for longer durations of oral contraceptive use, although there was no steady dose-response pattern [106]. In this cohort, there were incoherent mildly elevated risks for duration of use among women of all ages. In two subgroups, women ages 30-34 and women who were parity 1, much higher risks were seen as high as a 10-fold excess risk for use of 10 or more years [106]. The Oxford cohort in the United Kingdom has seen no evidence of increased risk related to OCP use [80]. All three of these cohorts may have been initiated too early to include many women born recently enough to have had the chance to use OCPs at a young age or for a long duration. Since most of the studies increased risk was confined to 40-50 years. We confined our study age groups should also be in the same range. Since there was no consistent

pattern in duration of use and BC risk we recruited our study population of having minimum of cumulative six month duration of OCP use.

3.2.7 Oral Contraceptive use before first full-term pregnancy or before age 25

Since of the increasing incidence of use of the OCP at young ages, and because of the possibility of increased vulnerability of breast tissue to hormonal exposures during young ages when breast development is still continuing and when endogenous hormone concentrations are still increasing, there has been growing interest in the estimation of BC risk in relation to OCP use at young ages. Findings regarding any relationship between use of OCP before the first full-term pregnancy (FFTP) or before age 25 have been not consistent. A factor that further complicates the picture is that some reaseachers report on use before FFTP only among parous women, some regard as parous and nulliparous women combined, and others investigate only nulliparous women.

Summary of risk estimates for oral contraceptive use before first full-term pregnancy.

The vast majority of investigations of the relationship between OCP use prior to FFTP and breast cancer risk have focused on women under age 45. Pike's 1981 study was the first to report an undesirable effect from OCP use prior to FFTP, observing an RR of 2.3 for 5-8 years of OCP use preceding FFTP and an RR of 3.5 for more than 8 years use before FFTP. Meirik and associates reports on Swedish and Norwegian women revealed an increased risk for eight or more years of use before FFTP in the cumulative as well as in both nulliparous and parous women when assessed separately (all women: RR = 2.0, confidence interval (CI) = 1.8-4.2; nulliparous women: RR =

4.3, CI = 1.4-13.1; parous women: RR = 1.7, CI = 0.7-4.2). A threefold excess risk was reported by McPherson and his associates for use exceeding four years of duration before FFTP and was accompanied by a dose-response relationship. The McPherson teams 1987 report examined a twofold excess risk of BC for one to four years of use before FFTP and a 2.6-fold excess risk for use exceeding four years of duration prior to FFTP among nulliparous and parous women combined. A number of analyses found no hint of increased BC risk for OCP use before FFTP [107-109]. Overall consideration of OCP use before FFTP in the complete Cancer and Steroid Hormone study data discovered no suggestion of excess BC risk. [110]. In present study we though had many of the subjects used OCPs and at younger age and probably before FFTP, But we did not consider these factors for evaluation. A major challenge in interpreting many of the investigations of use at a young age lies in separating the effects related to use early in life from effects associated with longer durations of exposure.

3.2.8 Duration since first use of oral contraceptives (latency)

It has been evaluated that long-term latent effects that have been missed might be the different explanation for many of the research with negative findings. More than 10 studies have presented such analyses with no demonstration of a reliable latency pattern [111,112]. It is possible, however, that these studies were carried out too early to see such an effect.

Use of oral contraceptives in high-risk subgroups

Although many investigators adjust for high-risk factors e.g., family history of BC, history of benign breast disease in their analyses, only a subset include examined OCP

use within each of these strata. Furthermore, of the few studies that have investigated OCP use in each strata, the majority have limited their definition of use to "ever/never" and their definition of the high-risk subgroups to fairly rough delineations. These approaches are untoward because they may well miss important modifying relationships that cannot be noticed at such a crude level.

With regard to family history of BC, the bulk of the studies have detected no sizeable differences in the risk related to OCP use for women with and without this factor [113]. Brinton and colleagues (1982) found no differences in women with and without a mother with BC but did observe differences in women with and without a sister with BC. Elevated risks have been observed among OCP users with a history of benign breast disease (114, 115]; however more work is needed to assess specific histologic types of benign breast disease in terms of both BC risk and relationship to use of the OCP. Some past analyses were unsuccessful to distinguish between OCP use before and after the diagnosis of benign breast disease. In addition, not much has been done to scrutinize histologic subgroups and molecular subtypes of BC for the possibility of differential relationships with OCP exposure.

3.2.9 Steroidal Potency of Various Formulations

The hormonal contents of oral contraceptives have been checked by a number of classification schemes related to potency, brand, and type of [116, 117, 118]. Not one of these approaches, however, has consistently exhibited a relationship with BC risk. However, in present study we presumed that most of the patients were on combined or classical oral pills Mala D and Mala N.

3.2.10 Issues to Consider in Reviewing the Epidemiological Evidence

In attempting to evaluate the gathered research, several issues should be considered. First, the design and conduct of each study should be evaluated to detect possible limitations that could have affected the outcome. Specific factors such as the basic design case-control or follow-up study and the ratio of eligible subjects, who participated in the study, or in a follow-up design, are relevant to interpretation of the findings. Several of the formerly mentioned studies suffered from low response rates or hefty losses to follow-up. If these losses are great, the validity of the case-control study is negotiated because of the possibility of differential exposure allocations in the responders versus the nonresponders. In a follow-up study, analogous questions arise concerning the possible differential distribution of disease occurrence. The sample size of a study must be good enough to allow the detection of an outcome or to rule out with a certain amount of confidence the presence of an effect. A number of previous studies may not have had adequate power to estimate the relationship of OCPs and BC. In addition, sample sizes need to be much larger to inspect the interrelationships of other risk factors with OCP use. It is necessary to understand and integrate into analyses the other important breast cancer risk factors, such as age of first full-term pregnancy, age of menarche, number of live births, family history of breast cancer, and history of benign breast disease etc. Several of these risk factors may affect the choice to use OCPs, the age of first and last use, and the lifetime duration of use. These and other factors may alter or be modified by the relationship of OCPs to BC risk. When birth year and opportunity for exposure to OCPs are examined in the context of the possible latency period for BC, it becomes obvious that

many of the already completed studies may have been not capable to evaluate the relationship of OCPs and BC, particularly in view of use at younger ages as well as premenopausal disease onset. Presuming OCPs have a promotional effect, but the time interval until a detectable lesion is still unidentified. Studies that include women diagnosed before a certain point in time may not have permited an adequate interval between exposure to OCPs and onset of BC. In this instance, even if an association were present, it might not be identifiable in these women.

Another understudied facet of the association between OC use and BC risk is the potential variation in risk by molecular subtype of BC. More than 90% of TNBC tumors fall within the basal like subgroup, when called for its gene expression profile they mimics basal epithelial cells in other parts of the body and a characteristic morphology that includes high proliferative rate, central necrosis. Basal-like BC is associated with aggressive histology, unresponsiveness to typical endocrine therapies, poor prognosis, and BRCA1-related BC. Western population which is considered to be socio-economically of higher status, OCP use is high. This may be the reason why they have more preponderance develop ER+ BC and less preponderance to develop TNBC. African and Asian population have less preponderance to develop ER+ and more preponderance to develop TNBC. The reason could be here less use of OCP. There are proofs that estrogen decreases the proliferation of TNBC by non-genomic action. These cumulative effects could be reason why western population has less number of TNBC. There is paucity in the studies whether OCP use associated with increased/reduced risk of especially with TNBC in Indian population particularly in younger age group. Addressing these issues is of public health importance given the high prevalence of use OC among Indian women and the greater aggressiveness of

breast cancer in younger women especially Triple negative breast cancer. In order to characterize the association between OCP use, and risk of different breast cancer subtypes among Indian young women, since these biological subtypes of breast cancers this particular study was conducted.

3.3.1 Oncogenic intracellular signaling in breast cancer

Intracellular signalling which drives a normal cell to cancerous is very complex process. It makes a cell to divide indefinitely even in the absence of growth factors or in presence of growth inhibiting factors. Upregulation and/or mutations that augment the function of oncogenes, or the loss or inactivation of tumour suppressor genes, can enhance cancerogenesity of the cell. This means that any cell at any time can behave badly under the pressure of single mutational event. As a result, there are checkpoint and repair mechanisms to thwart this possibility. If repair is not possible, cells invoke self-destruct programmed cell death pathways to get rid of damaged cells which might otherwise go on to form a tumour. However, if the tumour succeeds, it must find ways to escape these death mechanisms and this make ups a second change in intracellular signaling. Apart from uncontrolled, purposeless growth of cells, the continued existence of these cells due to faulty apoptosis or cell death has been regarded as a major contributor to their transformed state. Hence, mutations that give rise to excessive proliferation and a complementary disrupt ion of survival signalling pathways make sure the persistence of these hyper proliferative cells. This can be alternatively exploited for successful treatment of cancer. Any tumor cell with a lesion gather speed proliferation can be strategically designed to disengage the survival promoting mechanisms and drive itself into apoptosis. In addition, in the later steps towards the journey of neoplastic transformation, there are a number of

characteristics that need to be acquired by the cells, for example, the abilities to invade and to exist without normal stromal support, and the capacity to induce neovascularisation. Although these changes are considered mostly extracellular in nature, they also result from changes in intracellular events and add to the intricate nature of signalling in malignant cells. Tumor invasion is a complex biological process, during which tumor cells detach from the primary tumor and infiltrate the neighboring tissue. This course requires loss of cell contacts between tumor cells, active cell migration, adhesion to the extracellular matrix and proteolytic degradation of the extracellular matrix. Since cancer was assessed as a 'systems biology disease' by hence insights into these intricated intracellular processes have exposed many new cancer targets for which therapeutic agents may be developed. The present in-vitro study was devised to understand the effect estrogen on expression of EGFR in ER positive and triple negative cancer cell lines. Where we tried to understand effect of estrogen on EGFR whether estrogen has a differential action on EGFR, whether it increases the synthesis of EGFR or degrades it, if degrades what is the pathway of degradation?

Tumor is a result of multi factorial; multigenic disease hence targeting one at a time (single-hits) may alter the target but may not bring desired effects in a complex setting. Cells may activate backup systems against this targeting. These make advent of newer approaches with multiple targeting simultaneously a necessity and need of the hour. Our study was also devised in a way that if up gradation or degradation pathways get activated they can be targeted. Therapeutic networking becomes an important tool in studying intracellular oncogenic networks.

3.3.2 EGFR signalling

Uncharacteristic epidermal growth factor receptor (EGFR) signaling is a major characteristic of many human malignancies including BC. Since the discovery of EGF in 1960 and its receptor in 1980, researchers understanding of the EGF/EGFR pathway have been significantly advanced and consequently, EGFR is considered as a major oncogenic factor and an attractive therapeutic target. The well-established conventional function of EGFR is to send extra-cellular mitogenic signals, such as EGF and transforming growth factor-alpha (TGF-alpha), through activating a number of downstream signaling cascades. These include signaling units that involve phospholipase-C, Ras, and phosphatidylinositol-3 kinase (PI-3K). In cancer cells, following the activation of the EGFR-mediated downstream pathways which are altered gene activities, leads to uncontrolled tumor proliferation and apoptosis. Interestingly, up-and-coming evidences are claiming the existence of a direct mode of the EGFR pathway which is distinct from the traditional transduction pathway. This new mode of EGFR signaling involves transport of EGFR within the cell, from the cell-surface to the cell nucleus. This also involves organization of nuclear EGFR complex with gene promoters, and transcriptional regulation of the target genes. Although the nature and pathological consequences of the nuclear EGFR pathway remain indescribable, accumulating evidences suggest its association with increased tumor cell proliferation and poor survival rate in BC patients. While several anti-EGFR agents are being tested in BC patients clinically and others under pre-clinical development, a better understanding of the conventional and the nuclear EGFR pathways will make possible the identification of patients that are likely to respond to

these agents as well as future development of more effective anti-EGFR therapeutic interventions. Since EGFR is tried and tested molecule in the development of breast cancer we selected this molecule to understand the effect of estrogen in different BC subtypes.

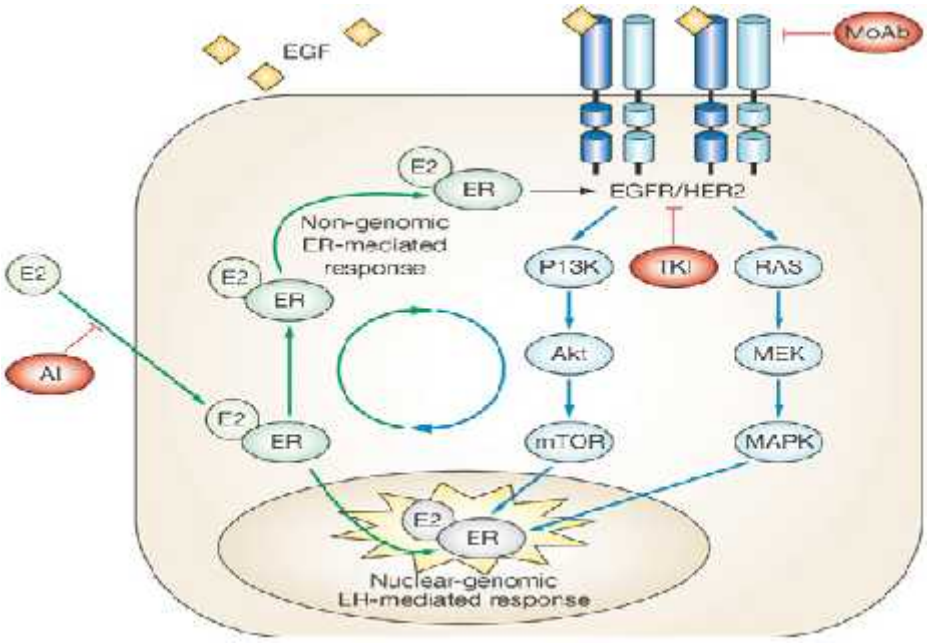


Figure 5: Estrogen activates nuclear ER (genomic pathway) and ER in or near the membrane (non-genomic pathway). Membrane associated ER binds to GF signaling components such as PI3K. E2 then activates GF signaling, activating key molecules such as Akt or RAS, and downstream molecules such as mTOR, Raf, MEK and MAPK, which promote cell proliferation and survival.

3.3.3 Oncogenic activation of the epidermal growth factor receptor

EGFR is a membrane spanning glycoprotein, which frequently has been implicated in various cancer types. The mechanisms by which EGFR turns oncogenic are numerous and are often specific for each cancer type. In some of the tumors, EGFR is triggered

by autocrine/paracrine growth factor loops, whereas in others activating mutations that encourage EGFR signaling. Overexpression and/or amplification of the EGFR gene are ubiquitous in many cancer types leading to aberrant EGFR signaling. In addition, failure to attenuate receptor signal transduction by receptor downregulation can also lead to cellular maltransformation. Heterodimerization of EGFR with ErbB2 blocks downregulation of EGFR and thereby extends growth factor signalling duration. This as well indicates that cross-talk among EGFR and heterologous receptor systems serves as one more mechanism for oncogenic activation of EGFR. Since of EGFR has a role in tumor promotion, the EGFR has been intensively studied as a therapeutic target. However, tumorigenesis is a multi-step course of action involving several mutations that might explain why EGFR therapeutics has only been partially successful.

EGFR is a transmembrane receptor whose overexpression in BC predicts for poor prognosis and is inversely correlated with expression of estrogen receptor (ER). This study was designed to investigate whether estrogen plays an active role in activation or suppression of EGFR expression in different molecular subtypes of BC.

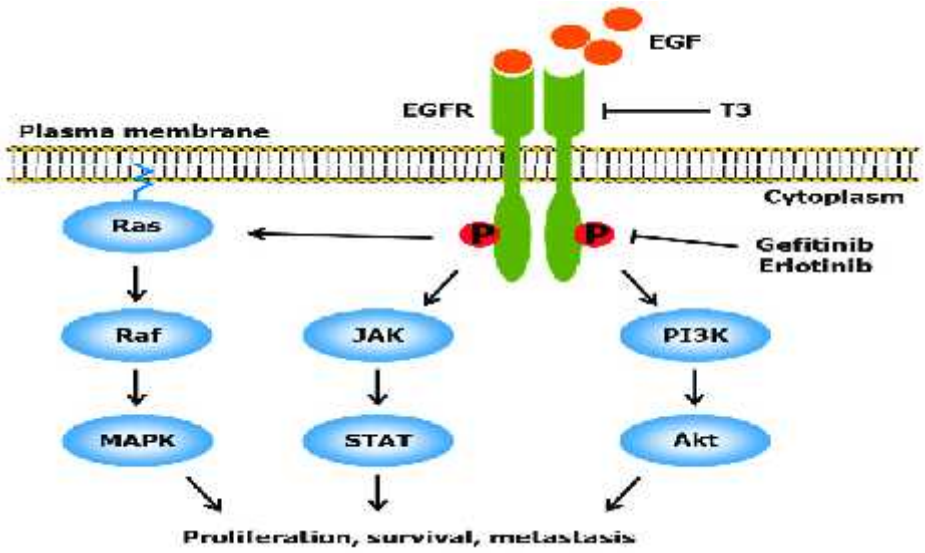


Figure 6: EGFR downstream signalling in cancer. These include signaling modules that involve phospholipase-C, Ras, JAK and phosphatidylinositol-3 kinase (PI-3K).

3.4 Cycloheximide chase for evaluation of protein degradation

Proteins perform crucial functions in virtually every cellular functioning. Many physiological processes need the presence of a specific protein for a defined point of time or under particular circumstances. Organisms therefore check and regulate protein abundance to meet cellular needs [119]. For example, cyclins are present at specific phases of the cell cycle, and the absence of regulated cyclin levels has been associated with malignant tumor formation [120]. In addition to regulating protein levels to serve cellular needs, our cells employ degradative quality control mechanisms to get rid of unassembled, misfolded, or otherwise abnormal protein molecules [121]. Regulation of protein abundance involves control of both macromolecular synthesis and degradation. Impaired or excessive protein degradation contributes to multiple pathologies, including cancer, neurodegenerative conditions, cystic fibrosis and cardiovascular disorders [122-126]. Study of proteins at a single time point by a western blot [127], a flow cytometry [128], or a fluorescence microscopy [129] gives a snapshot of steady state protein abundance without divulging the relative contributions of synthesis or degradation. Similarly growth-based reporter assays reflect steady state protein levels over an extended time period without affecting the influences of synthesis and degradation [129-134]. It is possible to make interpretation of the contribution of degradative processes to steady state protein levels by comparing abundance before and after blocking specific components of the degradative mechanism *e.g.,* by pharmacologically blocking the proteasome [135] or knocking out a gene hypothesized to be essential for degradation. A change

in steady state protein levels after inhibiting degradative pathways provides strong substantiation for the contribution of proteolysis to the control of protein abundance. However, such studies still do not provide sufficient information regarding the kinetics of protein turnover. Cycloheximide chase run followed by a western blotting overcomes these weaknesses by allowing researchers to visualize protein degradation over period of time [136-138]. Further, because protein detection following cycloheximide chase run is typically performed by a western blotting, radioactive isotopes and lengthy immunoprecipitation steps are not essential, unlike several commonly used pulse chase techniques, which are also performed regularly to visualize protein degradation [139].

Cycloheximide was first to be identified as a compound with anti-fungal properties formed by the gram-positive bacterium Streptomyces griseus [140, 141]. It is a cell-permeable molecule that specifically inhibits eukaryotic cytosolic translation by impairing ribosomal translocation [142-145]. In a cycloheximide chase run experiment, cycloheximide is added to cells, and aliquots of cells are gathered immediately and at specific time interval points following addition of the compound. Cells are lysed, and protein abundance at each time point interval is analyzed, typically by a western blot. Decrease in protein abundance following the adding up of cycloheximide can be confidently attributed to protein degradation. An unsteady protein will decrease in abundance over period of time, while a relatively stable protein will exhibit change in abundance that much. Mechanisms involved in selective protein degradation have been highly conserved to Eukarya. Much of what is understood about protein degradation was first learnt in the model unicellular eukaryote, Saccharomyces cerevisiae [146-150]. Studies with yeast are likely to continue to provide knowledge, novel and important insights into protein degradation.

Cycloheximide chase run is appropriate for analyzing protein stability over a relatively short time course that is in minutes. Over longer time courses two hours to days, cycloheximide, a global inhibitor of translation, which is toxic to cells, likely due to exhaustion of ubiquitin [151]. Additionaly, analyses of protein stability over longer time intervals are more likely to be affected by indirect effects of globally impaired protein synthesis on the degradation of the protein of interest (*e.g.*, degradation of a short-lived protein involved in the degradation of the protein of interest). Therefore, other techniques, such as pulse chase metabolic labeling experiments, are appropriate for studying the degradation of long-lived proteins and may be performed to corroborate results obtained in cycloheximide chase experiments.

The timing of adding up of cycloheximide and collection of cells is an important consideration in this procedure. Precision and perfection is especially important in the analysis of very short-lived proteins, as small deviations in the time elapsed from cycloheximide adding up to cell harvest can have a substantial impact on apparent degradation kinetics. Further, without a clear plan for executing these time-bound-sensitive steps, an experiment may rapidly devolve into chaos and frustration. For this even reason, it is recommended to add up cycloheximide to culture plates at planned, regular intervals. Different intervals were adapted in our study to match the comfort level and dexterity of the investigation.

Cycloheximide chase experiment may be implemented to estimate the degradation of a diversity of yeast proteins and may be modified to study protein stability in other eukaryotic cells. As described in our study protocol, cycloheximide treatment is followed by cell lysis and detection of protein abundance by a western blot analysis.

Depending on the experiment and application, however, protein abundance aftermath cycloheximide treatment may be assessed by a range of techniques, as appropriate as convenient as research objectives. For example, when protein size is not a relevant factor for analysis, cell lysates may be subjected to a dot blot or a enzyme-linked immunosorbent assay (ELISA) analysis, which report on protein abundance, nevertheless not on apparent molecular weight [152]. For proteins which are located on the cell surface flow cytometry may be utilized to quantify protein abundance of samples at different times after cycloheximide addition. Fluorescence microscopy following cycloheximide adding up would provide information on both protein abundance and localization. The range of substrates, organisms, and downstream applications amenable to cycloheximide chase experiment makes the technique a highly versatile and informative means of studying protein degradation in detail.

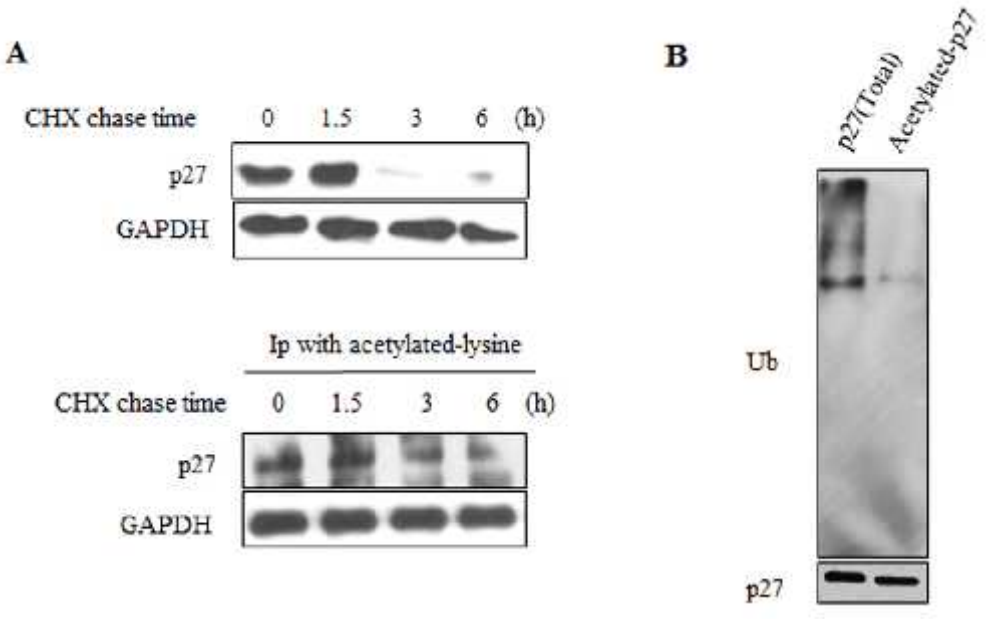


Figure 7: Example showing to how cycloheximide chase is executed by utilizing western blot technique.

3.5.1 Ubiquitination a one of the pathway for degradation of proteins.

Intracellular proteins and many extracellular proteins are constantly turning over. They are hydrolyzed toward their constituent amino acids and replaced by newly synthesed amino acids. The continual destruction of process serves several important homeostatic functions. Individual proteins whether they are in the nucleus or cytosol, wether in the endoplasmic reticulum (ER) or mitochondria are degraded at widely differing rates. They take minutes for some regulatory enzymes, days or weeks for proteins such as actin, myosin in skeletal muscle and months for hemoglobin in the red cell. Cells made up of numerous proteolytic systems to carry out the degradation process and complex regulatory mechanisms to ensure that these continual proteolytic processes are highly selective. The excessive breakdown of cell components is also prevented at the same time. Overall, the pace of protein synthesis and degradation in each cell must be balanced precisely because even a small decrease in synthesis or a small acceleration of production, if at all they sustained, can result in a marked loss of function in the organism [153].

In tissues, the majority of intracellular proteins are degraded by the ubiquitin–proteasome pathway (UPP) [154]. But, some extracellular proteins and some cell surface proteins are taken up by endocytosis and degraded within lysosomes. These organelles contain several acid-optimal proteases, including cathepsins, and several other acid hydrolases. Some cytosolic proteins are degraded in lysosomes after being engulfed in autophagic vacuoles that blend with lysosomes [155,156]. There are further cytosolic proteolytic systems in mammalian cells. The Calcium-induced ATP-

independent proteolytic route involves the cysteine proteases termed calpains. These proteases seem to be activated when cells are injured and cytosolic calcium rises, so they may play an important role in tissue injury, necrosis, and autolysis [157]. These enzymes, which are cysteine proteases, are critical in destruction of cell components during apoptosis [158].

Ubiquitin Proteasome Pathway (UPP) has become important in our understanding of the control of protein turnover. The UPP consists of concerted actions of enzymes that connect chains of the polypeptide co-factor, Ubiquitin b (Ub), onto proteins to mark them for degradation [159, 160]. This tagging process guides to their detection by the 26S proteasome, a very large multi-catalytic protease complex that degrades ubiquitinated proteins to small peptides [161]. Three enzymatic components are required to connect chains of Ub onto proteins that are destined for degradation. Ub-activating enzyme (E1) and Ub-carrier or conjugating proteins (E2s) arrange Ub for conjugation, but the key enzyme in the process is the E3 (Ub-protein ligase), because it identifies a specific protein substrate and catalyzes the shift of activated Ub to it. Since the initial narrative of the UPP as a protein tagging and demolition mechanism, knowledge in this area has exploded, with thousands of proteins shown to be degraded by the system and additional new functions for Ub conjugation being uncovered.

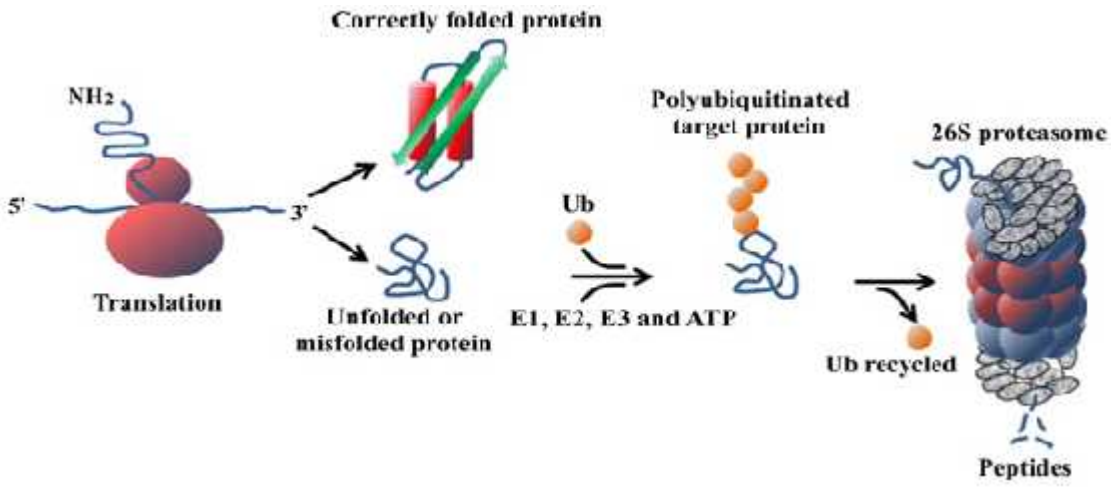


Figure 8: Ubiquitin Proteasome Pathway. Shows different stages of ubiquitination.

3.5.2 Rapid Removal of Proteins

Not like most regulatory mechanisms, protein degradation is intrinsically irreversible. Destruction of a protein can lead to a complete, fast, and sustained termination of the physiological process involving that protein and as well as a change in cell composition. The fast degradation of specific proteins permits adjustment to new physiologic surroundings.Control of Gene Transcription Ub conjugation affects transcription by multiple mechanisms [162]. A lot of transcription factors by the proteasome system are ubiquitinated and degraded. In fact, in lots of cases, transcriptional activation domains and signals for Ub conjugation directly have common pathways. Ubiquitination and proteolysis of activators still may stimulate transcriptional activity by removing “spent” activators and reorganizing a promoter for further rounds of transcription [163]. In addition, transcription factors can be regulated by alterations in their location. For example, NF-γB, the proinflammatory transcriptional activator, is set aside out of the nucleus by its interaction with IκB. IκB degradation is triggered by its phosphorylation, which leads it to be recognized by the E3 -transducin repeat containing protein (TRCP). IκB then is ubiquitinated and

rapidly degraded, and the released NF-γB translocates to the nucleus. This step is important in acceleration of the inflammatory response [164].

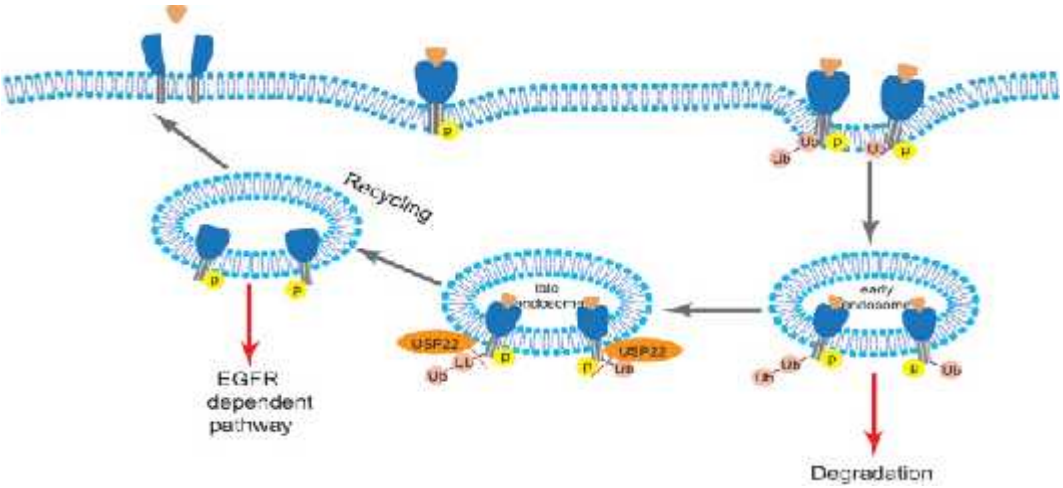


Figure 9: The diagram shows different pathways of degradation of EGFR.

3.5.3 Quality Control Mechanism

The UPP specifically removes abnormally folded, altered or damaged proteins that have arisen by nonsense or missense mutations, damage by oxygen radicals or biosynthetic errors or by denaturation. In cystic fibrosis, the mutant form of the transmembrane conductance regulator protein (CFTR) is selectively degraded and therefore falls short to reach the cell surface [165, 166]. Since the Ub conjugation and degradation mechanism are cytoplasmic, the destruction of CFTR demonstrates that the UPP degrades misfolded or secreted proteins. In the process of ER-related degradation, many misfolded proteins within the ER are retro-translocated out of that compartment into the cytosol by a series of ER membrane associated Ub conjugating proteins; these then are objected to cytosolic proteasomes [167]. Functioning of the Immune System Antigen presentation on MHC class I molecules is reliant on proteasomal function. Other Functions of Ub which is not linked with proteolysis is it

also can be conjugated to proteins as a monomer. This type of tagging initiates internalization of cell surface proteins into the endocytic pathway [168] and also can be used in the regulation of transcription [169].

3.5.4 Ub is linked to Substrates through an Enzymatic Cascade

Ub is composed of 76 amino acids. C-terminus of Ub is a important that is necessary for its conjugation to other Ub molecules and substrates, and it contains internal lysine residues which are used in the formation of polyubiquitin chains. Ub is not expressed frankly as free Ub but rather as linear fusions either to itself or to definite ribosomal protein subunits. These extremely unusual Ub-fusion precursors are subjected quickly by deubiquitinating enzymes, yielding monomeric, Ub moieties; this is one way in which Ub is produced quickly in times of cellular stress. Ub conjugation to cellular proteins also can be reverted by the many deubiquitinating enzymes in cells. Consequently these enzymes release Ub from precursor fusion proteins; they also catalyze the disassembly of Ub chains. Most likely, they function to recycle monomeric Ub for use in new conjugation reactions and avoid the accumulation of polyubiquitin chains that could fight with the binding of ubiquitinated substrates to the proteasome. The first step in conjugation of Ub onto proteins is the activation of Ub at its C-terminus by Ub-activating enzyme E1. This abundant enzyme uses ATP to generate a Ub thiolester, a extremely reactive form of Ub [170]. In mammalian organisms, only a single functional E1 enzyme has been detected, unlike the large number of E2s and E3s in cells [171]. Once it is activated, the Ub that is bound to E1 via the thiolester linkage is shifted to a sulfhydryl group of one of the 30-40 Ub carrier proteins or E2s [172]. The E2s generally are very small proteins that share a inner conserved core that includes the cysteine that forms a thiolester linkage with the

triggered Ub. The huge number of E2s helps to create the specificity of the ubiquitination system, because specific E2s function in the degradation of a variety of types of substrates, and they can conjugate with different E3s in a specific manner.

3.5.5 E3s Recognize the Cellular Proteins That Undergo Ub Conjugation

The major specificity factor in the UPP is E3 enzyme. There are thousand E3s in cells that link Ub to proteins in a highly regulated pattern. E3s catalyze the transfer of the activated Ub from an E2 originally to a lysine in the target protein and afterward to lysines that are present in Ub, yielding a substrate attached chain of Ub molecules. Generally, E3s id divided into two broad structural classes: They are either homologous to homologous to E6-AP carboxy-terminus (HECT) domains or RING fingers [173]. HECT domain proteins are large monomeric E3s that contains of two functionally distinct domains [174]. The C-terminal HECT domain accepts the triggered Ub from the E2s by forming a thiol ester linkage with Ub, making it to be transferred to the substrate. HECT-domain E3s directly unite activated Ub and are real components of the enzymatic conjugation cascade [175]. The classical member of this family is the E6-associated protein (E6-AP) [176]. Absence of this enzyme causes Angelman's syndrome, an inherited neurologic disorder [177]. Nedd4, one more HECT-E3, targets the epithelial sodium channel for internalization and degradation [178] by identifieng specific residues in the channel's cytoplasmic tails. When these proteins cannot act together, either as a result of mutations in the sodium channel or the E3, the channel becomes more stable, resulting in increased sodium reabsorption, hypervolemia, hypertension, metabolic alkalosis a genetic defect known as Liddle's syndrome [179]. The majority of E3s consists of RING finger domains. These 40-60 residue zinc-binding motifs consist of core amino acids, cysteine, and histidine [180].

RING finger E3s can be monomeric enzymes or multisubunit composites. As a whole, they seem to serve up as scaffolds that bring the substrate and the E2 nearer, an optimal condition for Ub conjugation [180-182]. Monomeric RING finger E3s includes the oncoprotein Mdm2, a physiologic regulator of p53 stability in cells [183], and c-Cbl, that catalyzes ubiquitination of certain cell surface receptors. Two E3s that critical in the pathology of muscle atrophy are muscle ring finger-1 (MuRF-1), E3 belong to this particular group; This E3 was among the first of the E3s to be biochemically discovered. It detects protein substrates on the basis of their N-terminal amino acid. Proteins that begin with basic or hydrophobic residues are objected for ubiquitination and degradation by E3. This "N-end rule" pathway is important in the destruction of cohesions [184], few signaling molecules like regulator of G-protein signaling 4 [RGS4] [185]. The other RING-finger E3s contain various subunits that serve as scaffolds to bring together the substrate and an E2 conjugated to a triggered Ub. The largest most complex E3 is the anaphase-promoting complex and it is essential in ubiquitination of mitotic cyclins and other proteins that are involved in regulation of cell-cycle. Cullin-RING Ub ligases are the largest group of E3s. The basic core of these E3s is long, rigid cullin subunit. On the one end of these subunits attach the RING component and the E2, whereas at the other side, the substrate-interacting protein is bound, often through another adaptor protein. The large number of cullins and substrate-binding subunits, the identical organization using the same basic method can recognize and ubiquitinate a large number of varied proteins. The best studied group of cullin-RING ligases are the Skp1–Cul1–F-box (SCF) complexes. The F-box protein is the subunit which contains the substrate-binding motif. It binds to an adaptor, Skp1, through an approximately 45 amino acid F-box motif. Substrates of SCF complexes E3s are numerous key molecules that control

inflammation and cell growth and cell cycle–induced proteins. In many cases, phosphorylation leads to attachment of substrate to the F-box subunit and subsequent Ub conjugation. Regulated expression of F-box proteins can lead to tissue and disease specific Ub conjugation of target proteins. For example, the F-box protein atrogin-1/MAFbx is expressed at very high levels specifically in atrophying skeletal muscle and cardiac muscle [186].

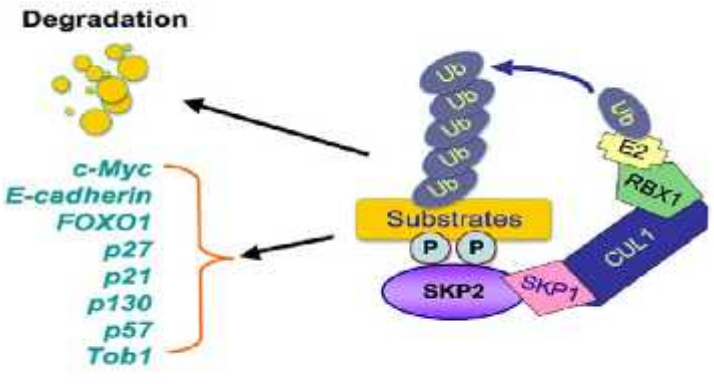


Figure 10: The diagram shows different pathways of cullin-RING ligases are the Skp1–Cul1–F-box (SCF) complexes in degradation of EGFR.

In addition with phosphorylation, other types of posttranslational protein modifications can excite ubiquitination. For example, oxygen concentration in the cell is sensed by the Von HippelLindau (VHL)-containing VHL-elongin BC (VBC) E3 that specifically recognizes hydroxyproline. When oxygen levels are sufficient in cells, the hypoxia-inducible factor 1 (HIF-1) transcriptional activator subjected prolyl hydroxylation and ubiquitination by this E3. When oxygen pressure falls, the unchanged HIF-1 is not recognized by VHL and is not degraded, hence it triggers

transcription of genes for angiogenesis [187]. The VBC complex isone more cullin-RING ligase, made up of Cul2 and a substrate-interacting domain which is made up of VHL and the adaptors elongin B and elongin C. VHL mutations are linked with highly vascular tumors in the kidney, presumably as a consequence at least in part of the presence of stable, active HIF-1. Other protein modifications that have been made known to recruit E3s include glycosylation, nitrosylation, and deacetylation. Substrate modification adds on another layer of regulation to the UPP by integrating cell signaling and metabolic pathways along the conjugation-degradation machinery. Recently, a new group of enzymes with Ub ligase activity have been described: The U-box domain proteins, like Ub fusion degradation protein 2 (UFD2) and the C-terminal of Hsp70-interacting protein (CHIP). These E3 enzymes consist of atypical RING finger motifs [188]. CHIP is very important for the removal of unusual proteins such as the abnormal misfolded CFTR in cystic fibrosis and tau protein of polyglutamine repeat proteins which are present in several neurodegenerative diseases [189]. Degradation of these proteins begins when they are attached by specific molecular chaperones followed by binding of the E3s. This leads to selective ubiquitination of the chaperone-bound substrate. In our study if ostreogen degrades EGFR by ubiquitination it would be interesting to study the effect of estrogen at different steps of ubiquitination so as that pathway or molecules can be targeted for therapeutic approach.

3.5.6 Cell Proteins Are Degraded by the 26S Proteasome

The rapid degradation of ubiquitinated proteins is mediated by the 26S proteasome. This structure is present in the nucleus and the cytosol of all cells and makes approximately 1 to 2% of cell mass [190]. The 26S particle is consists of

approximately 60 subunits and therefore is approximately 50-100 times larger than the usual proteases that function in the extracellular environment and differs in critical ways. The most elementary difference is that it is a proteolytic machine in which protein degradation is related to ATP hydrolysis. The 26S complex is composed of a central barrel-structure 20S proteasome in the company of a 19S regulatory particle at either or both of its ends [190, 191]. The 20S proteasome is a void cylinder which contains the mechanisms for protein digestion. It is made up of four stacked, hollow rings, each containing seven distinct but connected subunits. The two outer rings are alike, as are the two inner rings. Three of the subunits in the rings have the proteolytic active sites that are positioned on the interior part of the cylinder. The outer subunits of the 20S particle surround a narrow, central, and gated aperture through which substrates enter and products exit [192]. Substrate access is a complex process which is catalyzed by the 19S particle. This complex architecture evolved to segregate proteolysis within a nano-sized partition and prevents the nonspecific destruction of cell proteins. One can observe protein ubiquitination and the functioning of the 19S particle as mechanisms that make certain proteolysis as an exquisitely selective process; only selective molecules get degraded within the 20S proteasome [193]. The 19S regulatory particles on the ends of the 20S proteasome are consists of at least 18 subunits.The base contains six homologous ATPases in a ring and link ups the outer ring of the 20S particle. These ATPases attach the proteins to be degraded and utilize ATP hydrolysis to unfold and translocate the protein into the 20S particle [194]. The 19S ptotein outer lid contains subunits that attach the polyubiquitin chains plus two deubiquitinating enzymes which disassemble the Ub chain so that the Ub can be reutilized in the degradation of other proteins. There is growing proof that additional factors related with the 19S particle and actually help bring ubiquitinated proteins into

the particle [195]. Although the 26S complex catalyzes the degradation of ubiquitinated proteins, they can digest certain proteins without ubiquitination; it remains unclear how important this activity is in-vivo [196].

About proteasomes multistep system much has been learned by which a ubiquitinated protein is degraded. After it attaches to the 19S component, the polyubiquitin string is cleaved off the substrate and disassembled. The protein is unfolded one way or another by the six ATPases in the base of the particle. Linearization of the folded protein is necessary for it to be translocated through the gated entry channel into the 20S particle because this hole, even in its open state, is too slender for globular proteins to enter. The ATPases also act as a key in a lock to lead opening of the gated, substrate entry channel of the 20S outer ring and into its innermost degradative chamber [197]. Linearizing and delivering the substrate uses significant energy, perhaps one third as much ATP as the ribosome would utilize in synthesizing the protein, but it make sures the efficient and regulated removal of the protein. Once the substrate enters the 20S central chamber, the polypeptide is sliced by its six proteolytic sites on the inner part of the chamber, forming small peptides that range from 3 to 25 residues in length [198]. Contrasting traditional proteases, that cut a protein once and free the fragments, the proteasome digests the substrates all the way to small peptides that leave the particle. Peptides which are released by the proteasome only exist in the cell for few seconds, because they are rapidly digested into amino acids by the plentiful cytosolic endopeptidases and aminopeptidases. The amino acids can be reused to synthesize fresh proteins or metabolized, yielding energy [199, 200]. Although the proteasome principally catalyzes the total hydrolysis of cell proteins, in a few cases, the 26S proteasome degrades proteins only partially, yielding a biologically dynamic fragment. An example of such activity is the

generation of a subunit of the transcription factor NF-γB [201]. For NF-γB to be efficient in inflammatory processes, the proteasome have to digest an inactive precursor molecule and let go one half, which functions in transcriptional regulation.

3.6.1 Proteasome Inhibitors and Cancer Therapy

One interesting thing which the proteasomes active site cannot remove is repeated sequences of glutamines. This exception is significant in the pathogenesis of certain neurodegenerative diseases e.g., Huntington's disease which result from a genetically connected series of polyglutamines within certain proteins [202]. Actually the glutamines are poorly degraded and mount up as toxic, intracellular inclusions. The active sites in the proteasome cut peptide bonds by a unique mechanism; peptide bonds are cleaved by the hydroxyl group on a important threonine residue. Because the proteolytic mechanism is novel, highly specific inhibitors of the active sites have been manufactured. These inhibitors e.g., MG132, epoxymycin lactacystin, have been used widely as research tools that have facilitated investigators to discover many of the impotant functions of the UPP. In our study MG-132 was utilized to study whether EGFR was ubiquitinated with or without estrogen in TNBC cell line. Bortezomib a synthetic protease inhibitor has emerged as an imperative new anticancer drug. Bortezomib has been approved by the US Food and Drug Administration and is extensively used for the treatment of multiple myeloma and clinical trials against a variety of other cancers are under active investigation [203]. The proteasome inhibitors at first were synthesized in an attempt to develop agents that could inhibit the excessive breakdown of muscle proteins in different cachectic states, but it was discovered that they also could inhibit the activation of NF-γB, the important transcription factor which mediates production of several inflammatory

cytokines. NF-γB also has key antiapoptotic roles that could inhibit the death of cancer cells. However, blocking of the proteasome was found to induce apoptosis, especially in neoplastic cells and transplanted tumors. Surprisingly, these agents have therapeutic efficacy, even when protein degradation by the proteasome in cancer cells is only partly affected. Actually, the myeloma cells are particularly dependent on NF-γB for genetation of essential growth factors. However, promising responses have been observed in patients with other hematologic malignancies, and Bortezomib in amalgamation with other chemotherapeutic agents is being tested against new malignancies in clinical trials. In short, the development of proteasome blockers that exhibit numerous biologic properties emphasizes the enormous benefits that are emerging.

3.6.2 Protease inhibitor- MG-132

Enzyme-specifc blockers of proteases are usually short peptides connected to a pharmacophore, generally present at its C-terminus. The pharmacophore act together with a catalytic residue with the formation of reversible or irreversible covalent adduct, while the peptide portion specially join together with the enzymes substrate attaching pocket in the active site. Although the proteasome has several active sites, inhibition of all of them is not necessary to significantly reduce protein breakdown. In fact, blocking of the chymotrypsin-like site or its inactivation by mutation by itself causes a large reduction in the pace of protein breakdown [204, 205]. In contrast, blocking of trypsinlike or caspase-like sites will have little effect on overall proteolysis [205, 206]. In addition, most blockers of chymotrypsin-like sites are very much hydrophobic and consequently much more cell-permeable than blockers of the trypsinor caspase-like sites, that contain charged residues. As a result, almost all the

synthetic and natural blockers of the proteasome act primarily on the chymotrypsin-like activity but also have a little, usually much weaker, effects on the two other sites. Even though cleavages by the chymotrypsin-like sites appear to be rate-limiting in protein breakdown, the amount of inhibition of chymotrypsin-like activity cannot be directly associated to the reduction in protein breakdown as inhibition of this site may lead to the increased cleavages by two other sites. Unluckily, inhibitor potency has often been calculated only against the chymotrypsin-like action along with purified proteasomes and synthetic peptide substrates. The range of reports concerning the potencies of various proteasome inhibitors in cells are difficult to contrast because deferent investigators have used different cell lines and assays, that were generally only indirectly related to the speed of protein breakdown. The chymotrypsin-like site of proteasomes cleaves principally after large hydrophobic residues, similar to the fondness of intracellular cysteine proteases such as cytosolic calpains and several lysosomal cathepsins [207]. For that reason high selectivity of proteasome inhibition by peptide based compounds would be difficult to achieve just by simply manipulating the peptide part of the inhibitor. As an alternative, the use of a pharmacophore with preference for the proteasomes N-terminal threonine is required. Based on pharmacophores, proteasome inhibitors can be classified into several groups in them peptide aldehydes are most important.

3.6.3 Peptide aldehydes

Peptide aldehydes were the initial proteasome inhibitors to be developed [208] and are still the most widely accepted inhibitors. Aldehyde inhibitors of the chymotrypsin-like site are slow-binding [208], but they gain entry in to the cells rapidly and are reversible. These blockers have fast dissociation rates, are rapidly oxidized into

inactive acids by cells and are carried out of cell. Accordingly, in experiments concerning cultured mammalian cells and yeast, results of these inhibitors can be rapidly reverted by removal of the inhibitor [209]. As discussed above, peptide aldehydes are known inhibitors of cysteine and serine proteases, and therefore can inhibit such proteases in vivo. For example, ALLN, which was utilized in earlier studies, was first described as a calpain inhibitor I [210], and is 25-fold more effective against cathepsin B. Several other peptide aldehydes have been synthesized [211, 212], but only some of them are now used widely. MG132 (Z-Leu-Leu-Leu-al, also termed Cbz-LLL or z-LLL) is not only appreciably more potent than ALLN against the proteasome [213], but is much more selective, as demonstrated by the fact that inhibition of calpains and cathepsins need at least 10-fold higher concentrations [214]. Another peptide aldehyde, PSI (Z-Ile-Glu (OtBu)-Ala-Leu-al), blocks the proteasome 10-fold better than calpain but is less effective than MG132. Finally, the dipeptide aldehyde CEP1612 appears as good as MG132 in potency and selectivity, but is not accessible commercially. Since MG132, PSI, MG115 (Z-Leu-Leu-nVal-al) and ALLN can all inhibit calpains and variety of lysosomal cathepsins in addition to the proteasome, when using these blockers in cell culture it is essential to perform control experiments to confirm that the observed effects are because of inhibition of the proteasome. In yeast, digestive vacuoles contain mainly serine, not cysteine proteases, phenylmethylsulfonyl fluoride can be used to block these enzymes without affecting proteasomes. Additionally, with any effect sensitive to MG-132 or other aldehydes, involvement of the proteasome can be confirmed or ruled out by use of several more specific inhibitors of the proteasome, such as epoxomicin, lactacystin and boronate MG-262 which would be too expensive for most investigators to utilize in routine studies.

Even though the availability of these blockers, MG132, due to its low price and the rapid reversibility of its action, still remains in our opinion the first choice to study proteasome involvement in a process in cell cultures or tissues, provoided appropriate controls are used. As the most potent and selective of commercially available aldehydes, MG132 is first choice compared to ALLN, MG115 (Z-Leu-Leu-nVal-al), or even PSI. On the other hand, the least selective inhibitor, ALLN, for the reason that of its ability to inhibit most major proteases in mammalian cells, is most likely the best tool for prevention of unwanted proteolysis, for example during separation of proteins from mammalian cells.

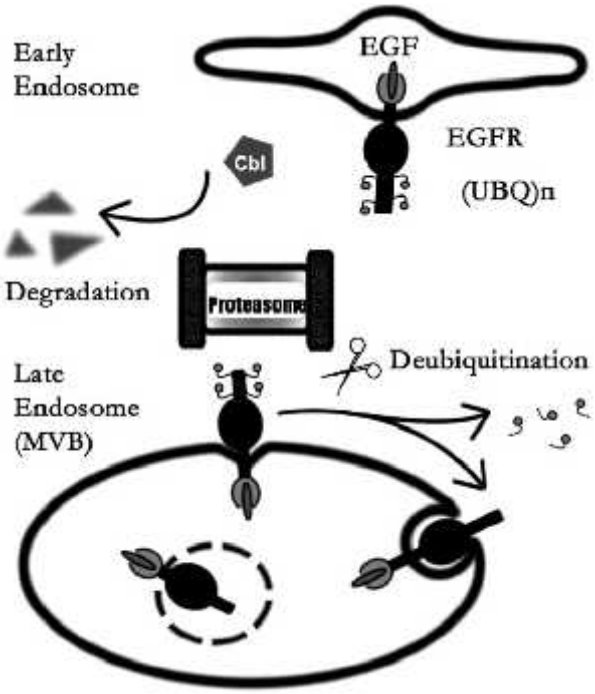


Figure 11: Diagram showing the mechanism of action of MG-132 inhibiting ubiquitination of EGFR.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov; 68(6):394-424.

2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021; 71(3):209-249.

3. 3Garcia M, Jemal A, Ward EM, Center MM, Hao Y, Siegel RL, Thun MJ. (Eds). (2007): Global Cancer Facts and Figures. Atlanta, GA: American Cancer Society. 2007; 1-3.

4. Fackenthal JD and Olopade O.: Breast cancer risk associates with BRCA1 and BRCA2 mutations in diverse populations. Nat Rev Cancer. 2007; 7: 937-947.

5. Easton DF, Pooley KA, Dunning AM, pharoah PDP, Thompson D, Ballinger D, et al. Genomewide asociated study identifies novel breast cancer susceptibility loci. Nature. 2007; 447: 1087-10944.

6. Horn-Ross PL, Lee M, John EM, Koo J.Sources of phytoestrogen exposure among non-Asian women in California.Cancer Causes and Control.2000; 11: 299-301.

7. Curado MP, Edwards B, Shin HR, Storm H, Ferlay J, Heanue M, Boyle P. (Eds). Cancer incidence in Five Continents. Lyon: Vol. IX IARC Scientific Publications, 2007; No. 160.

8. Ziegler R, Hoover RN, Pike MC, Hildesheim A, Nomura A, et al. Migration Patters and Breast Cancer Risks in Asian-American Women. JNCI.1993; 85; 1819- 1827.

9. Porter P. Westernizing women's risks? Bresat cancer in low income countries. New Eng J med. 2008; 358: 213-216.

10. Ginsburg OM and Love RR. Breast Cancer: a neglected disease for the majority of affected women worldwide. Breast J. 2011: 17: 289-295.

11. Li CL, Malone KE and Daling JR. Differences in breast cancer hormone receptor status and histology by race and ethnicity among women 50 years of age and older. Cancer Epidemiol Biomarkers Prev. 2002; 11: 601-607.

12. Uy GB, Laudico AV, Fernandez AM, Lim FG, et al. Immunohistochemical assay of hormone receptors in breast cancer at the Philippine General Hospital: Importance of early fixation of specimen. Philipp Jsurg Spec. 2007; 62:123-127

13. Tran D and Lawson J. (2004): Rates of estrogen receptor-alpha expression are no different in low-risk (Vietnam) and high-risk (Austarlian) breast cancer. Appl Immunohistochem Mol Morphol, 2004. 12:139-141.

14. Olopade OI, Ikpatt OF, Dignam JJ, Khramstov Z, Tetriakova M, Grushko T, et al. Intrinsic Gene Expression subtypes correlated with grade and morphometric

parameters reveal a high proportion of aggressive basal-like tumors among black women of African ancestry. Proc Am Soc Clin Oncol. 2004; 23: 9509.

15. Deng CX. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. Nucleic Acids Res. 2006;34:1416-1426

16. Dine J, Deng CX. Mouse models of BRCA1 and their application to breast cancer research. Cancer Metastasis Rev. 2013;32:25-37

17. Tan-Wong SM, French JD, Proudfoo NJ. et al. Dynamic interactions between the promoter and terminator regions of the mammalian BRCA1 gene. P Natl Acad Sci USA. 2008;105:5160-5165

18. Hegan DC, Lu Y, Stachelek GC. et al. Inhibition of poly(ADP-ribose) polymerase down-regulates BRCA1 and RAD51 in a pathway mediated by E2F4 and p130. Proc Natl Acad Sci U S A. 2010;107:2201-2206

19. Sanchez H, Paul MW, Grosbart M. et al. Architectural plasticity of human BRCA2-RAD51 complexes in DNA break repair. Nucleic Acids Res. 2017;45:4507-4518

20. Martinez JS, von Nicolai C, Kim T. et al. BRCA2 regulates DMC1-mediated recombination through the BRC repeats. Proc Natl Acad Sci U S A. 2016;113:3515-3520

21. Bane AL, Beck JC, Bleiweiss I. et al. BRCA2 mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. Am J Surg Pathol. 2007;31:121-128

22. Balmana J, Diez O, Rubio IT. et al. BRCA in breast cancer: ESMO Clinical Practice Guidelines. Ann Oncol. 2011;22(Suppl 6):31-34

23. Paluch-Shimon S, Cardoso F, Sessa C. et al. Prevention and screening in BRCA mutation carriers and other breast/ovarian hereditary cancer syndromes: ESMO Clinical Practice Guidelines for cancer prevention and screening. Ann Oncol. 2016;27:103-110

24. 32. Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol. 2007;25:1329-1333

25. Shih C, Padhy LC, Murray M. et al. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature. 1981;290:261-264

26. Harbeck N, Gnant M. Breast cancer. The Lancet. 2017;389:1134-1150

27. Davis NM, Sokolosky M, Stadelman K. et al. Deregulation of the EGFR/PI3K/PTEN/Akt /mTORC1 pathway in breast cancer: possibilities for therapeutic intervention. Oncotarget. 2014;5:4603-4650

28. Elizalde PV, Cordo Russo RI, Chervo MF. et al. ErbB-2 nuclear function in breast cancer growth, metastasis and resistance to therapy. Endocr Relat Cancer. 2016;23:243-257

29. Appert-Collin A, Hubert P, Cremel G. et al. Role of ErbB Receptors in Cancer Cell Migration and Invasion. Frontiers in pharmacology. 2015;6:283

30. Ali R, Wendt MK. The paradoxical functions of EGFR during breast cancer progression. Signal transduction and targeted therapy. 2017;2:16042

31. Alanazi IO, Khan Z. Understanding EGFR Signaling in Breast Cancer and Breast Cancer Stem Cells: Overexpression and Therapeutic implications. Asian Pacific journal of cancer prevention: APJCP. 2016;17:445-453

32. Zhang D, LaFortune TA, Krishnamurthy S. et al. Epidermal growth factor receptor tyrosine kinase inhibitor reverses mesenchymal to epithelial phenotype and inhibits metastasis in inflammatory breast cancer. Clin Cancer Res. 2009;15:6639-6648

33. Kim A, Jang MH, Lee SJ. et al. Mutations of the Epidermal Growth Factor Receptor Gene in Triple-Negative Breast Cancer. Journal of breast cancer. 2017;20:150-159

34. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. Nat Rev Cancer. 2011;11:761-774

35. Siewertsz van Reesema LL, Lee MP, Zheleva V. et al. RAS pathway biomarkers for breast cancer prognosis. Clinical laboratory international. 2016;40:18-23

36. Hoenerhoff MJ, Chu I, Barkan D. et al. BMI1 cooperates with H-RAS to induce an aggressive breast cancer phenotype with brain metastases. Oncogene. 2009;28:3022-3032

37. Fernandez-Medarde A, Santos E. Ras in cancer and developmental diseases. Genes & cancer. 2011;2:344-358

38. Ray A, Ray BK. Induction of Ras by SAF-1/MAZ through a feed-forward loop promotes angiogenesis in breast cancer. Cancer medicine. 2015;4:224-234

39. 65. Brewer HR, Jones ME, Schoemaker MJ. et al. Family history and risk of breast cancer: an analysis accounting for family structure. Breast Cancer Res Treat. 2017;165:193-200

40. Washbrook E. Risk factors and epidemiology of breast cancer. Women's Health Medicine. 2006;3:8-14

41. Horn J, Vatten LJ. Reproductive and hormonal risk factors of breast cancer: a historical perspective. International journal of women's health. 2017;9:265-272

42. Dall GV, Britt KL. Estrogen Effects on the Mammary Gland in Early and Late Life and Breast Cancer Risk. Front Oncol. 2017;7:110

43. Horn J, Asvold BO, Opdahl S. et al. Reproductive factors and the risk of breast cancer in old age: a Norwegian cohort study. Breast Cancer Res Treat. 2013;139:237-243

44. Rosato V, Bosetti C, Negri E. et al. Reproductive and hormonal factors, family history, and breast cancer according to the hormonal receptor status. Eur J Cancer Prev. 2014;23:412-417

45. Endogenous H, Breast Cancer Collaborative G, Key TJ. et al. Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies. Lancet Oncol. 2013;14:1009-1019

46. Soroush A, Farshchian N, Komasi S. et al. The Role of Oral Contraceptive Pills on Increased Risk of Breast Cancer in Iranian Populations: A Meta-analysis. Journal of cancer prevention. 2016;21:294-301

47. Bethea TN, Rosenberg L, Hong CC. et al. A case-control analysis of oral contraceptive use and breast cancer subtypes in the African American Breast Cancer Epidemiology and Risk Consortium. Breast Cancer Res. 2015;17:22

48. Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet. 2003;362:419-427

49. Liu J-Y, Chen T-J, Hwang S-J. The Risk of Breast Cancer in Women Using Menopausal Hormone Replacement Therapy in Taiwan. International journal of environmental research and public health. 2016;13:482

50. Narod SA. Hormone replacement therapy and the risk of breast cancer. Nature reviews. Clinical oncology. 2011;8:669-676

51. Fahlen M, Fornander T, Johansson H. et al. Hormone replacement therapy after breast cancer: 10 year follow up of the Stockholm randomised trial. Eur J Cancer. 2013;49:52-59

52. Ravdin PM, Cronin KA, Howlader N. et al. The decrease in breast-cancer incidence in 2003 in the United States. N Engl J Med. 2007;356:1670-1674

53. Hamajima N, Hirose K, Tajima K. et al. Alcohol, tobacco and breast cancer-collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. Br J Cancer. 2002;87:1234-1245

54. Jung S, Wang M, Anderson K. et al. Alcohol consumption and breast cancer risk by estrogen receptor status: in a pooled analysis of 20 studies. International journal of epidemiology. 2016;45:916-928

55. Makarem N, Chandran U, Bandera EV. et al. Dietary fat in breast cancer survival. Annu Rev Nutr. 2013;33:319-348

56. Knight JA, Fan J, Malone KE. et al. Alcohol consumption and cigarette smoking in combination: A predictor of contralateral breast cancer risk in the WECARE study. Int J Cancer. 2017;141:916-924

57. Catsburg C, Miller AB, Rohan TE. Active cigarette smoking and risk of breast cancer. Int J Cancer. 2015;136:2204-2209

58. Gaudet MM, Carter BD, Brinton LA. et al. Pooled analysis of active cigarette smoking and invasive breast cancer risk in 14 cohort studies. International journal of epidemiology. 2017;46:881-893

59. McKenzie F, Ellison-Loschmann L, Jeffreys M. et al. Cigarette smoking and risk of breast cancer in a New Zealand multi-ethnic case-control study. PLoS One. 2013; 8:e63132

60. Kispert S, McHowat J. Recent insights into cigarette smoking as a lifestyle risk factor for breast cancer. Breast Cancer: Targets and Therapy. 2017; 9:127-132

61. Andrew M. Kaunitz, MD, reviewing Karlsson T et al. Use of Oral Contraceptives and Cancer Risks: New Data Cancer Res. 2020

62. Fisher, E.R., Wang, J., Bryant, J., Fisher, B., Mamounas, E. and Wolmark, N. Pathobiology of preoperative chemotherapy. Cancer. 2002; 95: 681-695.

63. Mukai H, Takashima T, Hozumi Y, Watanabe T, Murakami S, Masuda N, Mitsuyama S, Ohmura T, Yajima T, Ohashi Y. Randomized study of taxane

versus TS-1 in women with metastatic or recurrent breast cancer (SELECT BC). Jpn J Clin Oncol. 2010; 40(8):811-4.

64. Broxterman HJ, Georgopapadakou NH. New cancer therapeutics: target-specific in, cytotoxics out? Drug Resist Updat. 2004; 7(2):79-87.

65. Lessene G, Czabotar PE, Colman PM. BCL-2 family antagonists for cancer therapy. Nat Rev Drug Discov. 2008; 7(12):989-1000.

66. Pandit P, Patil R, Palwe V, Gandhe S, Patil R, Nagarkar R. Prevalence of Molecular Subtypes of Breast Cancer: A Single Institutional Experience of 2062 Patients. Eur J Breast Health. 2019; 16(1):39-43.

67. Mane A, Khatib KI, Deshmukh SP, Nag SM, Sane SP, Zade BP. A Comparison of Clinical Features, Pathology and Outcomes in Various Subtypes of Breast Cancer in Indian Women. J Clin Diagn Res. 2015; 9:PC01–PC4.

68. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. Clin Med Res. 2009; 7:4–13.

69. Mid-Atlantic Division of the Cooperative Human Tissue Network. [Accessed December 15, 2012]. Available from:
RL: http://www.cdp.nci.nih.gov/breast/prognostic_dm.html.

70. Ragaz J, Olivotto IA, Spinelli JJ, Phillips N, Jackson SM, Wilson KS, Knowling MA, Coppin CM, Weir L, Gelmon K, Le N, Durand R, Coldman AJ, Manji M. Locoregional radiation therapy in patients with high-risk breast cancer receiving

adjuvant chemotherapy: 20-year results of the British Columbia randomized trial. J Natl Cancer Inst. 2005; 97:116–126.

71. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, Cheang MC, Nielsen TO, Moorman PG, Earp HS, Millikan RC. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006; 295:2492–2502.

72. Olson JE, Ingle JN, Ma CX, Pelleymounter LL, Schaid DJ, Pankratz VS, Vierkant RA, Fredericksen ZS, Wu Y, Couch FJ, Vachon CM, Sellers TA, Weinshilboum RM. A comprehensive examination of CYP19 variation and risk of breast cancer using two haplotype-tagging approaches. Breast Cancer Res Treat. 2007; 102:237–247.

73. Tischkowitz M, Brunet JS, Begin LR, Huntsman DG, Cheang MC, Akslen LA, Nielsen TO, Foulkes WD. Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. BMC Cancer. 2007; 7:134.

74. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M, Perou CM. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res. 2004; 10:5367–5374.

75. Henderson, B. E., D. Powell, I. Rosario, et al.. An epidemiologic study of breast cancer. Journal of the National Cancer Institute. 1974; 53:609–614.

76. Sartwell, P. E., F. G. Arthes, and J. A. Tonascia. Exogenous hormones, reproductive history and breast cancer. Journal of the National Cancer Institute. 1997; 59:1589–1595.

77. Ravnihar, B., D. G. Seigel, and J. Lindtner. An epidemiologic study of breast cancer and benign breast neoplasias in relation to the oral contraceptive and estrogen use. European Journal of Cancer. 1979; 15:395–405.

78. Vessey, M. P., K. McPherson, D. Yeates, and R. Doll. Oral contraceptive use and abortion before first term pregnancy in relation to cancer risk. British Journal of Cancer. 1982; 45:327–331.

79. Kelsey, J. L., L. R. Holford, C. White, E. S. Mayer, S. E. Kilty, and R. M. Acheson.. Oral contraceptives and breast disease. American Journal of Epidemiology. 1978; 107:236–244.

80. Vessey, M. P., R. Doll, K. Jones, K. McPherson, and D. Yeates. An epidemiologic study of oral contraceptives and breast cancer. British Medical Journal. 1979; 1:1755–175

81. Brinton, L. A., R. Hoover, M. Szklo, and F. J. Fraumeni, Jr. Oral contraceptives and breast cancer. International Journal of Epidemiology. 1982; 11:316–322.

82. Janerich, D. T., A. P. Polednak, D. M. Glebatis, and C. E. Lawrence. Breast cancer and oral contraceptive use: A case-control study. Journal of Chronic Diseases. 1983; 36(9): 639–646.

83. Hennekens, C. H., F. E. Speizer, R. J. Lipnick, B. Rosner, C. Bain, C. Belanger, M. Stampfer, W. Willett, and R. Peto. A case-control study of oral contraceptive use and breast cancer. Journal of the National Cancer Institute. 1984; 72:39–42.

84. Rosenberg, L., D. R. Miller, D. W. Kaufman, S. P. Helmrich, P. D. Stolley, D. Schottenfeld, and S. Shapiro. Breast cancer and oral contraceptive use. American Journal of Epidemiology. 1984; 119:167–176.

85. Stadel, B. V., and J. J. Schlesselman. Oral contraceptive use and the risk of breast cancer in women with a prior history of benign breast disease. American Journal of Epidemiology. 1986; 123(3):373–382.

86. Talamini, R., C. La Vecchia, S. Franceschi, F. Colombo, et al. Reproductive, hormonal factors and breast cancer in a northern Italian population. International Journal of Epidemiology. 1985; 14:70–74.

87. Paffenbarger, R. S., J. B. Kampert, and H. G. Chang. Characteristics that predict risk of breast cancer before and after the menopause. American Journal of Epidemiology. 1980; 112(2):258–268.

88. Pike, M. C., B. E. Henderson, J. T. Casagrande, I. Rosario, and G. E. Gray. Oral contraceptive use and early abortion as risk factors for breast cancer in young women. British Journal of Cancer. 1981; 43:72–76.

89. Trapido, E. J. A prospective study of oral contraceptives and breast cancer. Journal of the National Cancer Institute. 1981; 67:1011–1015.

90. McPherson, K., and J. Drife. The pill and breast cancer: Why the uncertainty. British Medical Journal. 1986; 293(6549):709.

91. Olsson, H., M. Landin-Olsson, T. Moller, J. Ranstam, and P. Holm. Oral contraceptive use and breast cancer in young women in Sweden. Lancet. 1985; 30:748–749.

92. Ellery, C., R. MacLennan, G. Berry, and R. P. Sherman.. A case-control study of breast cancer in relation to the use of steroid contraceptive agents. Medical Journal of Australia. 1986; 144:173–176.

93. La Vecchia, C., A. Decarli, M. Fasoli, S. Franceschi, A. Gentile, E. Negri, F. Parazzini, and G. Tognoni. Oral contraceptives and cancers of the breast and the female reproductive tract. Interim results from a case-control study. British Journal of Cancer. 1986; 54(2):311–317.

94. Lipnick, R. J., J. E. Buring, C. H. Hennekens, B. Rosner, W. Willet, C. Bain, M. P. Stampfer, G. Colditz, R. Peto, and F. E. Speizer. Oral contraceptives and breast cancer: A prospective cohort study. Journal of the American Medical Association. 1986; 255:58–61.

95. Romieu I, W. C. Willett, G. A. Colditz, M. J. Stampfer, B. Rosner, C. H. Hennekens, and F. E. Speizer. Prospective study of oral contraceptive use and risk of breast cancer in women. Journal of the National Cancer Institute. 1989; 81(17):1313–1321.

96. Stanford, J. L, L. A. Brinton, and R. N. Hoover. Oral contraceptives and breast cancer: Results from an expanded case-control study. British Journal of Cancer. 1989; 60:375–381.

97. Meirik, O, E. Lund, H. O. Adami, R. Bergstrom, T. Christofferson, and P. Bergsjo. Oral contraceptive use and breast cancer in young women. A joint national case-control study in Sweden and Norway. Lancet. 1986;2:650–654.

98. Paul, C., D. C. G. Skegg, G. H. Spears, and J. M. Kaldor. Oral contraceptives and breast cancer: A national study. British Medical Journal. 1986; 293:723–726.

99. Miller, D. R., L. Rosenberg, D. W. Kaufman, D. Schottenfeld, P. D. Stolley, and S. Shapiro. Breast cancer risk in relation to early oral contraceptive use. Obstetrics and Gynecology. 1986; 68:863–868.

100. U.K. National Case-Control Study Group. Oral contraceptive use and breast cancer risk in young women. Lancet. 1989; 8645:973–982.

101. Piper, J. M., and D. L. Kennedy. Oral contraceptives in the US: Trends in content and potency. International Journal of Epidemiology. 1987; 16(2):215–221.

102. Olsson, H, T. R. Moller, and J. Ranstam. Early oral contraceptive use and breast cancer among premenopausal women: Final report from a study in southern Sweden. Journal of the National Cancer Institute. 1989; 81(13):1000–1004.

103. Miller, D. R., L. Rosenberg, D. W. Kaufman, P. D. Stolley, M. E. Warshaver, and S. Shapiro. Breast cancer before age 45 and oral contraceptive use: New findings. American Journal of Epidemiology. 1989; 129:269–280.

104. (CASH) Cancer and Steroid Hormone Study of the Centers for Disease Control and the National Institute of Child Health and Human Development: Long-term

oral contraceptive use and the risk of breast cancer. Journal of the American Medical Association. 1983; 249:1591–1595.

105. Meirik, O., T. M. Farley, E. Lund, H. O. Adami, T. Christofferson, and P. Bergsjo. Breast cancer and oral contraceptives: Patterns of risk among parous and nulliparous women—further analysis of the Swedish-Norwegian material. Contraception. 1989; 39(5):471–475.

106. Kay, C. R., and P. C. Hannaford. Breast cancer and the pill—A further report from the Royal College of General Practitioners' oral contraception study. British Journal of Cancer. 1988; 58:675–680.

107. Jick, S. S., A. M. Walker, A. Stergachis, and H. Jick. Oral contraceptives and breast cancer. British Journal of Cancer. 1989; 59:618–621.

108. (WHO) World Health Organization Collaborative Study of Neoplasia and Steroid Contraceptives. Breast cancer and combined oral contraceptives: Results from a multinational study. British Journal of Cancer. 1990; 61:110–119.

109. Peto, J. Oral contraceptives and breast cancer: Is the CASH study really negative? Lancet. 1989; 1:552.

110. Stadel, B. V., G. L. Rubin, L. A. Webster, J. J. Schlesselman, and P. A. Wingo. Oral contraceptives and breast cancer in young women. Lancet. 1985; 2:970–973.

111. Lee, N. C., L. Rosero-Bixby, M. W. Oberle, C. Grimaldo, A. S. Whatley, and E. Z. Rovira. A case-control study of breast cancer and hormonal contraception in Costa Rica. Journal of the National Cancer Institute. 1987; 79(6):1247–1254.

112. Schlesselman, J. J., B. V. Stadel, P. Murray, S. Lai. Breast cancer in relation to type of estrogen contained in oral contraceptives. Contraception. 1987; 36(6):595–612.

113. Murray, P. P., B. V. Stadel, and J. J. Schlesselman. Oral contraceptive use in women with a family history of breast cancer. Obstetrics and Gynecology. 1989; 73:977–983.

114. Fasal, E., and R. S. Paffenbarger.Oral contraceptives as related to cancer and benign lesions of the breast. Journal of the National Cancer Institute. 1975; 55:767–773.

115. Lees, A. W., P. E. Burns, and M. Grace. Oral contraceptives and breast disease in premenopausal northern Albertan women. International Journal of Cancer. 1978; 22:700–707.

116. Kelsey, J. L., D. B. Fischer, T. R. Holford, V. A. Livolsi, E. D. Moston, I. S. Goldenberg, and C. White. Exogenous estrogens and other factors in the epidemiology of breast cancer. Journal of the National Cancer Institute.1981; 67:327–333.

117. Ravnihar, B, M. P. Zakelj, K. Kosmelj, and J. Stare. A case-control study of breast cancer in relation to oral contraceptive use in Slovenia. Neoplasma. 1988; 35:109–121.

118. Spirtas, R. Breast cancer before age 45 and oral contraceptive use: New findings (letter). American Journal of Epidemiology. 1989; 130:1255–1256.

119. Jankowska E, Stoj J, Karpowicz P, Osmulski PA, Gaczynska M. The proteasome in health and disease. Cur Pharm Des. 2013;19(6):1010–1028.

120. Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer. 2006;6(5):369–381.

121. Amm I, Sommer T, Wolf DH. Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. Biochim Biophys Acta. 2014;1843(1):182–196.

122. Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature. 2003;426(6968):895–899.

123. Guerriero CJ, Brodsky JL. The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. Physiol Rev. 2012;92(2):537–576.

124. Pagan J, Seto T, Pagano M, Cittadini A. Role of the ubiquitin proteasome system in the heart. Circ Res. 2013;112(7):1046–1058.

125. Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. 2006;443(7113):780–786.

126. Turnbull EL, Rosser MF, Cyr DM. The role of the UPS in cystic fibrosis. BMC Biochem. 2007;8 Suppl 1:S11.

127. Crowder JJ, et al. Rkr1/Ltn1 Ubiquitin Ligase-Mediated Degradation of Translationally Stalled Endoplasmic Reticulum Proteins. J Biol Chem. 2015;290(30):18454–18466.

128. Gardner RG, et al. Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J Cell Biol. 2000; 151(1):69–82.

129. Metzger MB, Maurer MJ, Dancy BM, Michaelis S. Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. J Biol Chem. 2008; 283(47):32302–32316.

130. Watts SG, Crowder JJ, Coffey SZ, Rubenstein EM. Growth-based Determination and Biochemical Confirmation of Genetic Requirements for Protein Degradation in Saccharomyces cerevisiae. J Vis Exp. 2015; e52428.

131. Cohen I, Geffen Y, Ravid G, Ravid T. Reporter-based growth assay for systematic analysis of protein degradation. J Vis Exp. 2014; e52021.

132. Ravid T, Kreft SG, Hochstrasser M. Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. EMBO J. 2006; 25(3):533–543.

133. Kohlmann S, Schafer A, Wolf DH. Ubiquitin ligase Hul5 is required for fragment-specific substrate degradation in endoplasmic reticulum-associated degradation. J Biol Chem. 2008; 283(24):16374–16383.

134. Medicherla B, Kostova Z, Schaefer A, Wolf DH. A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. EMBO Rep. 2004; 5(7):692–697.

135. Habeck G, Ebner FA, Shimada-Kreft H, Kreft SG. The yeast ERAD-C ubiquitin ligase Doa10 recognizes an intramembrane degron. J Cell Biol. 2015; 209(2):261–273.

136. Tran JR, Brodsky JL. Assays to measure ER-associated degradation in yeast. Methods Mol Biol. 2012; 832:505–518.

137. Kao SH, et al. GSK3beta controls epithelial-mesenchymal transition and tumor metastasis by CHIP-mediated degradation of Slug. Oncogene. 2014; 33(24):3172–3182.

138. Hampton RY, Rine J. Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J Cell Biol. 1994; 125(2):299–312.

139. Hochstrasser M, Varshavsky A. In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. Cell. 1990; 61(4):697–708.

140. Schneider-Poetsch T, et al. Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nat Chem Biol. 2010; 6(3):209–217.

141. Whiffen AJ, Bohonos N, Emerson RL. The Production of an Antifungal Antibiotic by Streptomyces griseus. J Bacteriol. 1946; 52(5):610–611.

142. Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem. 1971; 246(1):174–181.

143. Ennis HL, Lubin M. Cycloheximide: Aspects of Inhibition of Protein Synthesis in Mammalian Cells. Science. 1964; 146(3650):1474–1476.

144. Kerridge D. The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis. J Gen Microbiol. 1958; 19(3):497–506.

145. Chakrabarti S, Dube DK, Roy SC. Effects of emetine and cycloheximide on mitochondrial protein synthesis in different systems. Biochem J. 1972; 128(2):461–462.

146. Seufert W, Jentsch S. In vivo function of the proteasome in the ubiquitin pathway. EMBO J. 1992; 11(8):3077–3080.

147. Varshavsky A. Discovery of the biology of the ubiquitin system. JAMA. 2014; 311(19):1969–1970.

148. Heinemeyer W, Kleinschmidt JA, Saidowsky J, Escher C, Wolf DH. Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. EMBO J. 1991; 10(3):555–562.

149. Sommer T, Jentsch S. A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. Nature. 1993; 365(6442):176–179.

150. Hiller MM, Finger A, Schweiger M, Wolf DH. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science. 1996; 273(5282):1725–1728.

151. Hanna J, Leggett DS, Finley D. Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. Mol Cell Biol. 2003; 23(24):9251–9261.

152. Hornbeck PV. Enzyme-Linked Immunosorbent Assays. Curr Protoc Immunol. 2015; 110:2.1.1–2.1.23.

153. Mitch WE, Goldberg AL: Mechanisms of muscle wasting: The role of the ubiquitin-proteasome system. N Engl J Med. 1996; 335: 1897–1905

154. Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL: Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class 1 molecules. Cell. 1994; 78: 761–771

155. Baehrecke EH: Autophagy: Dual roles in life and death? Nat Rev Mol Cell Biol. 2005; 6: 505–510

156. Lardeux BR, Mortimore GE: Amino acid and hormonal control of macromolecular turnover in perfused rat liver. Evidence for selective autophagy. J Biol Chem. 1987; 262: 14514 – 14519

157. Goll DE, Thompson VF, Li H, Wei W, Cong J: The calpain system. Physiol Rev. 2003; 83: 731– 801

158. Salvesen GS, Dixit VM: Caspases: Intracellular signaling by proteolysis. Cell 1997; 91: 443– 446

159. Glickman MH, Ciechanover A: The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. Physiol Rev. 2002; 82: 373– 428

160. Pickart CM: Back to the future with ubiquitin. Cell. 2004; 116: 181–190

161. Baumeister W, Walz J, Zuhl F, Seemuller E: The proteasome: Paradigm of a self-compartmentalizing protease. Cell. 1998; 92: 367–380

162. Muratani M, Tansey WP: How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol. 2003; 4: 192– 201

163. Lipford JR, Smith GT, Chi Y, Deshaies RJ: A putative stimulatory role for activator turnover in gene expression. Nature. 2005; 438: 113–116

164. Karin M, Ben-Neriah Y: Phosphorylation meets ubiquitination: The control of NF-kappaB activity. Annu Rev Immunol. 2000; 18: 621– 663

165. Jensen TJ, Loo MA, Pind S, Williams DB, Goldberg AL, Riordan JR: Multiple proteolytic systems, including the proteasome contribute CFTR processing. Cell. 1995; 83: 129 –135

166. Ward CL, Omura S, Kopito RR: Degradation of CFTR by the ubiquitin-proteasome pathway. Cell. 1995; 83: 121–127

167. Meusser B, Hirsch C, Jarosch E, Sommer T: ERAD: The long road to destruction. Nat Cell Biol. 2005; 7: 766 –772

168. Hicke L, Dunn R: Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu Rev Cell Dev Biol. 2003; 19: 141–172

169. Sigismund S, Polo S, Di Fiore PP: Signaling through monoubiquitination. Curr Top Microbiol Immunol. 2004; 286: 149 –185

170. Haas AL, Rose IA: The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis. J Biol Chem. 1982; 257: 10329 –10337

171. Handley PM, Mueckler M, Siegel NR, Ciechanover A, Schwartz AL: Molecular cloning, sequence, and tissue distribution of the human ubiquitin-activating enzyme E1. Proc Natl Acad Sci U S A. 1991; 88: 258 –262

172. Jentsch S: The ubiquitin-conjugation system. Annu Rev Genet. 1992; 26: 179 – 207

173. Jackson PK, Eldridge AG, Freed E, Furstenthal L, Hsu JY, Kaiser BK, Reimann JD: The lore of the RINGs: Substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. 2000; 10: 429 – 439

174. Ingham RJ, Gish G, Pawson T: The Nedd4 family of E3 ubiquitin ligases: Functional diversity within a common modular architecture. Oncogene. 2004; 23: 1972–1984

175. Scheffner M, Nuber U, Huibregtse JM: Protein ubiquitination involving an E1–E2-E3 enzyme ubiquitin thioester cascade. Nature. 1995; 373: 81– 83

176. Huibregtse JM, Scheffner M, Beaudenon S, Howley PM: A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A 92: 5249, 1995

177. Albrecht U, Sutcliffe JS, Cattanach BM, Beechey CV, Armstrong D, Eichele G, Beaudet AL: Imprinted expression of the murine Angelman syndrome gene, Ube3a, in hippocampal and Purkinje neurons. Nat Genet 17: 75–78, 1997

178. Malik B, Schlanger L, Al-Khalili O, Bao H-F, Yue G, Price SR, Mitch WE, Eaton DC: ENaC degradation in A6 cells by the ubiquitin-proteasome proteolytic pathway. J Biol Chem 276: 12903–12910, 2001

179. Kamynina E, Debonneville C, Hirt RP, Staub O: Liddle's syndrome: A novel mouse Nedd4 isoform regulates the activity of the epithelial Na channel. Kidney Int 60: 466 – 471, 2001

180. Petroski MD, Deshaies RJ: Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol 6: 9 –20, 2005

181. Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM: RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc Natl Acad Sci U S A 1999: 11364 –11369.

182. Seol JH, Feldman RM, Zachariae W, Shevchenko A, Correll CC, Lyapina S, Chi Y, Galova M, Claypool J, Sandmeyer S, Nasmyth K, Deshaies RJ, Shevchenko A, Deshaies RJ: Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. 1999: 13: 1614 –1626,

183. Elenbaas B, Dobbelstein M, Roth J, Shenk T, Levine AJ: The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. Mol Med 1996; 2: 439 – 451.

184. Varshavsky A, Turner G, Du F, Xie Y: Felix Hoppe-Seyler Lecture 2000: The ubiquitin system and the N-end rule pathway. Biol Chem. 2000; 381: 779 –789.

185. Rao H, Uhlmann F, Nasmyth K, Varshavsky A: Degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability. Nature 2001; 410: 955–959.

186. Hu RG, Sheng J, Qi X, Xu Z, Takahashi TT, Varshavsky A: The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators. Nature. 2005; 437: 981–986.

187. Lecker SH, Jagoe RT, Gomes M, Baracos V, Bailey JL, Price SR, Mitch WE, Goldberg AL: Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. FASEB J. 2004; 18: 39 –51.

188. Kaelin WG Jr: How oxygen makes its presence felt. Genes Dev 16: 1441–1445.

189. Hatakeyama S, Nakayama KI: U-box proteins as a new family of ubiquitin ligases. Biochem Biophys Res Commun. 2003; 302: 635– 645.

190. Li HH, Kedar V, Zhang C, McDonough H, Arya R, Wang DZ, Patterson C: Atrogin-1/muscle atrophy F-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an SCF ubiquitin ligase complex. J Clin Invest. 2004; 114: 1058 –1071.

191. Voges D, Zwickl P, Baumeister W: The 26S proteasome: A molecular machine designed for controlled proteolysis. Annu Rev Biochem. 2000; 68: 1015–1068.

192. Kisselev AF, Goldberg AL: Proteasome inhibitors: From research tools to drug candidates. Chem Biol. 2001; 8: 739 –758.

193. Groll M, Bajorek M, Kohler A, Moroder L, Rubin DM, Huber R, Glickman MH, Finley D: A gated channel into the proteasome core particle. Nat Struct Biol. 2000; 7: 1062–1067.

194. Goldberg AL: Nobel committee tags ubiquitin for distinction. Neuron 45: 339 – 344, 2005

195. Benaroudj N, Zwickl P, Seemuller E, Baumeister W, Goldberg AL: ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. Mol Cell 11: 69 –78, 2003

196. Schmidt M, Hanna J, Elsasser S, Finley D: Proteasomeassociated proteins: Regulation of a proteolytic machine. Biol Chem. 2005; 386: 725–737.

197. Goldberg AL: Protein degradation and protection against misfolded or damaged proteins. Nature.2003; 426: 895– 899.

198. Smith DM, Kafri G, Cheng Y, Ng D, Walz T, Goldberg AL: ATP binding to PAN or the 26S ATPases causes association with the 20S proteasome, gate opening, and translocation of unfolded proteins. Mol Cell. 2005; 20: 687– 698.

199. Kisselev AF, Akopian TN, Woo KM, Goldberg AL: The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. J Biol Chem. 1999; 274: 3363–3371.

200. Reits E, Neijssen J, Herberts C, Benckhuijsen W, Janssen L, Drijfhout JW, Neefjes J: A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation. Immunity. 2004; 20: 495–506.

201. Saric T, Graef CI, Goldberg AL: Pathway for degradationof peptides generated by proteasomes: A key role for thimet oligopeptidase and other metallopeptidases. J Biol Chem. 2004; 279: 46723– 46732.

202. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T: The ubiquitin-proteasome pathway is required for processing the NF-kappaB1 precursor protein and the activation of NF-kappaB. Cell. 1994; 78: 773–785.

203. Venkatraman P, Wetzel R, Tanaka M, Nukina N, Goldberg AL: Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing proteins. Mol Cell.2004; 14: 95–104.

204. W. Heinemeyer, M. Fischer, T. Krimmer, U. Stachon, D.H. Wolf, The active sites of the eukaryotic 20S proteasome and their involvement in subunit precursor processing, J. Biol. Chem. 1997;272: 25200-09.

205. C.S. Arendt, M. Hochstrasser, Identi¢cation of the yeast 20S proteasome catalytic centers and subunit interactions required for active-site formation, Proc. Natl. Acad. Sci. USA. 1997; 94: 7156-61.

206. H.A. Chapman, R.J. Riese, G.P. Shi, Emerging roles for cysteine proteases in human biology, Annu. Rev. Physiol. 1997; 59:63-88.

207. Vinitsky, C. Michaud, J.C. Powers, M. Orlowski, Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteinase complex, Biochemistry. 1992; 31: 9421-28.

208. D.H. Lee, A.L. Goldberg, Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae, J. Biol. Chem. 1996; 271: 27280-84.

209. K.K. Wang, P.W. Yuen, Calpain inhibition : an overview of its therapeutic potential, Trends Pharmacol. Sci. 1994; 15: 412-419.

210. J. Adams, M. Behnke, S. Chen, A.A. Cruickshank, L.R. Dick, L. Grenier, J.M. Klunder, Y.T. Ma, L. Plamondon, R.L. Stein, Potent and selective inhibitors of the proteasome: dipeptidyl boronic acids, Bioorg. Med. Chem. Lett. 1998; 8: 333-38.

211. C.V. Harding, J. France, R. Song, J.M. Farah, S. Chatterjee, M. Iqbal, R. Siman, Novel dipeptide aldehydes are proteasome inhibitors and block the MHC-I antigen-processing pathway, J. Immunol. 1995; 155:) 1767-75.

212. V.J. Palombella, O.J. Rando, A.L. Goldberg, T. Maniatis, The ubiuitin-proteasome pathway is required for processing the NF-kappaB1 precursor protein and the activation of NF-kappa-B, Cell. 1994; 773-85.

213. S. Tsubuki, Y. Saito, M. Tomioka, H. Ito, S. Kawashima, Di¡erential inhibition of calpain and proteasome activities by peptidaldehydes of di-leucine and tri-leucine, J. Biochem. 1996; 119: 576.

214. M.E. Figueiredo-Pereira, K.A. Berg, S. Wilk, A new inhibitor of chymotrypsin-like activity of the multicatalytic proteinase complex (20S proteasome) induces accumulation of ubiquitin-protein congates in a neuronal cell, J. Neurochem. 1994; 63: 1578-81.

Chapter 4

Methedology

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

4. Methods

I. Human study

4.1TYPE OF STUDY **:** Prospective study :

4.2: STUDY DESIGN **:** Observational explorative study

4.3. DURATION OF COLLECTION OF DATA:

Prospective study –From august 2016 to Nov 2019

4.4: PLACE OF CONDUCT OF RESEARCH:

Human study: SDM collage of medical sciences and hospital, Dharwad.

4.5: STUDY POPULATION:

The selection of sample was carried out from the outpatient and inpatients department of our institution. 155 breast cancer individuals enrolled in the study. Whole enrolled population of breast cancer was divided in to two groups. Group-1consisted of 48 clinically diagnosed breast cancer patients with molecular sub-types with history of cyclical oral contraceptive pill use for at least 6 months of duration. Group-2 consisted of 107 age matched controls with breast cancer of different molecular subtypes with no history of oral contraceptive pill use.

4.6 SAMPLE SIZE CALCULATION

With 95% confidence level and margin of error of ±8%, a sample size of 151 subjects will allow the study to determine Association of use of oral contraceptive pill (OCP) with expression pattern of different molecular subtypes of breast cancer.

The calculation was done by using the formula: $\mathbf{n = z^2p(1-p)/d^2,}$

where Z= z statistic at 5% level of significance

d is margin of error

p is maximum anticipated prevalence rate of breast cancer.

4.7 Selection criteria

Each individual was briefed about the study before; its importance and written consent of participants were taken before interview. Subjects using contraceptives other than cyclical, Individuals taking Hormonal replacement therapy (HRT) were excluded from the study. Individuals with other associated malignancies were excluded from the study. Molecular sub-typing was done based on whether individuals are ER+/-, PR+/-or HER2 +/- or and also based upon American Pathologists/American Society of Clinical Oncology (CAP/ASCO) guidelines a new clinical classification Luminal A (LA), Luminal B (LB), Non Luminal (NL)/HER2+ enriched or Basal (BA)/ (TNBC) [14]. The basic parameters and detailed history were recorded. General check-up of pulse, blood pressure, height, weight, food habits, were recorded. Detailed obstetric history of gravidity, parity, age of menarche, family history of breast cancer and breast feeding were noted.

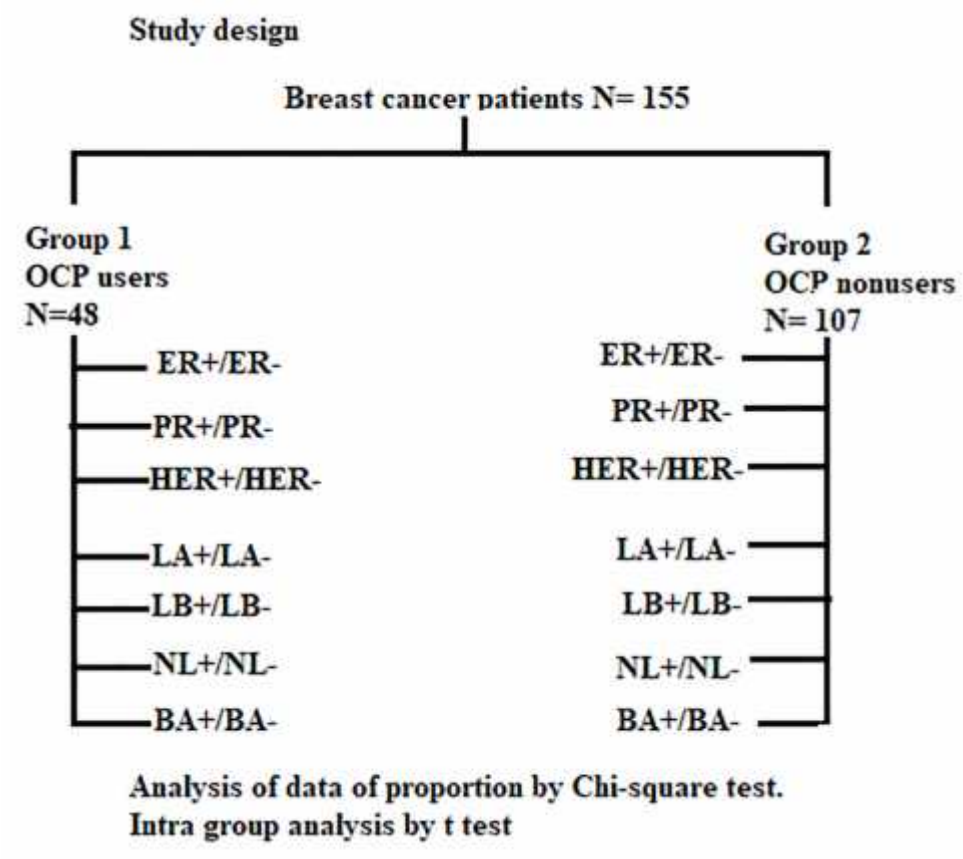


Figure 12: ER+:Estrogen Receptor+; PR+: Progesterone Receptor+; HER2+:Human Epidermal Growth Factor Receptor 2+; LA :Luminal A; LB: Luminal B; NL: Non luminal/ HER2 enriched; BA; Basal like/TNBC.

Immuno-histo-chemical Scoring System for ER, PR and HER2 and clinical classification

All records were collected from the hospital medical records. The histo-pathological and immune-histo-chemical (IHC) examination was performed in accordance with the College of American Pathologists/American Society of Clinical Oncology (CAP/ASCO) guidelines by a pathologist. ER and PR scoring for all cases were done using Allred scoring. [1]

Allred system of scoring for ER and PR: ER and PR are nuclear receptors. In Allred system of scoring, score 0-5 is given to the cells depending on the fraction of cells which are stained, proportion score [PS]) and score 0-3 is given depending on the intensity of staining, intensity score [IS]. By adding the PS and IS, we considered the final Allred score (PS + IS = AS).

Scoring for HER 2+/neu over expression: HER2+/neu is a cell membrane receptor. Depending on the intensity of staining a score of 0-3 is awarded to the cells. A positive HER2+/neu result is an immune-histo-chemical staining of 3+, uniform, intense membrane staining of >30% of invasive tumor cells. A negative HER2+/neu result is an immune-histo-chemical staining of 0 or 1+.

In Allred scoring system, only the invasive tumor cells should be evaluated ed as ER/PR staining is present in normal breast epithelial cells as well. Here, the normal adjucent breast epithelial cells act as internal positive control. According to ASCO/CAP guidelines, we classified breast cancer cases in 4 subtypes based on hormonal receptor and HER 2+ status. These were luminal A (ER+ and/or PR+/HER2−), luminal B (ER+ and/or PR+/HER2+), Non-luminal/ HER2-enriched (ER− and PR−/HER2+) and Basal like (ER− and PR−/HER2−). Those patients who had HER 2+ expression (Equivocal) were not included in molecular subtype analysis.

4.8 Statistical analysis

The results were summarized descriptively first. For continuous variables, the summary statistics of mean± standard deviation (SD) were used. For categorical data, the number and percentage were used in the data summaries and diagrammatic presentation. Chi-square ($\chi 2$) test was used for association between two categorical

variables. After confirming normality assumption, the data were analyzed by parametric test (student t test) which indicates the level of difference of means between two groups. Logistic linear regression analysis was employed to assess the effect of age on different subtypes of cancers after adjusting the effect of other background confounding variables. The software used was SPSS-20 (USA, Chicago) and Microsoft office 2007.

II. In-vitro study

4.1Cell culture

Human breast cancer cell lines MDA-MB-231, MCF-7 were obtained from the National Centre for Cell Sciences, Pune, India. Cells were grown in respective medium as prescribed by the supplier.MCF-7, MDA-MB-231 were cultured in Roswell Park Memorial Institute media (RPMI) containing phenol red and supplemented with 10% Fetal Bovine Serum (FBS). Cell lines were cultured in a 5% CO_2humidified atmosphere at 37°C. Cells were utilized up to a maximum of 28 passages and were subject to regular mycoplasma testing. β-estadiol, Cyclohexamide and MG-132 were obtained from Aldrisch sigma.

4.2 Effect of 17 β-estrdiol on MDA-MB-231 and MCF-7 cell lines on EGFR expression

EGFR is a transmembrane receptor whose over expression in breast cancer predicts for poor prognosis. This study was designed to investigate whether estrogen plays an active role in expression or suppression of EGFR in MCF-7 and MDA-MB-231 cell lines. We hypothesized that 100 nM of 17β-estradiol will lead to over expression of

EGFR in MCF-7 and suppresses in MDA-MB-231 cell lines. We standardised the prior the concentration of estrogen to be used i.e 100nM.

Technique: MDA-MB-231 and MCF-7 cell lines were cultured in large flask. At 70-80 percent confluence cell were trypsinized twice, centrifuged at 1200rcf for 4 min, supernatant was removed. FBS was added to approximately 4×10^6 cells/well were placed in 6 well plates and starved for 12 hours. Both cell lines were treated with 100 nM β-estradiol. Expression of EGFR at 0 and 3 hours time intervals was assessed by western blot.

4.3 Cycloheximide chase to assess the effect of 17 β-estradiol on MDA-MB-231 cells on EGFR expression

A difference in steady state protein levels after inhibiting degradative pathways provides strong proof for the contribution of proteolysis to the control of protein abundance [2]. However, such an analysis still does not furnish information regarding the kinetics of protein turnover. Cycloheximide chase run followed by western blotting overcomes this deficit by allowing researchers to visualize protein degradation over time [3-5]. Further, because protein detection following Cycloheximide chase is typically carried out by western blotting, radioactive isotopes and lengthy immune-precipitation measures are not needed for Cycloheximide chase, unlike other commonly used pulse chase techniques, that are also performed to visualize protein degradation [6]. Cycloheximide chase is suitable for analyzing protein stability over a short time course that are in minutes. Over longer time courses Cycloheximide, a global inhibitor of translation, is toxic to cells, likely due to exhaustion of ubiquitin [7]. Additionaly, analyses of protein stability over longer time

courses are more likely to be negotiated by indirect effects of globally decreased protein synthesis on the degradation of the protein of interest *e.g.*, degradation of a short-lived protein involvement in the degradation of the protein of interest. Other techniques, such as pulse chase metabolic labeling experiments, are therefore better suited for investigating the degradation of long-lived proteins and may be performed to corroborate results obtained in Cycloheximide chase experiments.

Technique: MDA-MB-231 cell lines were cultured in large flask. At 70-80 per cent confluence cells were trypsinized twice, centrifuged at 1200 rcf for 4 min, supernatant was removed. FBS added approximately 4×10^6 cells/well were placed in 6 well plates and starved for 12 hours. Cells were treated with 100 nM 17β-estradiol. Expression of EGFR at 0, 0.5, 1, 2, 3 and 4 hours' time intervals with or without 50µg cycloheximide was assessed by western blot.

4.4 EGFR degradation is due to ubiquitination.

The ubiquitin-proteasome system (UPS) is a major protein degradative pathway involved in the preservation of cellular structure and function [8,9]. While the 20S proteasome is involved in direct protein hydrolysis, degradation of ubiquitinated proteins by the 26S proteasome is a relatively more important process in protein turnover [10-12]. Ubiquitination of proteins devised for degradation is an ATP-dependent process and involves support of three ubiquitin ligase enzymes. Particularly, the ubiquitin moiety is transported by Ubiquitin-activating enzyme E1 to the Ubiquitin-conjugating enzyme E2 afterwards formation of ubiquitin chain ligation on target proteins by a substrate specific E3 ubiquitin ligase [13]. Selected components of the 26S cap proteins are associated with recognition and transport of ubiquitinated proteins for degradation by the 26S proteasome [14, 15]. MG-132 is a

very potent, reversible, and cell-permeable proteasome blocker. It decreases the degradation of ubiquitin-conjugated proteins in mammalian cells and permeable strains of yeast by the 26S complex without upsetting its ATPase or iso-peptidase activities.

Technique: MDA-MB-231 cell lines were cultured in large flask. At 70-80 percent confluence cells were trypsinized twice, centrifuged at 1200rcf for 4 min, supernatant was removed. FBS was added approximately 4×10^6 cells/well were placed in 6 well plates and starved for 12 hours. Cells were treated with 100 nM 17β-estradiol and 50 µg Cycloheximide. Expression of EGFR at 0, 0.5, 1, 2, 3 and 4 hours time intervals with or without MG-132 was assessed by western blot.

4.5 Total cell extraction and western blotting

Cells were obtained from 6-well plates after the treatment after adding lysate. Cell lysates were collected on ice bywashing x1 in ice-cold PBS then scraping in 100 µL of lysis buffer (150 mM NaCl, 50 mM Tris base pH 8, 1 %NP-40 containing protease and phosphatase inhibitors. The lysates were centrifuged at 14,000 rcf for 10 min at 4 °C and protein concentration of thesupernatants was determined by bicinchoninic acid assay (BCA) assay. For the expression analysis in different breast cancer cell lines, total protein was extracted and quantitated as described previously [16]. Total protein was separated on 10 % Bis–Tris PAGE gel using Tris–Hcl buffer and the proteins were transferred to nitrocellulose membranes (Himedia) using a transfer apparatus at 65 V for 90 min. The antibodies were used against EGFR (rabbit monoclonal, BD Biosciences, CA-9061) and GAPDH (Santa Cruz Biotechnology, CA-166574). Appropriate secondary antibodies conjugated to horseradish peroxidase (BioRad) were incubated with respective membranes for 2 h at room temperature. The

membranes were developed using ECL plus (BioRad), and the image was captured using enhanced Chemi-luminescence system, G: BOXChemi XX6/XX9. Immunoblot for GAPDH was considered as internal control for loading. The protein bands were quantified and normalized relatively as the control band with Image J, version 1.35d (National Institutes of Health Image software).

4.6 Wound healing assay

The wound healing assay is a standard *in vitro* technique for probing collective cell migration in two dimensions. In this assay, a cell-free area is created in a confluent monolayer by physical exclusion or by removing the cells from the area through mechanical, thermal or chemical damage. The exposure to the cell-free area induces the cells to migrate into the gap.

Technique: Briefly, the MDA-MB-231 cells (4×10^6 cells/well) were plated in 6-well plates for 48 h to a confluence of about 80%, then wounded by scratching with a p200 pipette tip. Thereafter, the debris was removed and we washed the cells once with 1 mL of the growth medium to assure the edges of the scratch were smoothed by washing. We took utmost care to make the wounds of the same dimensions, both for the experimental and control cells to minimize any possible variety resulting from a difference in scratch width. The cells were then incubated with DMEM medium containing 0.5% FBS and treated with 100 nM of 17β-estradiol. The control sample harboured the cells and a standard medium without any active agents. The MDA-MB-231 cell migration was assessed by gap closure migration assay, embedded by free ImageJ software (version 1.50i, National Institute of Health, Bethesda, MD, USA). The area of the initial wound was measured, followed by gap area measurements after 24 h. The migration factor was represented as the gap area value over the initial scratch area.

4.7 Statistical analysis

Statistical analysis was carried out by using Graph Pad Prism version 7.04 .Statistical analysis of expression of EGFR. $P<0.05$ was considered to be statistically significant. Results for normally distributed data were analysed using student t test and ANOVA.

Chapter 5

Results

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

5. Results

5.1 Human study

As per the Table-2, there was no significant difference in age among OCP users and non-users. The mean age of OCP users was 47.6 5 years and non-users was 49.8 years. There was no significant difference between height, weight and BMI among OCP users and non-users. There was no significant difference between parity, age of menarche (AOM), family history of breast cancer, menopausal status, breast feeding and stage of cancer on admission in OCP users and non-users. There was no significant difference in the stage of Basal like (TNBC) cases of OCP users compared to TNBC cases of non-users. (Table-3) The average duration of OCP intake in cases was 1year 3 months. There was no significant change in Ki-67 levels in vases of OCP users (31.4±18.3) compared to non users (33.4±21.3) (p=0.480).

A significant increase in the proportion of ER+ cases in OCP users (62.5%) compared to non-users (45.7%) with relative risk 1.97 was observed. There was significant increase in the proportion of PR+ cases in OCP users (58.3%) compared to non-users (36.4%) with relative risk 2.44. But, there was no significant difference in the proportion of HER2+ cases in OCP users (43.7%) compared to non-users (44.8%). (Table-4) (Fig-14) A significant increase in the proportion of Luminal B cases in OCP users (41.1%) compared to non-users (21.4%) was observed. But, there was no significant difference in the proportion of Basal like/ TNBC cases in OCP users (27.1%) compared to non-users (25.2%). (Table-4) (Fig-15)

There was a significant in decrease in the age at admission in ER+ cases of OCP users (45.3years) compared to non-users (52.2years) (Fig-16). On the contrary, there was significant higher age was observed at time of admission in TNBC cases of OCP users

(53.1years) when compared to non-users (45.5yeas) (Table-5) (Fig-16). Upon logistic regression among OCP users, the likelihood of ER+, PR+ and Luminal A was 11%, 10% and 13% less with 1 year of higher age respectively and among OCP users, the likelihood of TNBC was 18% more with 1 year of higher age. (Table-6)

Table-2: Demographic characters, parity and Age of menarche (AOM) of breast cancer patients in OCP users and non-users

	OCP users (N=48)	OCP non users (N=107)	T value	P value
Age (yrs)	47.6±8.4	49.8±8.1	-1.482	0.139
Height (cm)	147±31	138.2±44	1.322	0.188
Weight (Kgs)	57.4±9.8	58.3±3	-0.515	0.607
BMI (Kg/M^2)	24.1±3.6	25.3±3.3	-1.972	0.051
Parity	2.59±0.9	2.98±1.8	-1.273	0.206
AOM (yr)	13.02±0.5	13.19±0.6	-1.434	0.154

* ($p<0.05$); BMI: Body mass index; AOM: Age of menarche

Table-3: Reproductive history, family history of breast cancer (FHBC) and stage of breast cancer patients in OCP users and non-users.

		OCP users (N=48)	OCP non users (N=107)	Chi square value	p-value	Odds Ratio
FHBC		3 (6.6)/45	11 (10.2)/96	0.655	0.316	0.582 [0.155-2.188]
Nulliparity		7 (14.50/41	21 (19.6)/85	0.607	0.295	0.691 [0.272-1.757]
Menopause		38 (79.1)/9	91 (85)/15	0.433	0.289	0.696[0.280-1.727]
HOBF		40 (83.3)/8	85 (79.4)/21	0.214	0.412	1.235 [0.504-3.029]
Stage	2	9 (18.7)	19 (17.7)	3.352	0.187	
	3	28 (58.3)	48 (44.8)			
	4	11 (22.9)	40 (37.3)			

* ($p<0.05$); FHBC : Family history of breast cancer; HOBF: History of breast feeding. (Values in the brackets are in percentage)

A. B.

Figure-13: (A) Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+ in total number of cases. (B) Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2+ enriched and Basal like (BA)/TNBC in total number of cases. (Values are in percentage)

Table-4; Phenotype/ clinical sub- types status in OCP users and non-users in breast carcinoma

	OCP users (N=48)	OCP non users (N=107)	Chi square value	P value	Odds Ratio
ER+	30 (62.5)/18	49(45.7)/58	3.700	0.040*	1.973 [0.982-3.962]
PR+	28 (58.3)/20	39 (36.4)/68	6.467	0.009*	2.441 [1.217-4.895]
HER 2+	21 (43.7)/27	48 (44.8)/59	0.017	0.519	0.956 [0.482-1.898]
Luminal A	12 (25)/36	23 (21.4)/84	0.233	0.387	1.217 [0.547-2.709]
Luminal B	20 (41.6)/28	23 (21.4)/84	6.726	0.009*	2.609 [1.249-5.447]
Non-luminal	5 (10.4)/43	33 (30.8)/74	7.469	0.004*	0.264 [0.095-0.718]
Basal like	13 (27.1)/35	27 (25.2)/80	0.059	0.477	1.101 [0.509-2.381]

* ($p<0.05$); Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+); (Values in the brackets are in percentage)

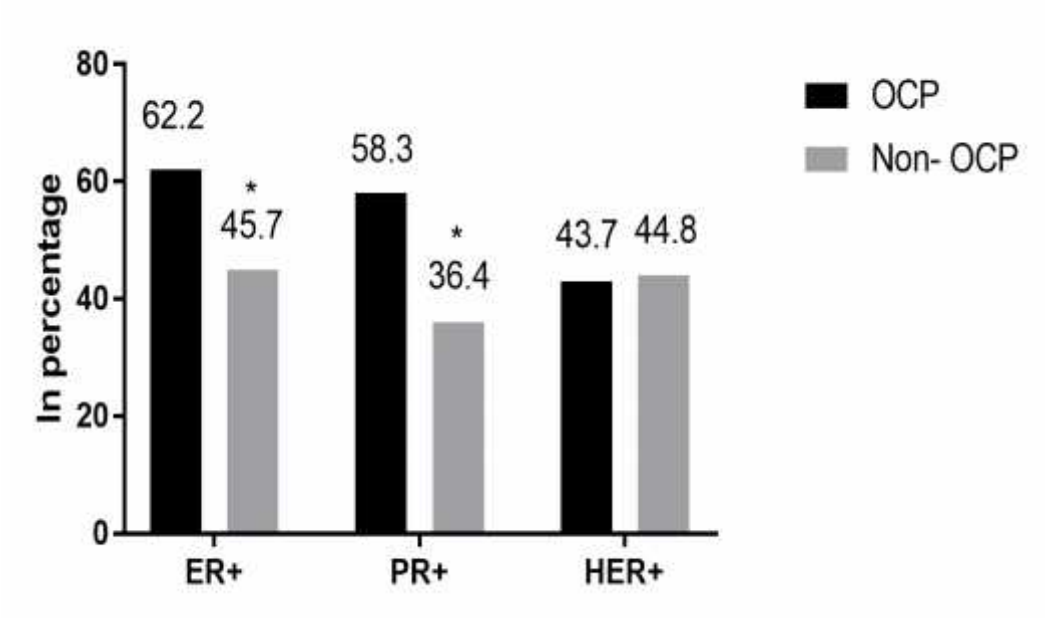


Figure-14: Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+) in OCP users, non-users and total number of cases. (Values are in percentages). *($p<0.05$);

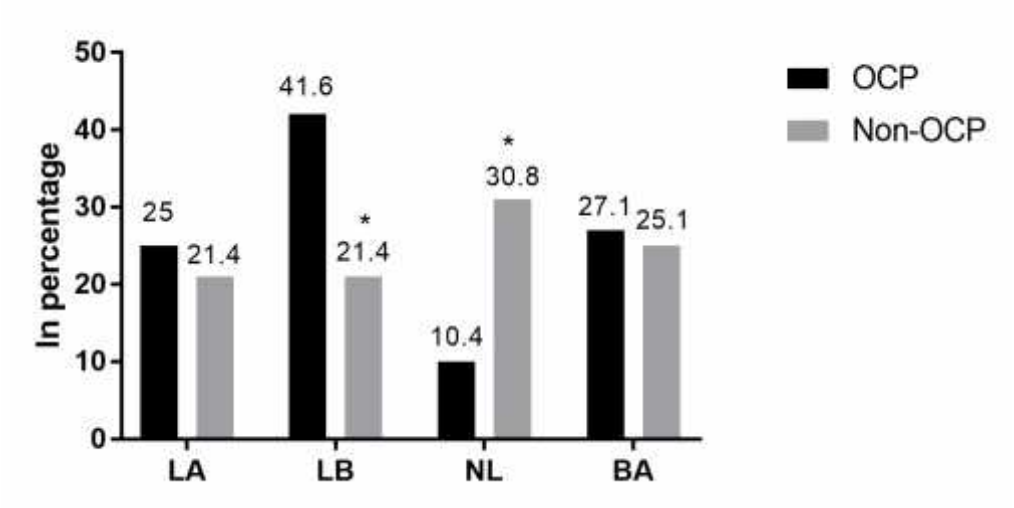


Figure-15: Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2 enriched and Basal like (BA)/TNBC in OCP users, nonusers and total number of cases. (Values are in percentages) *($p<0.05$);

Table-5: Age in Phenotypic sub types/ clinical sub types of breast carcinoma in OCP users and non-users.

Phenotype/ clinical subtypes	OCP users	Phenotype/ clinical subtypes	OCP nonusers	P value
ER + (30)	45.3±8.08	ER + (49)	52.2±8.2	0.001*
PR+ (28)	45.3±8.3	PR + (39)	51.3±8.6	0.001*
HER2 + (27)	46.19±8.5	HER2 + (49)	50.8±8.1	0.039*
Luminal A (12)	44±7.7	Luminal A (23)	51.2±8.6	0.030*
Luminal B (20)	45.5±8.2	Luminal B (23)	52.4±8.2	0.009*
Non-luminal (5)	48±9.2	Non-luminal (34)	50.4±8.1	0.564
Basal (13)	53.1±6.9	Basal (49)	45.5±6.07	0.001*

* ($p<0.05$); Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+);(Values in the brackets are number of cases)

Table-6: Logistic regression analysis of adjusted effect of age on selected parameters in OCP users and non-users

Phenotype/ clinical subtypes	OCP Users				Non OCP Users			
	Adjusted OR	p value	95% CI		Adjusted OR	p value	95% CI	
			Lower	Upper			Lower	Upper
ER +	0.89	0.032*	0.80	0.99	1.05	0.171	0.98	1.13
PR +	0.90	0.039*	0.81	1.00	1.04	0.276	0.97	1.12
HER2+	0.97	0.486	0.88	1.06	1.02	0.515	0.96	1.09
Luminal A	0.87	0.033*	0.76	0.99	1.08	0.068	0.99	1.18
Luminal B	0.97	0.559	0.89	1.07	1.00	0.899	0.91	1.08
Non-luminal	0.22	-	-	-	1.02	0.644	0.95	1.09
Basal	1.18	0.011*	1.04	1.34	0.92	0.026*	0.85	0.99

* ($p<0.05$); Odds Ratio are adjusted for BMI, AOM, parity and stage of breast carcinoma

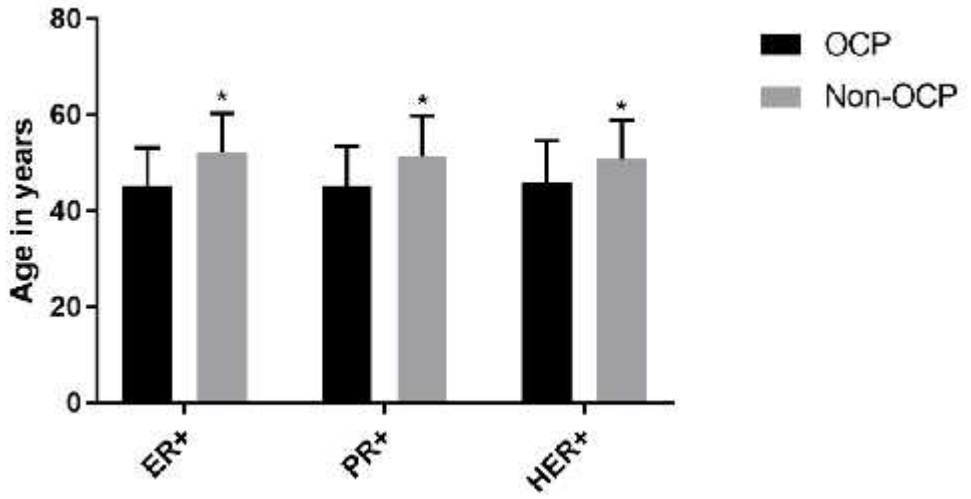


Figure-16: Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+) in OCP users, non-users and total number of cases. *($p<0.05$);

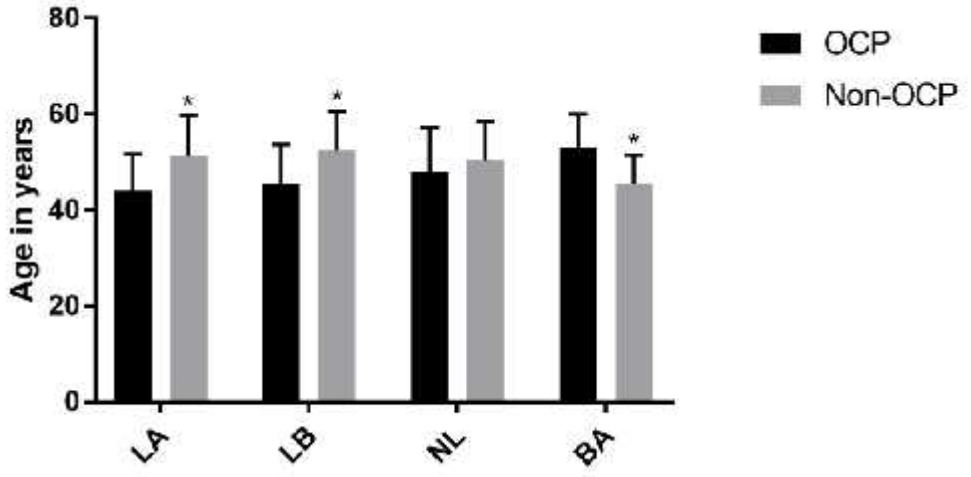


Figure-17: Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2 enriched and Basal like (BA)/TNBC in OCP users, nonusers and total number of case *($p<0.05$);

5. 2 In-vitro study: Results

5.2.1 Effect of 17β-estrdiol on MCF-7 and MDA-MB-231 cell lines on EGFR expression

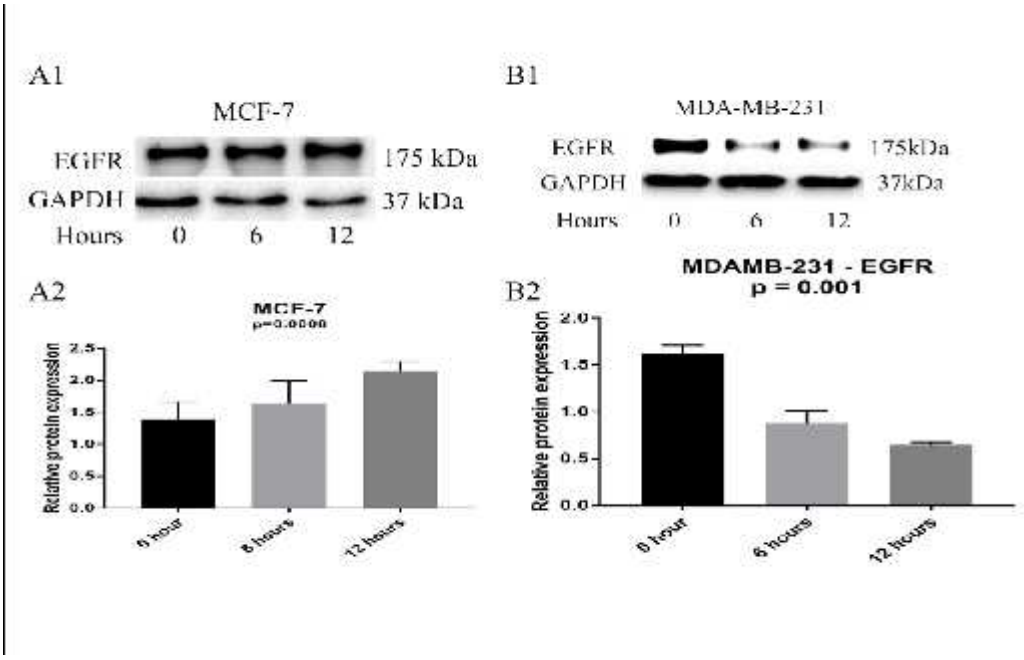


Figure-18: A1. Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours. (Representative blot) A2. Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours. Statistical analysis performed was ANOVA (p=0.0008). B1. Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours. (Representative blot) B2. Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours. Experiment was done thrice in triplicates. Statistical analysis performed was ANOVA (p=0.001).

There was increased expression of EGFR in 17β-estradiol treated MCF-7 cell lines at 0, 6 and 12 hours of interval. (P=0.0008) A statistical significant 1.12 and 1.4 fold increased expression of EGFR at 6 hours and 12 hours was observed respectively. There was reduced expression of EGFR in 17β-estradiol treated MDA-MB-231 cell lines at 0, 6 and 12 hours of interval. (P=0.0001) A statistical significant 0.41and 0.38 fold decreased expression of EGFR at 6 hours and 12 hours was observed respectively. (Fig-18)

5.2.2 Cycloheximide chase to assess the effect of β-estradiol on MDA-MB-231 cells on EGFR expression

There was reduced expression of EGFR at 3 hours in cells treated with Cycloheximide and 17β-estradiol compared to cells treated with Cycloheximide alone. With Cycloheximide alone expression of EGFR reduced significantly to 1.29 fold. With Cycloheximide and 17β-estrdiol expression was further reduced significantly 1.73 fold. (Fig-19)

5.2.3 Cycloheximide chase to assess Ubiquitin mediated EGFR degradation.

There was significant reduced expression of EGFR at 1, 2, 3 and 4 hours in cells treated with 17β-estradiol, Cycloheximide. (P= 0.001) There was 1.52 fold decreases in the expression of EGFR from 0 hour to 4 hours. There was no significant reduced expression of EGFR at 1, 2, 3, and 4 hours in cells treated with 17β-estradiol, Cycloheximide and MG-132. (P=0.05) There was meagre 0.7 fold decreases in the expression of EGFR from 0 hour to 4 hours. (Fig-20)

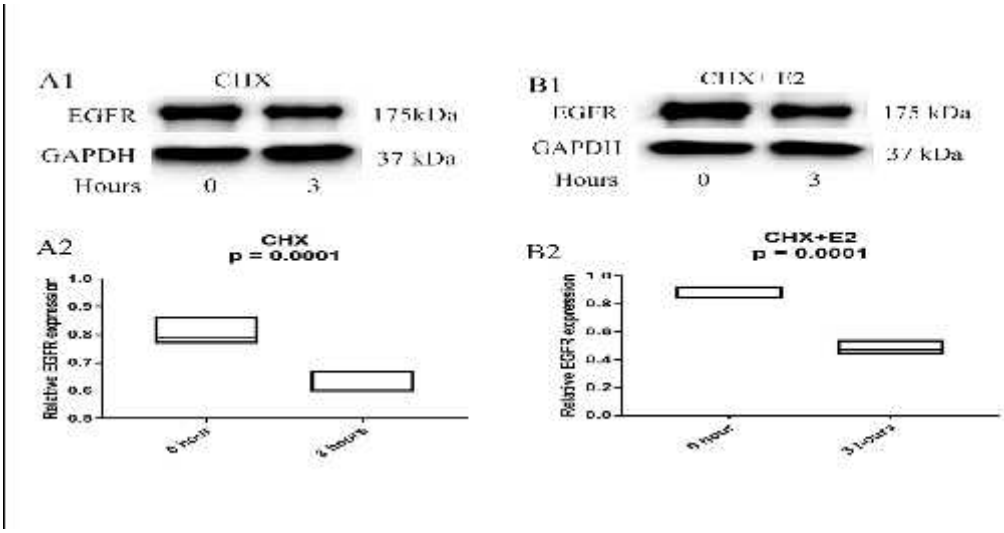


Figure-19: A1. Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours. (Representative blot) A2. Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours. Statistical analysis was performed by independent t test (p=0.0001). B1. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours. (Representative blot) B2. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours. Experiment was done thrice in triplicates. Statistical analysis was performed by independent t test (p=0.0001).

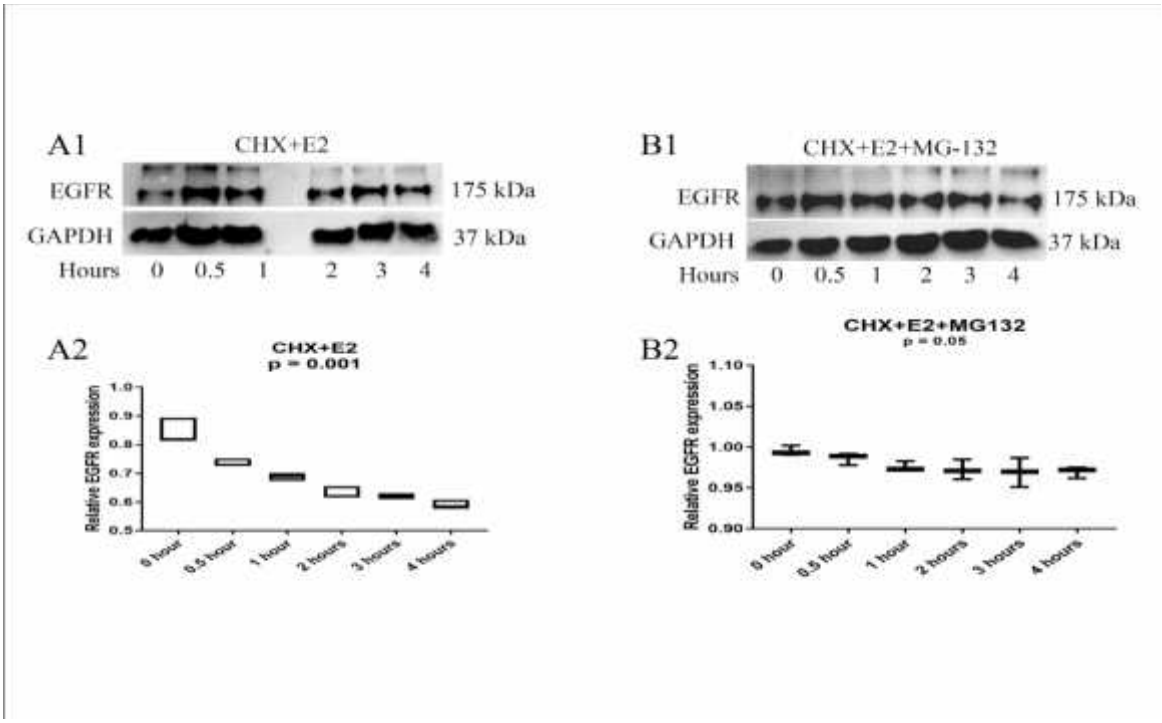


Figure-20: A1. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1,2, 3 and 4 hours. (Representative blot) A2. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1, 2, 3 and 4 hours. Statistical analysis performed by one way ANOVA (p=0.001). B1. Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide +MG-132at 0, 0.5, 1,2, 3 and 4 hours. (Representative blot) B2. Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide + MG-132at 0, 0.5, 1,2, 3 and 4 hours. Experiment was done thrice in triplicates. Statistical analysis performed by one way ANOVA (p=0.05).

5.2.4 Wound healing assay

The results of the wound healing assay are presented in Figure 4. In the control group cell migration was very dynamic and ratio of 0 hour to 24 hour gap was 1.09 after 24 hrs. Using a 100nM estrogen, the motility of the MDA-MB-231 cells was inhibited and ratio of 0 hour to 24 hour gap was 0.88. Therefore, it can be interpreted that 17β-estradiol promoted migration inhibition of the MDA-MB-231 cells. (Fig-4)

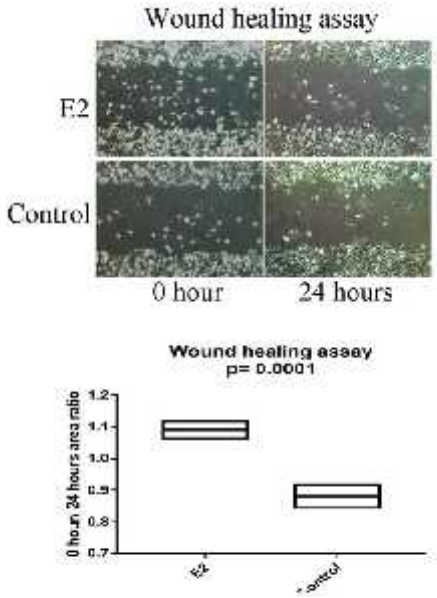


Figure-21: Effect of 100 nM 17β-estradiol on MDA-MB-231cellline with 100 nM of E2 on wound healing. Experiment was done thrice in triplicates. Statistical analysis was performed using unpaired t test (p=0.0001).

References

1. Allred D.C., Harvey J.M., Berardo M. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. 1998; 11:155–168.
2. Crowder JJ, Geigges M, Gibson RT, et al. Rkr1/Ltn1 Ubiquitin Ligase-mediated Degradation of Translationally Stalled Endoplasmic Reticulum Proteins. J Biol Chem. 2015; 290 (30):18454-18466.
3. Tran JR, Brodsky JL. Assays to measure ER-associated degradation in yeast. Methods Mol Biol. 2012; 832: 505–518.
4. Kao SH, Wang WL, Chen CY, Chang YL, Wu YY, Wang YT, Wang SP, Nesvizhskii AI, Chen YJ, Hong TM, Yang PC. GSK3β controls epithelial-mesenchymal transition and tumor metastasis by CHIP-mediated degradation of Slug. Oncogene. 2014;33(24):3172-82
5. Hampton RY, Rine J. Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J Cell Biol. 1994; 125(2):299–312.
6. Hochstrasser M, Varshavsky A. In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. Cell. 1990; 61(4):697–708.
7. Hanna J, Leggett DS, Finley D. Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. Mol Cell Biol. 2003; 23(24):9251–9261.

8. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev. 2002; 82(2):373-428.

9. Hershko A. The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. Cell Death Differ. 2005; 12(9):1191-1197.

10. Ravid T, Hochstrasser M. Diversity of degradation signals in the ubiquitin-proteasome system. Nat Rev Mol Cell Biol. 2008; 9:679–690.

11. Gallastegui N, Groll M. The 26S proteasome: assembly and function of a destructive machine. Trends Biochem Sci. 2010; 35(11):634-642.

12. Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem. 1983; 258(13):8206-8214.

13. Metzger M.B., Pruneda J.N., Klevit R.E., Weissman A.M. RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. Biochim. Biophys. Acta. 2014; 1843:47–60.

14. Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem. 2009; 78:477-513.

Chapter 6

Discussion

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

6. Discussion

6.1 Human study

In this study we observed that there was a significant increased proportion of ER+, PR+ tumours in OCP users according to our hypothesis with relative risk of 1.9 and 2.4. But, the proportion of TNBC cases did not alter with OCP us according to our hypothesis. We observed that there was significant higher age (18%) at admission in TNBC patients among OCP users compared to non-users, indicating OCP's have a role in delaying the progression of TNBC.

The study had several limitations. Our study had small sample size since it was hospital based study. Histories of oral contraceptive had no record and were verbal. We had to rely on their memory. Duration of use of OCP was cut down to six months in order to increase the sample size. There are studies even 'ever use' of OCP has increased risk of breast cancer [16]. An analysis of data from more than 150,000 women who participated in 54 epidemiologic studies showed that, overall, women who had ever used OCPs had a slight (7%) increase in the RR of breast cancer compared with women who had never used OCPs. That is the reason why we conducted the study with OCP use of even 6 months or more than 6 months. This investigation also revealed that women who were currently using OCPs had a 24% increase in risk that did not increase with the duration of use. Risk declined after use of OCP stopped, and no risk increase was evident by 10 years after use had stopped [1]. This was another limitation of our study that there were no current OCP users. Average age our subjects started using OCP was 19 years and average age after last use was 21 years. But, our strength of this case-case study was that we compared proportion of different subtypes of cancer in OCP users and matched non-users. There was no significant difference in the age, BMI, AOM, parity, breast feeding, and stage

between OCP users and non-users, all the cases were of same geographical area of South India of same genetic background which indicates many of the confounding factors were matched. We did not evaluate genetic factors involved significant mutations in *BRCA 1* and *2*, *CHEK2*, *TP53*, *LKB-1* and *PTEN* in our patients. In our opinion such studies are rarely available in the literature. Our study was designed as case-case study, where it is easy to quantify the exact risk for in subtypes comprehensively in collected data. However, some literature evaluated differences among breast cancer subtypes through case-case studies but not in Indian context.

Breast cancer is a global health matter among women. However, the incidence of breast cancer has increased significantly in Asian countries as compared to Western countries. Breast cancer accounts for the most commonly diagnosed cancer in Asian women. Although the incidence of breast cancer remains high in developed countries, there has been a shift in global distribution of breast cancer cases among women in South America, Africa, and Asia [2,3]. In a recent Indian study of 2062 breast cancer patients ranging between 22–100 years revealed the mean age of 51.18 years [4]. Our findings of distribution of different subtypes in total number of cases (155) were not similar to those reported by Prakash et al [3] and Mane et al. [4]. In our study, the incidence of all subtype cancers was much more evenly distributed. In our study, 22.4% of patients were luminal A, 27.5 were luminal B, 24.3% were HER2 rich, and 25.6% were basal-like in total number of cases. (Fig-13) In our study Luminal A and Luminal B cases were low and percentage Non Luminal and TNBC cases were more compared to other studies [4, 5] this is because we did not include more younger (<30 years) patients and not above the age of 60 years. Comparing the distribution of cases in OCP users and non-users it revealed that proportion of ER+, PR+, Luminal B and

Non-luminal cases were significantly high with significant relative risk in OCP users compared to non-users.

The age-specific incidence rates of breast cancer be different among Western and Asian population. Usually ER+ occurs in advancing age and TNBC is known to occur in early age group. In Asian population, breast cancer is characterized at an early age as contrast to advancing age among Western women. The age-specific incidence decreases or plateaus after 50 years in Asian women [6-9]. We did not observe significant age specificity in our study because age group we studied had a narrow range (30 to 60 years). Our study revealed that in OCP user and non-users age (years) at admission to the hospital was significantly reduced in ER+ (45.3 Vs. 52.2), PR+ (45.3 Vs. 51.3) HER+ (46.1 Vs. 50.8), Luminal A (44 Vs. 51.2) and Luminal B (45.5 Vs. 52.2) subtypes. These findings indicates that OCP use augments the progression of ER+, PR+, HER2+, Luminal A and Luminal B breast cancer. On the contrary, In OCP users age (years) at admission to the hospital was significantly high in TNBC (53.1 Vs. 45.5) compared to non-users. This difference (higher age) at the time of admission to the hospital when other risk factors are matched indicates that OCP use may delay the progression of TNBC.

No doubt breast cancer aetiology is multi-factorial and when it comes to subtypes it is still more complicated. In our study it is evident that proportion of ER+ and PR+ cases has increased significantly in OCP users compared to non-users. Our study revealed that age at admission to the hospital significantly reduced in ER+ and PR+ cancer in OCP users when compared to non-users taking consideration of other confounding factors like BMI, AOM, parity and stage of the cancer. Logistic regression among OCP users, the Likelihood of ER+, PR+ and Luminal A+ was 11%, 10% and 13% less with 1 year of higher age respectively. These findings are

consistent with other studies done globally. Conversely we found that proportion of TNBC has not increased in OCP users compared to non-user as reviews suggest. But, age at admission to the hospital was significantly high in TNBC cancer in OCP users when compared to non-users taking consideration of other confounding factors like BMI, AOM, parity and stage of the cancer and among OCP users. The likelihood of TNBC was 18% more with 1 year of higher age in OCP users. Our findings here differ with other studies done globally where in most of the studies OCP use is associated with increased risk of TNBC.

Both genetic and hormonal factors have been responsible for the genesis of breast cancer. Genetic factors involve major mutations in *BRCA 1* and *2, TP53, LKB-1, CHEK2* and *PTEN* in 5–10% of patients and lower risk mutations inferred by identical twin and genome wide association studies in others [10-12]. Epidemiologic and experimental data suggest estradiol as another contributing factor. It is discovered that estradiol, which normally stimulates the growth of cancer cells in tumours that express oestrogen receptor alpha (ERα) like in ER+ cancer. ER+ breast cancer development can be influenced by estrogen via ERα receptor independent mechanisms. Acting through ERα, it stimulates cell proliferation and initiates mutations that occur as a function of errors during DNA replication. The promotional effect of estrogen then maintains the growth of cells harboring mutations, that then accumulate until cancer ultimately results. But estrogen has the opposite effect in triple-negative breast cancer.

However, β-estradiol was only able to inhibit the growth of TNBC when estrogen receptor beta (ERβ) was present in excess and in TNBC presence of ER β is almost 25% [13].There are some studies reports that selective activation of ER β reduces the

metastatic potential of TNBC cells [14]. These putative evidences in favour of why in our study TNBC cases admitted to the hospital at higher age group.

Conclusions

Despite of extensive research in understanding effect of OCP in different subtypes of breast cancers there are many questions than answers. Our study concludes that prior use OCP increases the prevalence, relative risk and progression of the disease in ER+, PR+ and Luminal B type of breast cancer. Whereas prior OCP use do not increase prevalence, relative risk in TNBC. But, OCP use is associated with higher age at admission to the hospital. Therefore, it delays the progression of TNBC.

6.2 In-vitro study

In this study we observed that there was reduced expression of EGFR in MDA-MB-231 cell lines compared to MCF-7 cell lines upon treatment with 17β-estradiol. This indicates that estrogen behaves differently with ER+ and TNBC cell lines. There was reduced expression of EGFR in MDA-MB-231 cell lines upon treatment with β-estradiol and Cycloheximide when compared to cell lines treated with Cycloheximide alone. This further confirms that estrogen certainly degrades EGFR. We also observed that there was no reduced expression of EGFR in MDA-MB-231 cells treated with 17β-estradiol, Cycloheximide and MG-132 compared to cells treated with 17β-estradiol and Cycloheximide. These observations suggest that β-estradiol degrades EGFR in MDA-MB-231Cells and degradation is mediated by ubiquitination.

There were few limitations of the study. We did not use other types of TNBC cell lines. We did not perform RT-PCR to estimate mRNA levels. We did not change the levels of 17β-estradiol to at what exact concentration EGFR inhibited. We did no

elucidate the effect of estrogen antagonist. We did not study downstream molecules of EGFR.

While breast cancer subtypes are genetically linked environmental factors play a key role. Oral estrogen (OCP) consumption in western countries has been high and in developing countries is low. Accordingly incidence of ER+ breast cancer is high and low respectively. But, TNBC incidences are low and high respectively. This has leaded us to think that more usage of oral estrogens may lead to less incidences of TNBC. There are evidences that ER+ tumours and TNBC behaves indifferently in presence of estrogen. In our study we too tried to demonstrate that 17β-estradiol can act indifferently in different phenotypic breast cancers in vitro. We demonstrated that 17β-estradiol augment the proliferation of MCF-7 cells by increasing the expression of EGFR whereas β-estradiol decreases the proliferation of MDA-MB-231 cells by decreasing the production of EGFR.

TNBCs typically occur in younger women And African American women aswell as among some patients with BRCA1 gene defects [16, 17]. Population-based data show that African American women have a higher incidence of TNBC and present with more advanced stages than Caucasian women [17]. This cancer subtype also relates with adverse biological features including high mitotic count and very hostile behavior. Based on current data, estradiol regulates gene expression of EGFR and other several proteins by genomic and non-genomic inputs [18,19]. Genomic signals involve direct action of nuclear-localized ERα as an estradiol regulated transcription factor or co-regulator. By contrast, non-genomic signaling involves extra nuclear events mediated by extra nuclear ERs often in cooperation with co activator or adaptor proteins [20]. Thus estrogens promote progression of ER+ breast cancers through predominantly ERα. In TNBC second type of estrogen receptor, termed

estrogen receptor-beta (ERβ) are present. ERα and ERβ have reciprocal actions. Studies have demonstrated that ERβ1 inhibits epithelial mesenchymal transition (EMT) and invasion in basal-like breast cancer cells when they grow either in vitro or in vivo in zebrafish. EMT is also because of hypoxia known in cancer development [21]. As a consequence activation of ERβ in TNBC probably reduces the expression of EGFR [22]. EGFR expression can also be degraded by activation of non-genomic pathways. In our study we demonstrated that β-estradiol indeed causes degradation, also we tried to analyse how degradation occurs. There are various steps involved in degradation of EGFR.Upon activation, EGFR is tyrosine-phosphorylated, and subsequently recruits Cbl, an E3 ubiquitin ligase, and Grb2, an adaptor protein, for assembly of the ubiquitination complex, and interacts with Eps15 and AP-2, two endocytic adaptor proteins, to form clathrin-coated endocytic vesicles [23-26]. The endocytic vesicles or endosomes containing ubiquitinated EGFR are identified by the ubiquitin-binding protein Hrs and carried to multi-vesicular bodies (MVBs) [27, 28]. Finally, the MVBs blend with lysosomes to complete the process of degradation of EGFR. We hypothesized that estradiol inhibits ubiquitination. To test our hypothesis we treated MDA-MB-231 cells with cycloheximide and estradiol and observed for degradation of EGFR. There was 1.52 fold significantdecreases in the expression of EGFR. Subsequently to test our another hypothesis that degradation of EGFR occurs due to facilitation of ubiquitination we treated MDA-MB-231 cells with cycloheximide, estradiol and MG-132 where MG-132 effectively blocks the proteolytic activity of the 26S proteasome complex reduces the degradation of ubiquitin-conjugated proteins. We observed that there was statistically insignificant 0.7 fold decrease in EGFR expression. This indicates that degradation of EGFR occurs upon treatment with 17β-estradiol, where degradation is mainly mediated by

ubiquitination. We also tested our hypothesis on wound healing test. We treated MBA-MB-231 cells with or without estradiol we observed that without estradiol, cell migration was very dynamic, ratio of 0 hour to 24 hour gap was 1.09 after 24 hrs and motility of the MDA-MB-231 cells was inhibited and ratio of 0 hour to 24 hour gap was 0.88 with estradiol. This observation also proves that esrogen delays the proliferation of MDA-MB-231 cells.

EGFR is one of the receptors most commonly connected with human tumors and has been shown to correlate with the progression of numerous tumor types including breast tumors [29-31]. Although it is often associated with aspects of tumor growth i.e., proliferation, apoptosis, and cell survival, very little emphasis has been placed on the effects of EGFR on breast cancer cell migration. The complex process of cell migration is a critical component of many normal and patho-physiological processes, and its central role in the progression of tumors from a noninvasive to an invasive and metastatic phenotype is well known [32]. Epidermal growth factor receptor (EGFR) levels predict a poor outcome in human breast cancer and are most commonly associated with proliferative effects of epidermal growth factor. In this study we tried to demonstrate the effect of estrogen with EGFR expression in different subtypes of breast cancers mainly ER+ and TNBC tumours whether they respond differently. We found that estradiol degrades EGFR in MDA-MB-231and we could also demonstrate that degradation occurring through ubiquitination.The underlying mechanism of degradation appears related to the sorting of internalized EGFRs to either recycling or degradation [33]. Sorting EGFR requires conjugation of multiple ubiquitins, which mark the receptor for degradation [34]. Consequently, our study demonstrated that EGFR degraded by ubiquitination.

Several lines of evidence support the possibility that EGFR play a driver role in a large fraction of TNBC. For example, EGFR gene amplification is frequently identified in metaplastic breast carcinoma, a basal-like fraction of tumors [35]. Similarly, gene expression signatures correlated TNBC with modules comprising EGF-like ligands, EGFR, and several downstream effectors [36]. But, TNBC clinical trials using EGFR inhibitors treating cancer, including cetuximab, reported lack of clinical benefit [37, 38]. Our study offers an alternative strategy by degrading EGFR by estrogen. Experiments in which we used TNBC cell line and demonstrated that down regulation of EGFR through ubiquitination can retard motility and proliferation of TNBC cell line.

By adding estogen, we detected EGFR degradationand degradation occurs through ubiquitinationin MDA-MB-231 cellline. Our in vitro study require confirmation in animal models. Assuming confirmation in vivo, this study may help in understanding alternative pathway where degradation of EGFR by estrogen or other specific molecule receptors can be a targeted in the treatment of TNBC. In future estrogen like molecules may be used as adjunct in the treatment of TNBC.

References

1. Lander GC, Estrin E, Matyskiela ME, Bashore C, Nogales E, Martin A. Complete subunit architecture of the proteasome regulatory particle. Nature. 2012;482(7384):186-191.
2. Das S, Roth CP, Wasson LM, Vishwanatha JK Signal transducer and activator of transcription-6 (STAT6) is a constitutively expressed survival factor in human prostate cancer. Prostate. 2007; 67:1550–1564.
3. Collaborative Group on Hormonal Factors in Breast Cancer. Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53,297 women with breast cancer and 100,239 women without breast cancer from 54 epidemiological studies. Lancet 1996; 347(9017):1713–1727.
4. Sung H, Rosenberg PS, Chen WQ, Hartman M, Lim WY, Chia KS, et.al, Female breast cancer incidence among Asian and Western populations: more similar than expected. J Natl Cancer Inst 2015; 107: djv107.
5. Youlden DR, Cramb SM, Yip CH, Baade PD. Incidence and mortality of female breast cancer in the Asia-Pacific region. Cancer Biol Med 2014; 11: 101-115.
6. Pandit P, Patil R, Palwe V, Gandhe S, Patil R, Nagarkar R. Prevalence of Molecular Subtypes of Breast Cancer: A Single Institutional Experience of 2062 Patients. Eur J Breast Health 2020; 16(1): 39-43.11.

7. Mane A, Khatib KI, Deshmukh SP, Nag SM, Sane SP, Zade BP. A Comparison of Clinical Features, Pathology and Outcomes in Various Sub types of Breast Cancer in Indian Women. J ClinDiagn Res 2015; 9: 1-4.

8. Shin HR, Joubert C, Boniol M, Hery C, Ahn SH, Won YJ, et.al, Recent trends and patterns in breast cancer incidence among Eastern and Southeastern Asian women. Cancer Causes Control 2010 21: 1777 -1785.

9. Bray F, McCarron P, Parkin DM. The changing global patterns of female breast cancer incidence and mortality. Breast Cancer Res 2004; 6: 229- 239.

10. Liu L, Zhang J, Wu AH, Pike MC, Deapen D. Invasive breast cancer incidence trends by detailed race/ethnicity and age. Int J Cancer 2012; 130: 395-404.

11. Leong SP, Shen ZZ, Liu TJ, Agarwal G, Tajima T, Paik NS, et.al, Is Breast Cancer the Same Disease in Asian and Western Countries? World J Surg 2010; 34: 2308-2324.

12. Martin AM, Weber BL. Genetic and hormonal risk factors in breast cancer. J Natl Cancer Inst. 2000; 92:1126–35.

13. Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, et.al, Environmental and heritable factors in the causation of cancer-analyses of cohorts of twins from Sweden, Denmark, and Finland. New Engl J Med. 2000; 343:78–85.

14. Thompson D, Easton D. The genetic epidemiology of breast cancer genes. J Mammary Gland BiolNeoplasia. 2004; 9:221–36.

15. Hinsche, O, Girgert, R., Emons, G, Gründker C.Estrogen receptor β selective agonists reduce invasiveness of triple-negative breast cancer cells. International Journal of Oncology. 2015; 46(2): 878-884.

16. Girgert, R., Emons, G., Gründker, C. Inhibition of growth hormone receptor by Somavert reduces expression of GPER and prevents growth stimulation of triple-negative breast cancer by 17β-estradiol. Oncology Letters. 2018: 15(6); 9559-66.

17. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med. 2010; 363(20):1938-1948.

18. Gloria J M, Sashi N, Allan KT, Fran G, Yihuan X, Peter M, et.al,Differences in breast carcinoma characteristics in newly diagnosed African- American and Caucasian patients: a single-institution compilation compared with the national cancer institute's surveillance,epidemiology, and end results database.Cancer. 2007; 110(4):876-84.

19. Amirikia KC, Mills P, Bush J, Newman LA. Higher population-based incidence rates of triple-negative breast cancer among young African-American women: Implications for breast cancer screening recommendations. Cancer. 2011; 117(12):2747–2753

20. Pietras R. J, Márquez-Garbán D. C. Membrane-associated estrogen receptor signaling pathways in human cancers. Clinical Cancer Research. 2007; 13(16):4672–4676.

21. Hammes S. R., Levin E. R. Extranuclear steroid receptors: nature and actions. Endocrine Reviews. 2007; 28(7):726–741.

22. Yan Y, Li X, Blanchard A, Bramwell VH, Pritchard KI, Tu D, et. Al, Expression of both estrogen receptor-beta 1 (ER-beta1) and its co-regulator steroid receptor RNA activator protein (SRAP) are predictive for benefit from tamoxifen therapy in

patients with estrogen receptor-alpha (ER-alpha)-negative early breast cancer (EBC) Ann Oncol. 2013; 24:1986–93.

23. Aldo Pezzuto and Elisabetta Carico. Role of HIF-1 in Cancer Progression: Novel Insights. A Review. Current Molecular Medicine 2018; 18: 343.
24. Christoforos T, GayaniR, FotisN, RuixinH, Anne K, Catherine WM. ERβ1 represses basal-like breast cancer epithelial to mesenchymal transition by destabilizing EGFR. Breast Cancer Res. 2012; 14(6): R148.
25. Waterman H, Levkowitz G, Alroy I, Yarden Y. The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. J Biol Chem. 1999; 274:22151–4.
26. Jiang X, Huang F, Marusyk A, Sorkin A. Grb2 regulates internalization of EGF receptors through clathrin-coated pits. MolBiol Cell. 2003; 14:858–70.
27. Confalonieri S, Salcini AE, Puri C, Tacchetti C, Di Fiore PP. Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. J Cell Biol. 2000; 150:905–12.
28. Ramaekers CH, Wouters BG. Regulatory functions of ubiquitin in diverse DNA damage responses. Curr Mol Med. 2011; 11(2):152-69.
29. Lu Q, Hope LW, Brasch M, Reinhard C, Cohen SN. TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation. Proc Natl AcadSci USA. 2003; 100:7626–31.
30. Bache KG, Brech A, Mehlum A, Stenmark H. Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. J Cell Biol. 2003; 162:435–42.

31. Radinsky R, Risin S, Fan D, Dong Z, Bielenberg D R, Bucana CD, Fidler I J. Level and function of epidermal growth factor receptor predict the metastatic potentialof human colon carcinoma cells. Clin. Cancer Res. 1995: 1: 19–31.

32. Harris A L, Nicholson S, Sainsbury R, WrightC, Farndon J. Epidermalgrowth factor receptor and other oncogenes as prognostic markers. J. Natl. Cancer Inst. Monogr.1992:181–187.

33. Price JT, Bonovich M T, Kohn EC. The biochemistry of cancer dissemination.Crit. Rev. Biochem. Mol. Biol.1997; 32: 175–253.

34. Spangler JB, Neil JR, Abramovitch S, Yarden Y, White FM, Lauffenburger DA, Wittrup KD. Combination antibody treatment down-regulates epidermal growth factor receptor by inhibiting endosomal recycling. Proc Natl AcadSci U S A. 2010; 107(30):13252-7

35. Goh LK, Huang F, Kim W, Gygi S, Sorkin A. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol. 2010; 189(5):871-883.

36. Reis-Filho JS, et al. EGFR amplification and lack of activating mutations inmetaplastic breast carcinomas. J Pathol. 2006; 209(4):445–453.

37. Hoadley KA, et al. EGFR associated expression profiles vary with breast tumorsubtype. BMC Genomics. 2007; 8:258.

38. Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor intriple-negative and basal-like breast cancer: Promising clinical target or onlya marker? Cancer J. 2010; 16(1):23–32.

Chapter 7

Summary and Conclusion

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

7. Summary and Conclusion

Observations fro human study revealed a significant increase in prevalence of molecular sub-types ER+, Progesterone Receptor+ (PR+) and Luminal B breast cancers in OCP users was observed compared to non-users. There was considerable decrease in the age at the point of admission in ER+ cancer in OCP users (45.3 years) compared to non-users (52.2years). Whereas in OCP users age at the time of admission of Basal (TNBC) cancer patients (53.1 years) was higher when compared to non-users (45.4years). Logistic regression revealed the likelihood of ER+, PR+ and Luminal B in OCP users was 11%,10% and 13% less respectively with 1 year of higher age against the likelihood of TNBC among OCP users was 18% more and 8% less in non-users. In in-vitro study EGFR expression was reduced with β-estradiol treatment in MDA-MB-231 cell line with Cycloheximide chase. Upon Treatment with MG-132 and E2, EGFR expression did not reduce suggestive of that Estrogen degrades EGFR by ubiquitination pathway.

We concluded that OCP use may be allied with increase in the prevalence of ER+, PR+ and Luminal B breast cancer. On the contrary OCP use is may be related with delay in the progression of the TNBC. In-vitro study conclusion was that estrogen degrades EGFR in MDA-MB-231 cells and this degradation occurs by ubiquitination.

Limitations

Human study- Our study had small sample size since it was hospital based study. Histories of oral contraceptive had no record and were verbal. We had to rely on their memory. Duration of use of OCP was cut down to six months in order to increase the sample size.

In Vitro study (Cell line)- We did not use other types of TNBC cell lines. We did not perform RT-PCR to estimate mRNA levels. We did not change the levels of 17β-estradiol to at what exact concentration EGFR inhibited. We did no elucidate the effect of estrogen antagonist. We did not study downstream molecules of EGFR.

Future direction

Clinical trails are being conducted to establish the effect of estrogen on TNBC. Our aim in future will be to understand molecular mechanism by which estrogen suppress TNBC. We will be studying other non-genomic Wnt -signaling and Notch -signaling pathways in TNBC cell lines. This will help in choosing common pathway and a common molecule that can be targeted for therapy.

Annexures

Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer

INFORMED CONSENT

RISKS: There will not be any risk involved in the study.

BENEFITS: No direct benefit is guaranteed to you from participating in our study.

OPTIONS: If you decide not to participate in this study, the hospital will provide you the usual standard care and treatment.

PRIVACY AND CONFIDENTIALITY: All information collected about you during the course of the study will be kept confidential to the extent permitted by law. You will be identified in this research record by the code numbers. Information which identifies you personally will not be revealed without your written permission. However your records may be revealed to the sponsor of the study. Information from this study may be published but your identity will be confidential in any publication.

COST FOR PARTICIPATION: You will not be charged for the test to be carried out.

FINANCIAL INCENTIVE FOR PARTICIPATION: You will not receive any remuneration for participating in this study.

VOLUNTARY PARTICIPATION/WITHDRAWAL: If you decide not to participate in this study, it will not affect the quality of the medical care you receive at this institution.

You may withdraw from the study anytime. The researchers might use the information learned from the study in scientific journal articles or in presentations.

EMERGENCY PROVISION: If you have questions as a participant in our study, you can contact the study investigator Dr.Vitthal Khode, Mobile No. 9916821453.

CONSENT TO PARTICIPATE IN A RESEARCH TRIAL

“IMPLICATION OF ORAL CONTRACEPTIVE USE TO PHENOTYPIC EXPRESSION PATTERN OF RECEPTORS IN BREAST CANCER”

I unreservedly, in my full senses, give my complete and informed consent for microscopic study of placenta, for the purpose of research

I hereby confirm that I have been informed (in a language understood by me) that a study is being conducted on “**Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer**” The study has been explained to me in detail. I understand that the information regarding me, collected during the course of this study will remain confidential. I understand that my participation in this study is voluntary. I understand that the records maintained will be used only for research purpose.

The refusal of my participation will not affect my treatment in any way and I may withdraw at any time of the study.

The purposes of the study, the protocol and procedures have been explained to me to the best of my understanding and I am fully convinced that the tests are not harmful.

_____ _____

Signature of patient Date

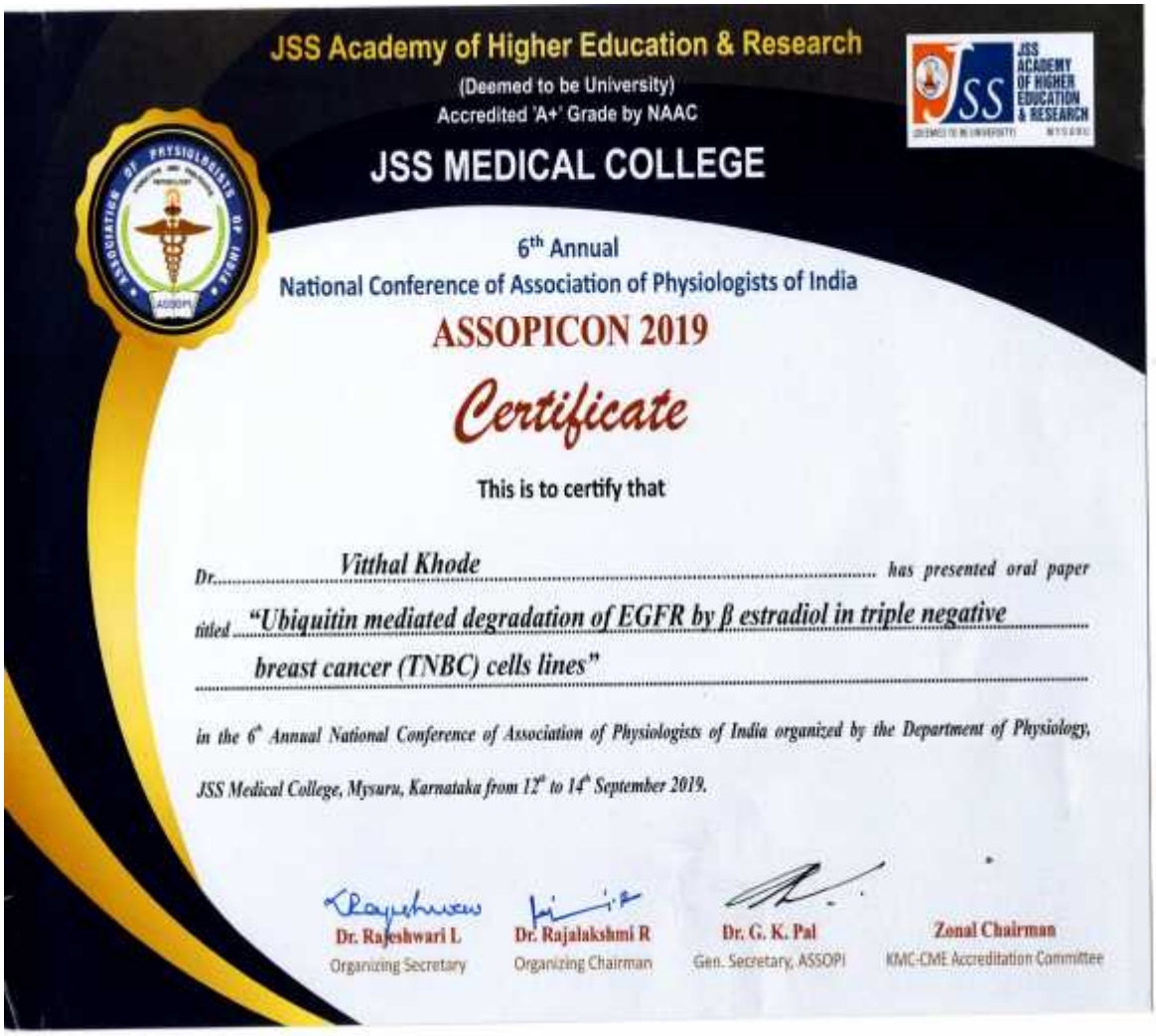
_____

Patients Name (Printed):

_____ _____

Signature of researchers or Date

Person obtaining consent



JSS Academy of Higher Education & Research

(Deemed to be University)

Accredited 'A+' Grade by NAAC

JSS MEDICAL COLLEGE

6th Annual

National Conference of Association of Physiologists of India

ASSOPICON 2019

Certificate

This is to certify that

Dr. Vitthal Khode has presented oral paper titled "Ubiquitin mediated degradation of EGFR by β estradiol in triple negative breast cancer (TNBC) cells lines"

in the 6th Annual National Conference of Association of Physiologists of India organized by the Department of Physiology, JSS Medical College, Mysuru, Karnataka from 12th to 14th September 2019.

Dr. Rajeshwari L, Organizing Secretary

Dr. Rajalakshmi R, Organizing Chairman

Dr. G. K. Pal, Gen. Secretary, ASSOPI

Zonal Chairman, KMC-CME Accreditation Committee

INFORMED CONSENT

RISKS: There will not be any risk involved in the study.

BENEFITS: No direct benefit is guaranteed to you from participating in our study.

OPTIONS: If you decide not to participate in this study, the hospital will provide you the usual standard care and treatment.

PRIVACY AND CONFIDENTIALITY: All information collected about you during the course of the study will be kept confidential to the extent permitted by law. You will be identified in this research record by the code numbers. Information which identifies you personally will not be revealed without your written permission. However your records may be revealed to the sponsor of the study. Information from this study may be published but your identity will be confidential in any publication.

COST FOR PARTICIPATION: You will not be charged for the test to be carried out.

FINANCIAL INCENTIVE FOR PARTICIPATION: You will not receive any remuneration for participating in this study.

VOLUNTARY PARTICIPATION/WITHDRAWAL: If you decide not to participate in this study, it will not affect the quality of the medical care you receive at this institution.

You may withdraw from the study anytime. The researchers might use the information learned from the study in scientific journal articles or in presentations.

EMERGENCY PROVISION: If you have questions as a participant in our study, you can contact the study investigator Dr.Vitthal Khode, Mobile No. 9916821453.

CONSENT TO PARTICIPATE IN A RESEARCH TRIAL

"IMPLICATION OF ORAL CONTRACEPTIVE USE TO PHENOTYPIC EXPRESSION PATTERN OF RECEPTORS IN BREAST CANCER"

I unreservedly, in my full senses, give my complete and informed consent for microscopic study of placenta, for the purpose of research

I hereby confirm that I have been informed (in a language understood by me) that a study is being conducted on "**Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer**" The study has been explained to me in detail. I understand that the information regarding me, collected during the course of this study will remain confidential. I understand that my participation in this study is voluntary. I understand that the records maintained will be used only for research purpose.

The refusal of my participation will not affect my treatment in any way and I may withdraw at any time of the study.

The purposes of the study, the protocol and procedures have been explained to me to the best of my understanding and I am fully convinced that the tests are not harmful.

_____ _____

Signature of patient Date

_____

Patients Name (Printed):

_____ _____

Signature of researchers or Date

Person obtaining consent

9916821453

"[redacted]"

[redacted], [redacted] [redacted] [redacted] [redacted] [redacted] [redacted], [redacted] [redacted]

"[redacted]" [redacted] [redacted] [redacted] [redacted] [redacted]

“IMPLICATION OF ORAL CONTRACEPTIVE USE TO PHENOTYPIC EXPRESSION PATTERN OF RECEPTORS IN BREAST CANCER **”**

PROFORMA FOR COLLECTION OF DATA:

In Human subjects-

Demographic characters and obstetric history.

1	Name	
2	Age in yrs	
3	Occupation and Education	
4	IP No	
5	Height in meters	
6	Weight in kgs	
7	BMI in kg/m2	
8	Alcohol/ smoking	
9	Family history of breast cancer	
10	Age of menarche	
11	Nulliparity	
12	Gravid	
13	Parity	
14	No of abortions	
15	Lactation	
16	Breast carcinoma- stage, ER, PR, HER Status, molecular subtype	

H/o Oral contraceptives use

1	OC use duration	
2	Type of OC	
3	Age at first use	
4	Year since last use	

BLDE (DEEMED TO BE UNIVERSITY)

PLAGIARISM VERIFICATION CIRTIFICATE

1. **Name of the Student: Dr Vitthal Khode Reg No:15PHD008**
2. **Title of the thesis: Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer**
3. **Department: Physiology**
4. **Name of the Guide and Designation: Dr.Sumangala Patil , Professor**
5. **Name of the Co-Guide and Designation: Dr. Praveenkumar Shetty, Professor**

The above thesis is verified for the similarity detection. The report is as follows

Software used: …… Date: ……..

Similarity index %: …… Total word count: ……..

The report is attached for the review by the Student and Guide.

The plagiarism report of above thesis has been reviewed by the undersigned.

The similarity index is below accepted norms, because of following reasons:

……
……
……

The thesis may be considered for submission to the university. The software report is attached.

Signature of the Guide Signature of Co-Guide Signature of the Student

Name & Designation Name & designation

Verified by (Signature)

Name & Designation

B.L.D.E. UNIVERSITY

(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act,1956)

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE, VIJAYAPURA

IEC Ref No- 192/2016-17

January 24, 2017

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 28th March 2016 at 11 AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.

Title: *"Implication of Oral Contraceptive use to phenotypic expression pattern of receptors in breast cancer".*

Name of Ph.D./ P. G. / U. G. Student / Faculty member: Dr.Vittalasa Khode

Name of Guide: Dr.Sumangala Patil, Professor, Dept. of Physiology.

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPURA– 586 103

INSTITUTIONAL ETHICAL COMMITTEE VIJAYAPURA BLDE UNIVERSITY

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
VIJAYAPURA – 586 103.

Member Secretary,
Institutional Ethical Committee,
BLDE University, BIJAPUR.

Following documents were placed before Ethical Committee for Scrutinization:

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant document's

ShriDharmasthalaManjunateshwara College of Medical Sciences and Hospital,
Manjushree Nagar, Sattur, Dharwad -580 009, Karnataka

Affiliated of Rajiv Gandhi University of Health Sciences, Bangalore & Recognized by Medical Council of India, New Delhi
GOI Notification No. U.12012/95/2001-ME (P-II) and MCI Notification No.MCI-34(41)/MED.2009/5527 dated 01.05.2009
Contact - Tel.No : +91836 2477574, 2477553, Tele fax: +91836 2461651 email: sdmcmshc@gmail.com Website: sdmmedicalcollege.org

Ref: SDMIAEC: : 2016 Date: 03/06/2016

To,
Dr. Vitthalsa Khode,
Associate Professor
Department of Physiology,
SDM College of Medical Sciences & Hospital,
Sattur, Dharwad.

Dear Dr. Vitthalsa Khode,

Sub : Institutional Animal Ethics Committee permission.

I am happy to inform you that **provisional permission** is granted to you to carry out your Study titled **"Implication of oral contraceptive use to phenotypic expression pattern of receptors in breast cancer."**

Thanking you,

Yours sincerely

Prof. J.V. Chowti
Principal & Chairman – IAEC

KLE University's

Re-Accredited "A" grade by NAAC | Placed in Category 'A' by MHRD (GoI)

Jawaharlal Nehru Medical College, Belagavi

"Environmental Physiology – High Altitude and Space"

Organized by : The Association of Physiologists and Pharmacologists of India, Belgaum Branch
Endorsed by : The Physiological Society of India & South Asian Association of Physiologists
Sponsored by : Indian Council of Medical Research, Medical Council of India, KLE University, Associations of Physicians of India and AD Instrument

Certificate

This is to certify that

Dr / Mr / Ms .....Vitthal.....Khode.....

From..SDMCMS&H..,.Dharwad..... has presented a Poster entitled .Comparison..of..risk factors..in..triple..negative..breast..cancer..(TNBC)..&..non TNBC. Hospital based cross sectional study. ..... at 17th National Seminar held on 19th August 2017 at Jawaharlal Nehru Medical College, Belagavi.

Dr. Aruna Bhavane
President, APPI
Belagavi Branch

Dr. Sanjay Bhalerao
Secretary, APPI
Belagavi Branch

Dr. (Mrs.) N. S. Mahantashetti
Principal,
KLE University's J N Medical College,
Belagavi

JSS Academy of Higher Education & Research

(Deemed to be University)
Accredited 'A+' Grade by NAAC

JSS MEDICAL COLLEGE

6th Annual
National Conference of Association of Physiologists of India

ASSOPICON 2019

Certificate

This is to certify that

Dr. *Vitthal Khode* has presented oral paper titled *"Ubiquitin mediated degradation of EGFR by β estradiol in triple negative breast cancer (TNBC) cells lines"*

in the 6th Annual National Conference of Association of Physiologists of India organized by the Department of Physiology, JSS Medical College, Mysuru, Karnataka from 12th to 14th September 2019.

Dr. Rajeshwari L	Dr. Rajalakshmi R	Dr. G. K. Pal	Zonal Chairman
Organizing Secretary	Organizing Chairman	Gen. Secretary, ASSOPI	KMC-CME Accreditation Committee

JSS Academy of Higher Education & Research

(Deemed to be University)
Accredited 'A+' Grade by NAAC

JSS MEDICAL COLLEGE

6th Annual
National Conference of Association of Physiologists of India

ASSOPICON 2019

Certificate

This is to certify that

Prof./Dr./Mr./Ms. ***Dr.Vitthal Khode*** ..... *has presented poster presentation titled* ***Association use of oral contraceptive pill with expression pattern of different molecular subtypes of breast cancer - Hospital based cross sectional study***

in the 6th Annual National Conference of Association of Physiologists of India organized by the Department of Physiology, JSS Medical College, Mysuru, Karnataka from 12th to 14th September 2019.

Dr. Rajeshwari L	Dr. Rajalakshmi R	Dr. G. K. Pal	Zonal Chairman
Organizing Secretary	Organizing Chairman	Gen. Secretary, ASSOPI	KMC-CME Accreditation Committee

Send Orders for Reprints to reprints@benthamscience.net

Current Women`s Health Reviews, 2021, *17*, 1-8

RESEARCH ARTICLE

Association of Use of the Oral Contraceptive Pill (OCP) with the Expression Pattern of Different Molecular Subtypes of Breast Cancer

Vitthal Khode[1,2,5], Sumangala Patil[1], Praveenkumar Shetty[3,4,*], U S Dinesh[6], Komal Ruikar[1,5], Anil Bargale[2] and Satish G. Patil[5]

[1]Department of Physiology, BLDE (Deemed to be University) Shri B M Patil Medical College Hospital and Research Centre, Vijaypur, India; [2]Central Research Laboratory, SDM College of Medical Sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India; [3]Department of Biochemistry, K S Hegde Medical Academy, Nitte (Deemed to be University) Mangalore, India; [4]Nitte University Centre for science Education and Research, Mangalore, India; [5]Department of Physiology, SDM College of Medical Sciences & Hospital, Shri Dharmasthala Manjunatheshwara University,Dharwad, India; [6]Department of Pathology, SDM College of Medical Sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India

Abstract: ***Background*:** Triple-negative breast carcinoma (TNBC) is a breast cancer subtype associated with high mortality and inadequate therapeutic options when compared to non-TNBC. Clinical data indirectly suggests where Oral Contraceptive Pill (OCP) usage is high, the prevalence of Estrogen Receptor+ (ER+) breast cancer is high, and the prevalence of TNBC is low. This has lead to our hypothesis that OCP use may increase the risk of ER+ breast cancer, and OCP use may reduce the risk of TNBC. We aimed to compare the prevalence and association of subtypes of breast cancer in OCP users with that of non-users among women 30 to 60 years of age.

***Methods*:** This hospital-based observational study of three-year duration included 155 subjects of primary invasive breast cancer who got admitted to our institution. The data was obtained for ER, PR, HER2 status, clinical classification, and data in relation to demographic factors, reproductive history, and history of OCP use. 155 subjects were divided into two groups. Group-1 included 48 patients with a history of OCP use, and group-2 included 107 patients who have not used OCP. Data was analysed using SPSS-20.

***Results*:** A significant increase in the prevalence of molecular subtypes ER+, Progesterone Receptor+ (PR+) and Luminal B breast cancer in OCP users was observed compared to non-users. There was a significant decrease in the age at the time of admission in ER+ cancer in OCP users (45.3 years) compared to non-users (52.2years). While age at the time of admission of Basal (TNBC) cancer patients in OCP users (53.1 years) was higher when compared to non-users (45.4years). Upon logistic regression, the likelihood of ER+, PR+ and Luminal B in OCP users was 11%,10% and 13% less, respectively, with 1 year of higher age and the likelihood of TNBC in OCP users was 18% more and 8% less in non-users.

***Conclusion*:** OCP use may be associated with increased prevalence of ER+, PR+ and Luminal B breast cancer. On the contrary, OCP use may be associated with a delay in the progression of the TNBC.

ARTICLE HISTORY

Received: June 30, 2020
Revised: November 20, 2020
Accepted: November 23, 2020

DOI:
10.2174/1573404817666210223165259

Keywords: Breast cancer, molecular subtype, oral contraceptive pill.

1. INTRODUCTION

Breast cancer is a heterogeneous disease with different clinical, pathological, and molecular features. Expression patterns and immune-histo-chemical markers can differentiate breast cancer subtypes and likely to reflect important differences in pathogenesis and aetiology [1, 2]. Epidemiologic studies strongly suggest that Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+), Human Epidermal Growth Factor Receptor 2+ (HER2+) and triple-negative breast cancers (TNBCs) may be distinct entities that the etiologic factors, clinical characteristics, and therapeutic possibilities may vary by molecular subtypes [3-5]. Several investigations propose that reproductive factors and exogenous hormones, especially estrogen use differently or even quite inversely, affect the risk of ER+ and TNBC [6-8]. Estrogen was used in the treatment of breast cancers in the past. Meta-analysis has shown that obstetric history and Oral Contraceptive pill (OCP) intake increases the risk of both ER+ and TNBC [9]. The role of some of the risk factors in the development of ER+ cancer is clear. Controversies concerning the exact role of risk factors in TNBC development and bio-

* Address correspondence to this author at the Nitte University Centre for Science Education and Research, Mangalore, India; Tel: 8971465632; E-mail: shettybp@hotmail.com

1573-4048/21 $65.00+.00

logical mechanisms behind the initiation of TNBCs are completely obscure. ER+ tumours more prevalent in elderly menopausal women and clinically, are responsive to hormonal treatment. TNBC constitutes a clinically challenging type of breast cancer that occurs more frequently in younger women (<50 years) and Asian women and is associated with significant aggressiveness as compared with other subtypes.

Western population, which is considered socio-economically of higher status, OCP use is high. Maybe because of this, they have more preponderance to develop ER+ breast cancer and less preponderance to develop TNBC. African and Asian populations have less preponderance to develop ER+ and more preponderance to develop TNBC. The reason could be the less use of OCP [10-12]. But, genetic and other environmental factors also play an important role in disease prevalence. ER +breast cancer development is influenced by estrogen via Estrogen Receptor α (ERα) receptors through independent mechanisms. Acting through ERα, it stimulates cell proliferation and initiates mutations that occur as a function of errors during DNA replication. But, estrogen has the opposite effect in TNBC when Estrogen Receptor β (ERβ) is present in excess. There are evidences estrogen decreases the proliferation of TNBC by non-genomic action [13]. These cumulative effects could be the reason why the western population has less number TNBC. There is paucity in the studies on whether OCP use is associated with increased/reduced risk of especially with TNBC in the Indian population, particularly in the younger age group. Therefore, in order to characterize the association between OCP use and risk of different breast cancer subtypes among Indian young women, since these biological subtypes of breast cancers have therapeutic implication, we hypothesized that there would be a preponderance of ER+ breast cancer in subjects exposed to OCPs prior and there will be a decrease in the prevalence in TNBC subjects exposed to OCP. Hence, we aimed at this hospital-based prospective cross-sectional study among women of younger age group (30-60 years) having different molecular subtypes of breast cancer.

2. METHODS

After getting the approval of the Ethical clearance committee of the institution, this prospective cross-sectional study was carried out over three years (June 2016 to June 2018). The sample size was decided based on the number of admissions of breast cancer in our hospital with the criteria of age between 30 to 60 years.

2.1. Sample Size Calculation

With a 95% confidence level and margin of error of ±8%, a sample size of 151 subjects will allow the study to determine the association of use of the oral contraceptive pill (OCP) with the expression pattern of different molecular subtypes of breast cancer. The calculation was done using the formula: $n = z^2p(1-p)/d^2$, where Z= z statistic at 5% level of significance, d is the margin of error, p is a maximum anticipated prevalence rate of breast cancer. The selection of the sample was carried out from the outpatient and inpatients departments of our institution. 155 breast cancer individuals were enrolled in the study. The whole enrolled population of breast cancer was divided into two groups. Group-1 consisted of 48 clinically diagnosed breast cancer patients with molecular sub-types with a history of cyclical oral contraceptive pill use for at least 6 months of duration. Group-2 consisted of 107 age-matched controls with breast cancer of different molecular subtypes with no history of oral contraceptive pill use (Fig. **1**). Individuals with other associated malignancies were excluded from the study. Molecular sub-typing was done based on whether individuals are ER+/-, PR+/-or HER2 +/- or and also based upon American Pathologists/American Society of Clinical Oncology (CAP/ASCO) guidelines-a new clinical classification Luminal A (LA), Luminal B (LB), Non-Luminal (NL)/HER2+ enriched or Basal (BA)/ (TNBC) [14]. Each individual was briefed about the study before; its importance and written consent of participants were taken before the interview. Subjects using contraceptives other than cyclical, Individuals taking Hormonal replacement therapy (HRT) were excluded from the study. The basic parameters and detailed history were recorded. General check-ups of pulse, blood pressure, height, weight and food habits were recorded. Detailed obstetric history of gravidity, parity, age of menarche, family history of breast cancer and breastfeeding were noted.

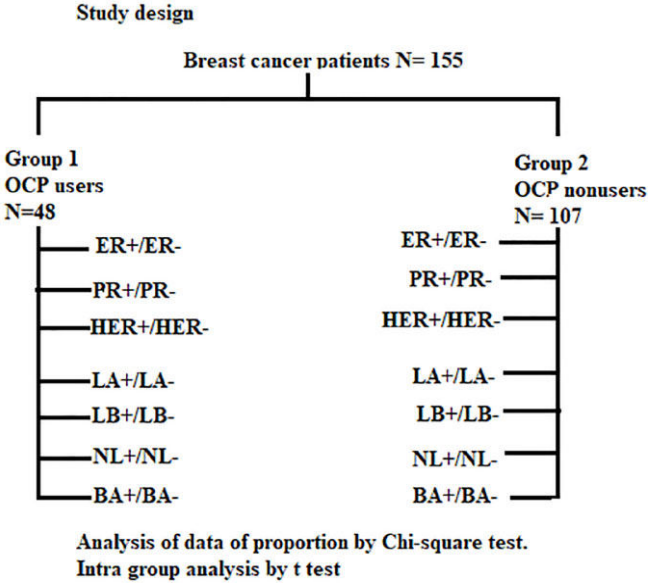


Fig. (1). ER+:Estrogen Receptor+; PR+: Progesterone Receptor+; HER2+:Human Epidermal Growth Factor Receptor 2+; LA:Luminal A; LB: Luminal B; NL: Non luminal/ HER2 enriched; BA; Basal like/TNBC.

2.2. Immuno-histo-chemical Scoring System for ER, PR and HER2 and Clinical Classification

All records were collected from the hospital's medical records. The histo-pathological and immune-histo-chemical

Table 1. Demographic characters, parity and Age of menarche (AOM) of breast cancer patients in OCP users and non-users.

	OCP Users (N=48)	OCP Non Users (N=107)	T value	P value
Age (yrs)	47.6±8.4	49.8±8.1	-1.482	0.139
Height (cm)	147±31	138.2±44	1.322	0.188
Weight (Kgs)	57.4±9.8	58.3±3	-0.515	0.607
BMI (Kg/M^2)	24.1±3.6	25.3±3.3	-1.972	0.051
Parity	2.59±0.9	2.98±1.8	-1.273	0.206
AOM (yr)	13.02±0.5	13.19±0.6	-1.434	0.154

* ($p<0.05$); BMI: Body mass index; AOM: Age of menarche.

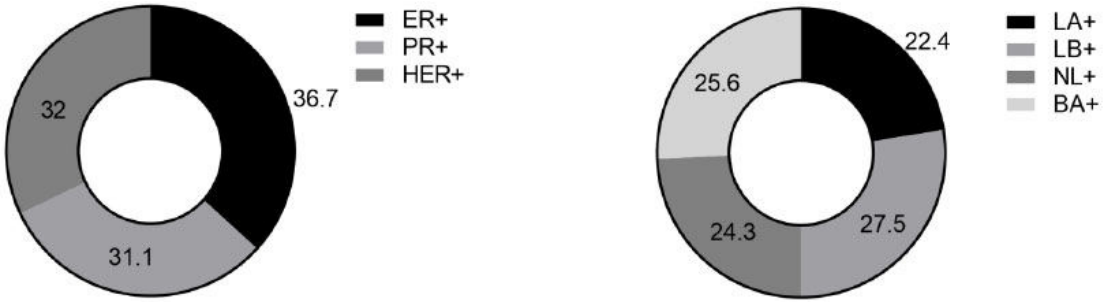


Fig. (2). (A) Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+ in a total number of cases. (B) Distribution of Luminal A (LA), Luminal B (LB), Non-luminal (NL)/ HER2+ enriched and Basal--like (BA)/TNBC in a total number of cases. (Values are in percentage).

(IHC) examination was performed in accordance with the College of American Pathologists/American Society of Clinical Oncology (CAP/ASCO) guidelines by a pathologist. ER and PR scoring for all cases were done using Allred scoring [15].

2.2.1. Allred System of Scoring for ER and PR

ER and PR are nuclear receptors. In the Allred system of scoring, score 0-5 is given to the cells depending on the proportion of cells that are stained (proportion score [PS]), and score 0-3 is given depending on the intensity of staining (intensity score [IS]). By adding the PS and IS, we calculated the final Allred score (PS + IS = AS).

2.2.2. Scoring for HER 2+/neu Overexpression

HER2+/neu is a cell membrane receptor. Depending on the intensity of staining, a score of 0-3 is given to the cells. A positive HER2+/neu result is immune-histo-chemical staining of 3+ (uniform, intense membrane staining of >30% of invasive tumor cells. A negative HER2+/neu result is immune-histo-chemical staining of 0 or 1+.

In Allred scoring system, only the invasive tumor cells should be assessed as ER/PR staining is present in normal breast epithelial cells as well. Here, the normal breast epithelial cells act as an internal positive control. According to ASCO/CAP guidelines, we classified breast cancer cases into 4 subtypes based on the hormonal receptor and HER 2+ status. These were luminal A (ER+ and/or PR+/HER2−), luminal B (ER+ and/or PR+/HER2+), Non-luminal/ HER2-enriched (ER− and PR−/HER2+) and Basal-like (ER− and PR−/HER2−). Those patients who had HER 2+ expression (Equivocal) were not included in molecular subtype analysis Fig. **2**.

2.3. Statistical Analysis

The results were summarized descriptively first. For continuous variables, the summary statistics of mean± standard deviation (SD) were used. For categorical data, the number and percentage were used in the data summaries and diagrammatic presentation. Chi-square ($\chi 2$) test was used for the association between two categorical variables. After confirming the normality assumption, the data were analyzed by parametric test (student t-test), which indicates the level of difference of means between two groups. Logistic linear regression analysis was employed to assess the effect of age on different subtypes of cancers after adjusting the effect of other background confounding variables. The software used was SPSS-20 (USA, Chicago) and Microsoft office 2007.

3. RESULTS

As per Table **1**, there was no significant difference in age among OCP users and non-users. The mean age of OCP users was 47.6 5 years, and non-users was 49.8 years. There was no significant difference between height, weight and BMI among OCP users and non-users. There was no significant difference between parity, age of menarche (AOM), family history of breast cancer, menopausal status, breast-feeding and stage of cancer on admission in OCP users and

Table 2. Reproductive history, family history of breast cancer (FHBC) and stage of breast cancer patients in OCP users and non-users.

	OCP Users (N=48)	OCP Non Users (N=107)	Chi Square Value	p-value	Odds Ratio
FHBC	3 (6.6)/45	11 (10.2)/96	0.655	0.316	0.582 [0.155-2.188]
Nulliparity	7 (14.50/41	21 (19.6)/85	0.607	0.295	0.691 [0.272-1.757]
Menopause	38 (79.1)/9	91 (85)/15	0.433	0.289	0.696[0.280-1.727]
HOBF	40 (83.3)/8	85 (79.4)/21	0.214	0.412	1.235 [0.504-3.029]
Stage 2	9 (18.7)	19 (17.7)	3.352	0.187	
3	28 (58.3)	48 (44.8)			
4	11 (22.9)	40 (37.3)			

* ($p<0.05$); FHBC: Family history of breast cancer; HOBF: History of breastfeeding. (Values in the brackets are in percentage). A. B.

Table 3. Phenotype/ clinical sub- types status in OCP users and non-users in breast carcinoma.

	OCP Users (N=48)	OCP Non Users (N=107)	Chi Square Value	P value	Odds Ratio
ER+	30 (62.5)/18	49(45.7)/58	3.700	0.040*	1.973 [0.982-3.962]
PR+	28 (58.3)/20	39 (36.4)/68	6.467	0.009*	2.441 [1.217-4.895]
HER 2+	21 (43.7)/27	48 (44.8)/59	0.017	0.519	0.956 [0.482-1.898]
Luminal A	12 (25)/36	23 (21.4)/84	0.233	0.387	1.217 [0.547-2.709]
Luminal B	20 (41.6)/28	23 (21.4)/84	6.726	0.009*	2.609 [1.249-5.447]
Non-luminal	5 (10.4)/43	33 (30.8)/74	7.469	0.004*	0.264 [0.095-0.718]
Basal like	13 (27.1)/35	27 (25.2)/80	0.059	0.477	1.101 [0.509-2.381]

* ($p<0.05$); Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+); (Values in the brackets are in percentage).

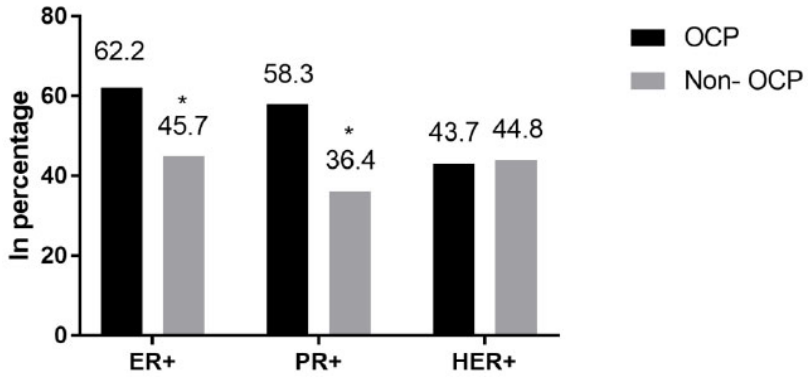


Fig. (3). Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+) in OCP users, non-users and total number of cases. (Values are in percentages). *($p<0.05$);

non-users. There was no significant difference in the stage of Basal-like (TNBC) cases of OCP users compared to TN-BC cases of non-users (Table **2**). The average duration of OCP intake in cases was 1year 3 months. There was no significant change in Ki 67 levels in vases of OCP users (31.4±18.3) compared to non-users (33.4±21.3) (p=0.480).

A significant increase in the proportion of ER+ cases in OCP users (62.5%) compared to non-users (45.7%) with a relative risk 1.97 was observed. There was a significant increase in the proportion of PR+ cases in OCP users (58.3%) compared to non-users (36.4%) with a relative risk 2.44. But, there was no significant difference in the proportion of HER2+ cases in OCP users (43.7%) compared to non-users (44.8%) (Table **3**) (Fig. **3**). A significant increase in the proportion of Luminal B cases in OCP users (41.1%) compared to non-users (21.4%) was observed. But, there was no significant difference in the proportion of Basal-like/ TNBC cases in OCP users (27.1%) compared to non-users (25.2%) (Table **3**) (Fig. **4**).

There was a significant decrease observed in the age at admission in ER+ cases of OCP users (45.3years) compared to non-users (52.2years) (Fig. **5**). On the contrary, a signifi-

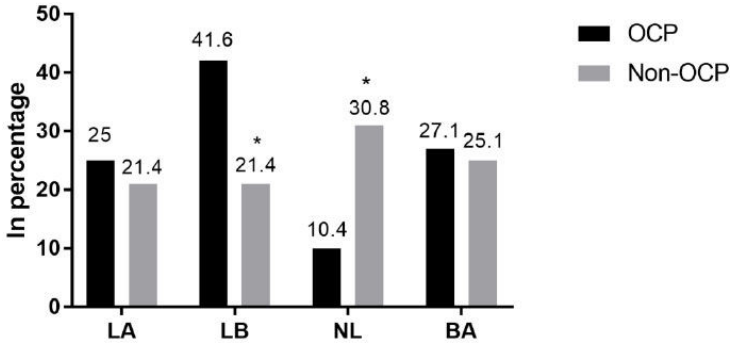


Fig. (4). Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2 enriched and Basal like (BA)/TNBC in OCP users, nonusers and total number of cases. (Values are in percentages) *($p<0.05$);

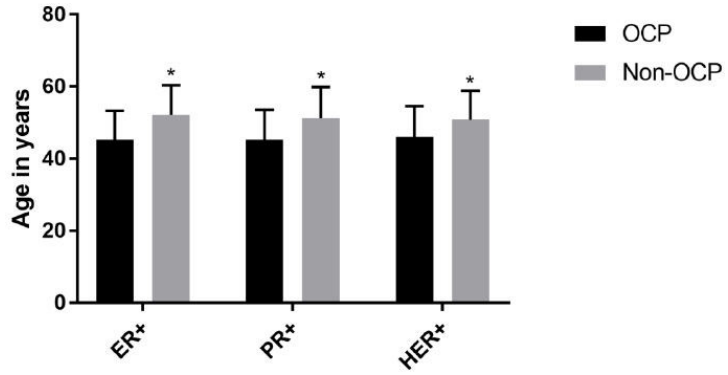


Fig. (5). Distribution of Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+) in OCP users, non-users and total number of cases. *($p<0.05$);

Table 4. Age in Phenotypic sub types/ clinical sub types of breast carcinoma in OCP users and non-users.

Phenotype/ Clinical Subtypes	OCP Users	Phenotype/ Clinical Subtypes	OCP Nonusers	P value
ER + (30)	45.3±8.08	ER + (49)	52.2±8.2	0.001*
PR+ (28)	45.3±8.3	PR + (39)	51.3±8.6	0.001*
HER2 + (27)	46.19±8.5	HER2 + (49)	50.8±8.1	0.039*
Luminal A (12)	44±7.7	Luminal A (23)	51.2±8.6	0.030*
Luminal B (20)	45.5±8.2	Luminal B (23)	52.4±8.2	0.009*
Non-luminal (5)	48±9.2	Non-luminal (34)	50.4±8.1	0.564
Basal (13)	53.1±6.9	Basal (49)	45.5±6.07	0.001*

* ($p<0.05$); Estrogen Receptor+ (ER+), Progesterone Receptor+ (PR+) and Human Epidermal Growth Factor Receptor 2+ (HER2+);(Values in the brackets are number of cases).

cantly higher age was observed at the time of admission in TNBC cases of OCP users (53.1years) when compared to non-users (45.5yeas) (Table **4**) (Fig. **6**). Upon logistic regression among OCP users, the likelihood of ER+, PR+ and Luminal A was 11%, 10% and 13% less with 1 year of higher age, respectively and among OCP users, the likelihood of TNBC was 18% more with 1 year of higher age (Table **5**).

4. DISCUSSION

In this study, we observed that there was a significantly increased proportion of ER+, PR+ tumours in OCP users according to our hypothesis with a relative risk of 1.9 and 2.4. But, the proportion of TNBC cases did not alter with OCP according to our hypothesis. We observed that there was a significantly higher age (18%) at admission in TNBC patients among OCP users compared to non-users, indicating that OCP plays an important role in delaying the progression of TNBC.

The study had several limitations. Our study had a small sample size since it was a hospital-based study. Histories of oral contraceptives had no record and were verbal. We had

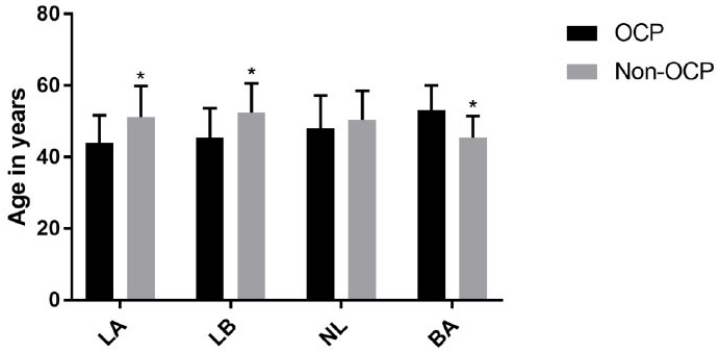


Fig. (6). Distribution of Luminal A (LA), Luminal B (LB), Non luminal (NL)/ HER2 enriched and Basal like (BA)/TNBC in OCP users, nonusers and total number of cases. *(p<0.05);

Table 5. Logistic regression analysis of the adjusted effect of age on selected parameters in OCP users and non-users.

Phenotype/ Clinical Subtypes	OCP Users				Non OCP Users			
	Adjusted OR	p value	95% CI		Adjusted OR	p value	95% CI	
			Lower	Upper			Lower	Upper
ER +	0.89	0.032*	0.80	0.99	1.05	0.171	0.98	1.13
PR +	0.90	0.039*	0.81	1.00	1.04	0.276	0.97	1.12
HER2+	0.97	0.486	0.88	1.06	1.02	0.515	0.96	1.09
Luminal A	0.87	0.033*	0.76	0.99	1.08	0.068	0.99	1.18
Luminal B	0.97	0.559	0.89	1.07	1.00	0.899	0.91	1.08
Non-luminal	0.22	-	-	-	1.02	0.644	0.95	1.09
Basal	1.18	0.011*	1.04	1.34	0.92	0.026*	0.85	0.99

* (p<0.05); Odds Ratio are adjusted for BMI, AOM, parity and stage of breast carcinoma.

to rely on their memory. The duration of use of OCP was cut down to six months in order to increase the sample size. There are studies that show that 'ever use' of OCP has increased the risk of breast cancer [16]. An analysis of data from more than 150,000 women who participated in 54 epidemiologic studies showed that, overall, women who had ever used oral contraceptives had a slight (7%) increase in the relative risk of breast cancer compared with women who had never used oral contraceptives. That is the reason why we conducted the study with OCP use of even 6 months or more than 6 months. This study also revealed that women who were currently using oral contraceptives had a 24% increase in risk that did not increase with the duration of use. Risk declined after use of oral contraceptives stopped, and no risk increase was evident by 10 years after use had stopped [16]. This was another limitation of our study that there were no current OCP users. The average age our subjects started using OCP was 19 years, and the average age after last use was 21 years. But, our strength of this case-to--case study was that we compared the proportion of different subtypes of cancer in OCP users and matched non-users. There was no significant difference in the age, BMI, AOM, parity, breastfeeding, and stage between OCP users and non-users; all the cases were of the same geographical area of South India of the same genetic background, which indicates that many of the confounding factors were matched. We did not evaluate genetic factors that involved significant mutations in *BRCA 1* and *2*, *CHEK2*, *TP53*, *LKB-1* and *PTEN* in our patients. In our opinion, such studies are rarely available in the literature. Our study was designed as a case□case study, where it is easy to quantify the exact risk for subtypes comprehensively in collected data. However, some literature evaluated differences among breast cancer subtypes through case□case studies but not in the Indian context.

Breast cancer is a global health issue among women. However, the incidence of breast cancer has increased significantly in Asian countries as compared to Western countries. Breast cancer accounts for the most frequently diagnosed cancer in Asian women. Although the incidence of breast cancer remains high in developed countries, there has been a shift in the global distribution of breast cancer cases among women in South America, Africa, and Asia [17, 18]. A recent Indian study of 2062 breast cancer patients ranging between 22–100 years revealed a mean age of 51.18 years [19]. Our findings of distribution of different subtypes in a total number of cases (155) were not similar to those reported by Prakash et al. [19] and Mane et al. [19]In our study, the incidence of all subtype cancers was much more evenly distributed. In our study, 22.4% of patients were luminal A, 27.5 were luminal B, 24.3% were HER2 rich, and 25.6% were basal-like in a total number of cases (Fig. **2**). In our study, Luminal A and Luminal B cases were low, and the

percentage Non-Luminal and TNBC cases were more compared to other studies [19, 20]; this is because we did not include more younger patients and not above the age of 60 years. Comparing the distribution of cases in OCP users and non-users revealed that the proportion of ER+, PR+, Luminal B and Non-luminal cases was significantly high with significant relative risk in OCP users compared to non-users.

The age-specific incidence rates of breast cancer vary among the Western and Asian populations. Usually, ER+ occurs in advancing age, and TNBC is known to occur in an early age group. In the Asian population, breast cancer is characterized at an early age in contrast to advancing age among Western women. The age-specific incidence decreases or plateaus after 50 years in Asian women [21-24]. We did not observe significant age specificity in our study because the age group we studied had a narrow range (30 to 60 years). Our study revealed that in OCP user and non-users age (years) at admission to the hospital was significantly reduced in ER+ (45.3 Vs. 52.2), PR+ (45.3 Vs. 51.3) HER+ (46.1 Vs. 50.8), Luminal A (44 Vs. 51.2) and Luminal B (45.5 Vs. 52.2) subtypes. These findings indicate that OCP use augments the progression of ER+, PR+, HER2+, Luminal A and Luminal B breast cancer. On the contrary, In OCP users, age (years) at admission to the hospital was significantly high in TNBC (53.1 Vs. 45.5) compared to non-users. This difference (higher age) at the time of admission to the hospital when other risk factors are matched indicates that OCP use may delay the progression of TNBC.

No doubt, breast cancer aetiology is multi-factorial, and when it comes to subtypes, it is still more complicated. In our study, it is evident that the proportion of ER+ and PR+ cases has increased significantly in OCP users compared to non-users. Our study revealed that age at admission to the hospital significantly reduced in ER+ and PR+ cancer in OCP users when compared to non-users taking consideration of other confounding factors like BMI, AOM, parity and stage of cancer. Logistic regression among OCP users, the Likelihood of ER+, PR+ and Luminal A+ was 11%, 10% and 13% less with 1 year of higher age, respectively. These findings are consistent with other studies done globally. Conversely, we found that the proportion of TNBC has not increased in OCP users compared to non-user, as reviews suggest. But, age at the time of admission to the hospital was significantly high in TNBC cancer in OCP users when compared to non-users taking into consideration of other confounding factors like BMI, AOM, parity and stage of cancer and among OCP users. The likelihood of TNBC was 18% more with 1 year of higher age in OCP users. Our findings here differ from other studies done globally wherein most of the studies, OCP use is associated with increased risk of TNBC.

Both genetic and hormonal factors have been implicated in the genesis of breast cancer. Genetic factors involve significant mutations in *BRCA 1* and *2*, *CHEK2*, *TP53*, *LKB-1* and *PTEN* in 5–10% of patients and lower risk mutations inferred by identical twin and genome-wide association studies in others [25-27]. Epidemiologic and experimental data implicate estradiol as another contributing factor. It is discovered that estradiol, which normally stimulates the growth of cancer cells in tumours that express estrogen receptor alpha (ERα) like in ER+ cancer. ER+ breast cancer development can be influenced by estrogen via ERα receptor-independent mechanisms. Acting through ERα, it stimulates cell proliferation and initiates mutations that occur as a function of errors during DNA replication. The promotional effect of estrogen then supports the growth of cells harboring mutations, which then accumulate until cancer ultimately results. But estrogen has the opposite effect in triple-negative breast cancer. However, β-estradiol was only able to inhibit the growth of TNBC when estrogen receptor beta (ERβ) was present in excess, and in TNBC, the presence of ER β is almost 25% [28]. There are some studies reports that selective activation of ER β reduces the metastatic potential of TNBC cells [29]. These putative evidences favour why, in our study, TNBC cases admitted to the hospital at higher age group.

CONCLUSION

Despite extensive research in understanding the effect of OCP in different subtypes of breast cancers, there are more questions than answers. Our study concludes that prior use of OCP increases the prevalence, relative risk and progression of the disease in ER+, PR+ and Luminal B types of breast cancer. At the same time, prior OCP use does not increase prevalence, the relative risk in TNBC. But is associated with higher age at the time of admission to the hospital. Therefore, it delays the progression of TNBC.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

HUMAN AND ANIMAL RIGHTS

Written consent from all the participants was taken before the study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

We have obtained the permission of the ethical committee from our institution to conduct the study.

CONSENT FOR PUBLICATION

The Author hereby grants the Publisher permission to publish the Work.

FUNDING

None.

CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Sorlie T, Tibshirani R, Parker J, Trevor Hastie JS. Marron, Andrew Nobel, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proceedings of the National Academy of Sciences of the United States of America. 8418-23.

[2] Sotiriou C, Neo SY, McShane LM, Edward L. Korn, Philip M Long, Amir Jazaeri,et al. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proceedings of the National Academy of Sciences of the United States of America. 10393-8.

[3] Trivers KF, Lund MJ, Porter PL, *et al.* The epidemiology of triple-negative breast cancer, including race. Cancer Causes Control 2009; 20(7): 1071-82.
http://dx.doi.org/10.1007/s10552-009-9331-1 PMID: 19343511

[4] Gaudet MM, Press MF, Haile RW, *et al.* Risk factors by molecular subtypes of breast cancer across a population-based study of women 56 years or younger. Breast Cancer Res Treat 2011; 130(2): 587-97.
http://dx.doi.org/10.1007/s10549-011-1616-x PMID: 21667121

[5] Yang XR, Sherman ME, Rimm DL, *et al.* Differences in risk factors for breast cancer molecular subtypes in a population-based study. Cancer Epidemiol Biomarkers Prev 2007; 16(3): 439-43.
http://dx.doi.org/10.1158/1055-9965.EPI-06-0806 PMID: 17372238

[6] Ma H, Wang Y, Sullivan-Halley J, *et al.* Use of four biomarkers to evaluate the risk of breast cancer subtypes in the women's contraceptive and reproductive experiences study. Cancer Res 2010; 70(2): 575-87.
http://dx.doi.org/10.1158/0008-5472.CAN-09-3460 PMID: 20068186

[7] Yang XR, Chang-Claude J, Goode EL, *et al.* Associations of breast cancer risk factors with tumor subtypes: a pooled analysis from the Breast Cancer Association Consortium studies. J Natl Cancer Inst 2011; 103(3): 250-63.
http://dx.doi.org/10.1093/jnci/djq526 PMID: 21191117

[8] Palmer JR, Boggs DA, Wise LA, Ambrosone CB, Adams-Campbell LL, Rosenberg L. Parity and lactation in relation to estrogen receptor negative breast cancer in African American women. Cancer Epidemiol Biomarkers Prev 2011; 20(9): 1883-91.
http://dx.doi.org/10.1158/1055-9965.EPI-11-0465 PMID: 21846820

[9] Li Li, Zhong Yahua, Zhang Hongyan, *et al.* Association between oral contraceptive use as a risk factorand triple-negative breast cancer: A systematic review and meta-analysis. Molecular and clinical oncology 2017; 7: 76-80.

[10] Warner ET, Tamimi RM, Boggs DA, *et al.* Estrogen receptor positive tumors: do reproductive factors explain differences in incidence between black and white women? Cancer Causes Control 2013; 24(4): 731-9.
http://dx.doi.org/10.1007/s10552-013-0153-9 PMID: 23380944

[11] Rosato V, Bosetti C, Negri E, Talamini R, Dal Maso L, Malvezzi M, *et al.* Reproductive and hormonal factors, family history, and breast cancer according to the hormonal receptor status. Eur J Cancer Prev 2013.
PMID: 23817433

[12] Phipps AI, Buist DS, Malone KE, *et al.* Reproductive history and risk of three breast cancer subtypes defined by three biomarkers. Cancer Causes Control 2011; 22(3): 399-405.
http://dx.doi.org/10.1007/s10552-010-9709-0 PMID: 21184265

[13] Yan Y, Li X, Blanchard A, *et al.* Expression of both estrogen receptor-beta 1 (ER-β1) and its co-regulator steroid receptor RNA activator protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with estrogen receptor-alpha (ER-α)-negative early breast cancer (EBC). Ann Oncol 2013; 24(8): 1986-93.
http://dx.doi.org/10.1093/annonc/mdt132 PMID: 23579816

[14] Hammond MEH, Hayes DF, Dowsett M, *et al.* American Society of Clinical Oncology; College of American Pathologists. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med 2010; 134(7): e48-72.
PMID: 20586616

[15] Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998; 11(2): 155-68.
PMID: 9504686

[16] Collaborative Group on Hormonal Factors in Breast Cancer. Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Lancet 1996; 347(9017): 1713-27.
http://dx.doi.org/10.1016/S0140-6736(96)90806-5 PMID: 8656904

[17] Sung H, Rosenberg PS, Chen WQ, *et al.* Female breast cancer incidence among Asian and Western populations: more similar than expected. J Natl Cancer Inst 2015; 107(7)djv107
http://dx.doi.org/10.1093/jnci/djv107 PMID: 25868578

[18] Youlden DR, Cramb SM, Yip CH, Baade PD. Incidence and mortality of female breast cancer in the Asia-Pacific region. Cancer Biol Med 2014; 11(2): 101-15.
PMID: 25009752

[19] Pandit P, Patil R, Palwe V, Gandhe S, Patil R, Nagarkar R. Prevalence of Molecular Subtypes of Breast Cancer: A Single Institutional Experience of 2062 Patients. Eur J Breast Health 2020; 16(1): 39-43.

[20] Mane A, Khatib KI, Deshmukh SP, Nag SM, Sane SP, Zade BP. A Comparison of Clinical Features, Pathology and Outcomes in Various Subtypes of Breast Cancer in Indian Women. J Clin Diagn Res 2015; 9(9): PC01-4.
http://dx.doi.org/10.7860/JCDR/2015/15253.6461 PMID: 26500944

[21] Shin HR, Joubert C, Boniol M, *et al.* Recent trends and patterns in breast cancer incidence among Eastern and Southeastern Asian women. Cancer Causes Control 2010; 21(11): 1777-85.
http://dx.doi.org/10.1007/s10552-010-9604-8 PMID: 20559704

[22] Bray F, McCarron P, Parkin DM. The changing global patterns of female breast cancer incidence and mortality. Breast Cancer Res 2004; 6(6): 229-39.
http://dx.doi.org/10.1186/bcr932 PMID: 15535852

[23] Liu L, Zhang J, Wu AH, Pike MC, Deapen D. Invasive breast cancer incidence trends by detailed race/ethnicity and age. Int J Cancer 2012; 130(2): 395-404.
http://dx.doi.org/10.1002/ijc.26004 PMID: 21351091

[24] Leong SP, Shen ZZ, Liu TJ, *et al.* Is breast cancer the same disease in Asian and Western countries? World J Surg 2010; 34(10): 2308-24.
http://dx.doi.org/10.1007/s00268-010-0683-1 PMID: 20607258

[25] Martin AM, Weber BL. Genetic and hormonal risk factors in breast cancer. J Natl Cancer Inst 2000; 92(14): 1126-35.
http://dx.doi.org/10.1093/jnci/92.14.1126 PMID: 10904085

[26] Lichtenstein P, Holm NV, Verkasalo PK, *et al.* Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med 2000; 343(2): 78-85.
http://dx.doi.org/10.1056/NEJM200007133430201 PMID: 10891514

[27] Thompson D, Easton D. The genetic epidemiology of breast cancer genes. J Mammary Gland Biol Neoplasia 2004; 9(3): 221-36.
http://dx.doi.org/10.1023/B:JOMG.0000048770.90334.3b PMID: 15557796

[28] Hinsche O, Girgert R, Emons G, Gründker C. Estrogen receptor β selective agonists reduce invasiveness of triple-negative breast cancer cells. Int J Oncol 2015; 46(2): 878-84.
http://dx.doi.org/10.3892/ijo.2014.2778 PMID: 25420519

[29] Girgert R, Emons G, Gründker C. Inhibition of growth hormone receptor by Somavert reduces expression of GPER and prevents growth stimulation of triple-negative breast cancer by 17β-estradiol. Oncol Lett 2018; 15(6): 9559-66.
http://dx.doi.org/10.3892/ol.2018.8521 PMID: 29805678

RESEARCH ARTICLE

Ubiquitin Mediated Degradation of EGFR by 17 β-estradiol in Triple Negative MDA-MB-231 (TNBC) Breast Cancer Cells Line

Vitthal Khode[1,2,3], Sumangala Patil[1], Vishwas Kaveeshwar[2], Komal Ruikar[3] Anil Bargale[2], Sarathkumar E.[4] and Satish Patil[3]

[1]Department of Physiology, Shri B M Patil Medical College, Hospital & Research Centre, BLDE (Deemed to be University), Vijaypur, India; [2]Central Research Laboratory, SDM College of Medical Sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India; [3]Department of Physiology, SDM College of Medical Sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India; [4]Nitte University Centre for Science Education and Research, Mangalore, India

ARTICLE HISTORY

Received: November 03, 2020
Revised: February 08, 2021
Accepted: February 16, 2021

DOI:
10.2174/1566524021666210729144713

Abstract: ***Background*****:** Triple Negative Breast Cancer (TNBC) commonly displays Epidermal growth factor receptor (EGFR). Effective EGFR degradation results in the suppression of tumor in various models. Studies have addressed the relevance of this strategy in the treatment of TNBC. In the present study, we examined the effect of 17 β-estradiol on EGFR expression in MDA-MB-231 (TNBC) cell line and assessed whether 17 β-estradiol degrades EGFR by ubiquitination pathway.

Objectives**:** The objectives of this study are to treat MDA-MB-231 cell lines with Cycloheximide with or without 17β-estrdiol to observe whether 17β-estradiol leads to EGFR degradation and to treat with MG-132 to assess whether degradation occurs through ubiquitination pathway.

Methods**:** MDA-MB-231 cells were treated with 17β-estradiol (E2) and EGFR expression was studied by western blotting at different intervals by using Cycloheximide chase. To assess ubiquitination pathway of degradation of EGFR in MDA-MB-231 cell line, MG-132 was used.

Results**:** EGFR expression was reduced with β-estradiol treatment in MDA-MB-231 cell line with Cycloheximide chase. Upon Treatment with MG-132 and E2, EGFR expression did not reduce, suggesting that Estrogen degrades EGFR by ubiquitination pathway.

Conclusion**:** Estrogen degrades EGFR in MDA-MB-231 cells and this degradation occurs by ubiquitination.

Keywords: Triple negative breast cancer, MDA-MB-231, estrogen, epidermal growth factor receptor, MG-132, ubiquitination.

1. INTRODUCTION

Breast cancer (BC) is the most common malignancy in women worldwide [1, 2]. Almost 70% of patients with breast cancer express estrogen receptor-α (ERα). Due to effective endocrine therapies, the mortality of patients with ERαtumors has reduced significantly in the past decade. Similarly, about 15% of patients have tumors that over express HER2 receptor and thus are candidates for HER2 targeted treatments. In contrast, Triple negative breast cancer (TNBC) occurs in 10–15% of patients, yet this subtype accounts for about 50% of all breast cancer deaths. TNBCs lack clinical expression of ERα, progesterone receptor, and HER2 over expression (ERα−/PR−/HER2−). Although heterogeneous, TNBCs typically occur in younger women and African American and Asian women as well as among some patients with BRCA1 gene defects [1, 2].

TNBC commonly displays epithelial growth receptor (EGFR). EGFR and human epidermal growth factor receptor 2 (HER2) are members of the ErbB family of RTKs that are of particular importance in breast cancer. Although estimates vary, EGFR over-expression is thought to present in approximately 30% of breast cancers, while HER2 positivity is detected in 25-30% of cases [3, 4]. High levels of EGFR and HER2 are associated with more aggressive cancer phenotypes and poorer prognosis [5]. When activated by ligand binding and dimerization, RTKs are internalized and

*Address correspondence to this author at the Depatment of Physiology, SDM College of Medical sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India;
Tel: 0836-2477280; E-mail: drkhoday@yahoo.co.n

1566-5240/XX $65.00+.00

pro-oncogenic intracellular signalling is initiated, primarily via the MAPK and PI3K/Akt pathways. The aberrant gene expression induced by enhanced expression/activation of EGFR or HER2 promotes cancer cell proliferation, survival, migration, and angiogenesis [6]. TNBC is more aggressive than other disease subtypes, and no molecular targeted agents are currently available for the treatment. Effective EGFR degradation results in the suppression of tumor in various models. Studies have addressed the relevance of this strategy in the treatment of TNBC. The ability of certain monoclonal antibody mixtures to enhance EGFR degradation raised the possibility that such a strategy would inhibit EGFR-driven tumors, including the most aggressive fraction of breast cancer [1]. Therefore, degradation of EGFR can be one of the potential targets for the treatment of TNBC.

In western countries, clinical data suggests that where OCP usage is high, prevalence of ER + breast cancer is high. In African and Asian countries where OCP usage is low, prevalence of TNBC is high [7, 8]. This clinical and molecular data have led to our hypothesis that estrogen may interact with EGFR in TNBCs and degrade it through one of the degradation pathways. The underlying mechanism hypothesis is probably related to the sorting of internalized EGFRs to either recycling or degradation [9]. Sorting requires the conjugation of multiple ubiquitins, which mark the receptor for degradation [10]. We hypothesized that estrogen is involved in this mechanism, which facilitates the ubiquitination process. Therefore, we chose MDA-MB-231 cell lines treated Cycloheximide with or without β-estrdiol to observe whether β-estradiol leads to EGFR degradation. Subsequently, cell lines were treated with MG-132 to assess whether degradation occurs through ubiquitination pathway.

2. MATERIALS AND METHODS

2.1. Cell Culture

Human breast cancer cell lines MDA-MB-231, MCF-7 were obtained from the National Centre for Cell Sciences, Pune, India. Cells were grown in the respective medium as prescribed by the supplier. MCF-7, MDA-MB-231 were cultured in Roswell Park Memorial Institute media (RPMI) containing phenol red and supplemented with 10% Fetal Bovine Serum (FBS). Cell lines were cultured in a 5% CO_2 humidified atmosphere at 37°C. Cells were used up to a maximum of 28 passages and were subject to regular mycoplasma testing. β-estadiol, Cyclohexamide and MG-132 were obtained from the Aldrisch sigma.

2.2. Effect of 17 β-estrdiol on MDA-MB-231 and MCF-7 Cell Lines on EGFR Expression

EGFR is a transmembrane receptor and its over expression in breast cancer predicts for poor prognosis. This study was designed to investigate whether estrogen plays an active role in the expression or suppression of EGFR in MCF-7 and MDA-MB-231 cell lines. We hypothesized that 100 nM of 17β-estradiol will lead to the over expression of EGFR in MCF-7 and suppresses in MDA-MB-231 cell lines. We standardised the prior concentration of estrogen to be used *i.e*. 100nM.

Technique: MDA-MB-231 and MCF-7 cell lines were cultured in a large flask. At 70-80% confluence cell were trypsinized twice, centrifuged at 1200rcf for 4 min, supernatant was removed. FBS was added to approximately 4×10^6 cells/well were placed in 6 well plates and starved for 12 hours. Both cell lines were treated with 100 nM β-estradiol. Expression of EGFR at 0 and 3 hours time intervals was assessed by western blot.

2.3. Cycloheximide Chase to Assess the Effect of 17 β-estradiol on MDA-MB-231 Cells on EGFR Expression

A difference in a steady state protein levels after inhibiting degradative pathways provides strong proof for the contribution of proteolysis to the control of protein abundance [11]. However, such an analysis still does not furnish information regarding the kinetics of protein turnover. Cycloheximide chase followed by western blotting overcomes this deficiency by allowing researchers to visualize protein degradation over time [12-14]. Further, because protein detection following Cycloheximide chase is typically carried out by western blotting, radioactive isotopes and lengthy immune-precipitation steps are not needed for Cycloheximide chase, unlike many commonly used pulse chase techniques, which are also performed to visualize protein degradation [15]. Cycloheximide chase is suitable for analyzing protein stability over a short time course (*i.e.,* up to two hours). Over longer time courses (*i.e.*, two hours to days), Cycloheximide, a global inhibitor of translation, is toxic to cells, likely due to depletion of ubiquitin [16]. Further, analyses of protein stability over longer time courses are more likely to be compromised by indirect effects of globally reduced protein synthesis on the degradation of the protein of interest (*e.g.,* degradation of a short-lived protein taking part in the degradation of the protein of interest). Other techniques, such as pulse chase metabolic labeling experiments, are therefore better suited for studying the degradation of long-lived proteins and may be carried out to corroborate results obtained in Cycloheximide chase experiments.

Technique: MDA-MB-231 cell lines were cultured in a large flask. At 70-80%, confluence cells were trypsinized twice, centrifuged at 1200 rcf for 4 min, supernatant was removed. FBS added approximately 4×10^6 cells/well were placed in 6 well plates and starved for 12 hours. Cells were treated with 100 nM 17β-estradiol. Expression of EGFR at 0, 0.5, 1, 2, 3 and 4 hours' time intervals with or without 50µg cycloheximide was assessed by western blot.

2.4. EGFR Degradation is Due to Ubiquitination

The ubiquitin-proteasome system (UPS) is a major protein degradative pathway involved in the

preservation of cellular structure and function [17, 18]. While the 20S proteasome is involved in direct protein hydrolysis, degradation of ubiquitinated proteins by the 26S proteasome is a relatively more important process in protein turnover [19-21]. Ubiquitination of proteins designed for degradation is an ATP-dependent process and involves cooperation of three ubiquitin ligase enzymes. In particular, the ubiquitin moiety is transferred by Ubiquitin-activating enzyme E1 to the Ubiquitin-conjugating enzyme E2 followed by formation of ubiquitin chain ligation on target proteins by a substrate specific E3 ubiquitin ligase [22]. Selected components of the 26S cap proteins are involved in the recognition and transport of ubiquitinated proteins for degradation by the 26S proteasome [23, 24]. MG-132 is a potent, reversible, and cell-permeable proteasome inhibitor. It reduces the degradation of ubiquitin-conjugated proteins in mammalian cells and permeable strains of yeast by the 26S complex without affecting its ATPase or iso-peptidase activities.

Technique: MDA-MB-231 cell lines were cultured in a large flask. At 70-80%, confluence cells were trypsinized twice, centrifuged at 1200rcf for 4 min, supernatant was removed. FBS was added to approximately 4 × 10^6 cells/well were placed in 6 well plates and starved for 12 hours. Cells were treated with 100 nM 17β-estradiol and 50 µg Cycloheximide. Expression of EGFR at 0, 0.5, 1, 2, 3 and 4 hours time intervals with or without MG-132 was assessed by western blot.

2.5. Total Cell Extraction and Western Blotting

Cells were obtained from 6-well plates after the treatment after adding lysate. Cell lysates were collected on ice by washing x1 in ice-cold PBS then scraping in 100 µL of lysis buffer (150 mM NaCl, 50 mM Tris base pH 8, 1 % NP-40 containing protease and phosphatase inhibitors. The lysates were centrifuged at 14,000 rcf for 10 min at 4 °C and protein concentration of the supernatants was determined by bicinchoninic acid assay (BCA) assay. For the expression analysis in different breast cancer cell lines, total protein was extracted and quantitated as described previously [25]. Total protein was separated on 10 % Bis–Tris PAGE gel using Tris–Hcl buffer and the proteins were transferred to nitrocellulose membranes (Himedia) using a transfer apparatus at 65 V for 90 min. The antibodies were used against EGFR (rabbit monoclonal, BD Biosciences, CA-9061) and GAPDH (Santa Cruz Biotechnology, CA-166574). Appropriate secondary antibodies conjugated to horseradish peroxidase (BioRad) were incubated with respective membranes for 2 h at room temperature. The membranes were developed using ECL plus (BioRad), and the image was captured using enhanced Chemi-luminescence system, G: BOXChemi XX6/XX9. Immunoblot for GAPDH was considered as an internal control for loading. The protein bands were quantified and normalized relatively as the control band with Image J, version 1.35d (National Institutes of Health Image software).

2.6. Wound Healing Assay

The wound healing assay is a standard *in vitro* technique for probing collective cell migration in two dimensions. In this assay, a cell-free area is created in a confluent monolayer by physical exclusion or by removing the cells from the area through mechanical, thermal, or chemical damage. The exposure to the cell-free area induces the cells to migrate into the gap.

Technique: Briefly, the MDA-MB-231 cells (4 × 10^6 cells/well) were plated in 6-well plates for 48 h to a confluence of about 80%, then wounded by scratching with a p200 pipette tip. Thereafter, the debris was removed and we washed the cells once with 1 mL of the growth medium to assure the edges of the scratch were smoothed by washing. We took utmost care to make the wounds of the same dimensions, both for the experimental and control cells, to minimize any possible variety resulting from a difference in scratch width. The cells were then incubated with DMEM medium containing 0.5% FBS and treated with 100 nM of 17β-estradiol. The control sample harboured the cells and a standard medium without any active agents. The MDA-MB-231 cell migration was assessed by gap closure migration assay, embedded by free ImageJ software (version 1.50i, National Institute of Health, Bethesda, MD, USA). The area of the initial wound was measured, followed by gap area measurements after 24 h. The migration factor was represented as the gap area value over the initial scratch area.

2.7. Statistical Analysis

Statistical analysis was carried out by using Graph Pad Prism version 7.04. Statistical analysis of expression of EGFR. $P<0.05$ was considered to be statistically significant. Results for normally distributed data were analysed using student t test and ANOVA.

3. RESULTS

3.1. Effect of 17β-estrdiol on MCF-7 and MDA-MB-231 Cell Lines on EGFR Expression

There was increased expression of EGFR in 17β-estradiol treated MCF-7 cell lines at 0, 6 and 12 hours of interval (P=0.0008). A statistical significant 1.12 and 1.4 fold increased expression of EGFR at 6 hours and 12 hours was observed, respectively. There was reduced expression of EGFR in 17β-estradiol treated MDA-MB-231 cell lines at 0, 6 and 12 hours of interval. (P=0.0001) A statistical significant 0.41and 0.38 fold decreased expression of EGFR at 6 hours and 12 hours was observed, respectively (Fig. **1**).

3.2. Cycloheximide Chase to Assess the Effect of β-estradiol on MDA-MB-231 Cells on EGFR Expression

There was reduced expression of EGFR at 3 hours in cells treated with Cycloheximide and 17β-estradiol compared to cells treated with Cycloheximide alone. With Cycloheximide alone expression of EGFR reduced significantly to 1.29 fold. With Cycloheximide

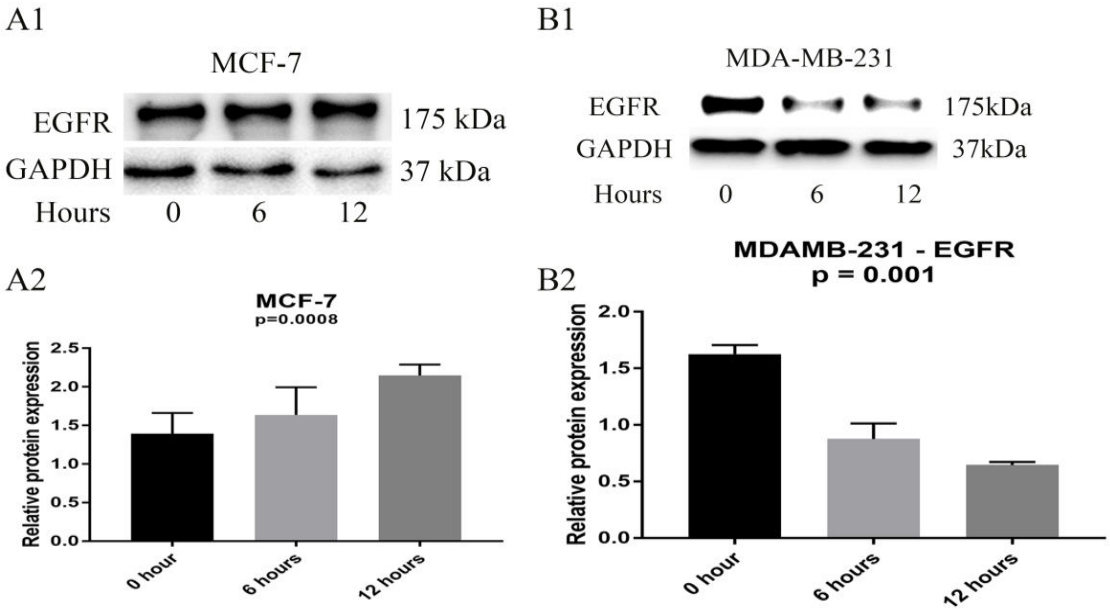


Fig. (1). A1. Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours. (Representative blot) **A2**. Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours. Statistical analysis performed was ANOVA (*p*=0.0008). **B1**. Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours. (Representative blot) **B2**. Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours. Experiment was done thrice in triplicates. Statistical analysis performed was ANOVA (*p*=0.001).

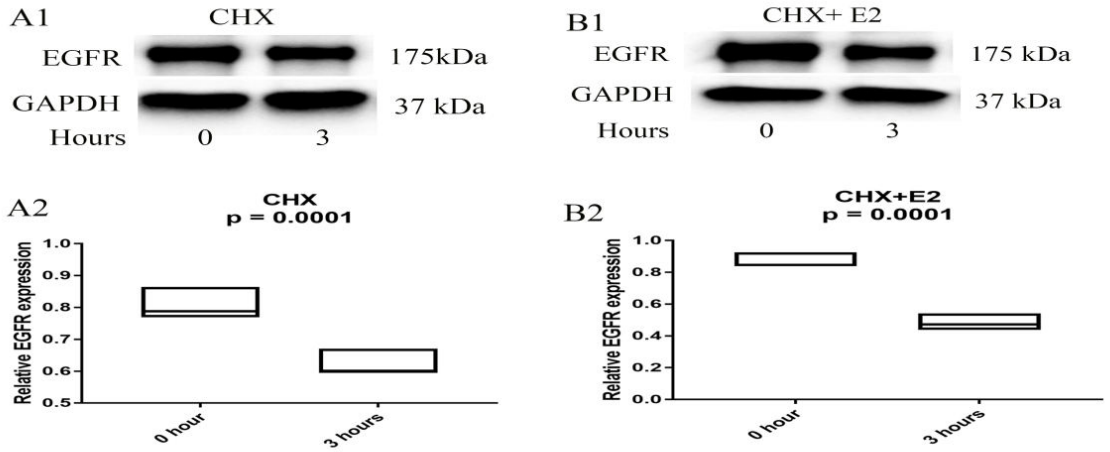


Fig. (2). A1. Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours. (Representative blot) **A2**. Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours. Statistical analysis was performed by independent t test (*p*=0.0001). **B1**. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours. (Representative blot) **B2**. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours. Experiment was done thrice in triplicates. Statistical analysis was performed by independent t test (*p*=0.0001).

and 17β-estrdiol expression was further reduced significantly 1.73 fold (Fig. **2**).

3.3. Cycloheximide Chase to Assess Ubiquitin Mediated EGFR Degradation

There was a significant reduced expression of EGFR at 1, 2, 3 and 4 hours in cells treated with 17β-estradiol, Cycloheximide (*P*= 0.001). There was a 1.52 fold decrease in the expression of EGFR from 0 hour to 4 hours. There was no significant reduced expression of EGFR at 1, 2, 3, and 4 hours in cells treated with 17β-estradiol, Cycloheximide and MG-132. (P=0.05) There was a meagre 0.7 fold decrease in the expression of EGFR from 0 hour to 4 hours (Fig. **3**).

3.4. Wound Healing Assay

The results of the wound healing assay are presented in Fig. **4**. In the control group, cell migration was very dynamic and the ratio of 0 hour to 24 hour gap was 1.09 after 24 hrs. Using a 100nM estrogen, the motility of the MDA-MB-231 cells was inhibited and the ratio of 0 hour to 24 hour gap was 0.88. Therefore,

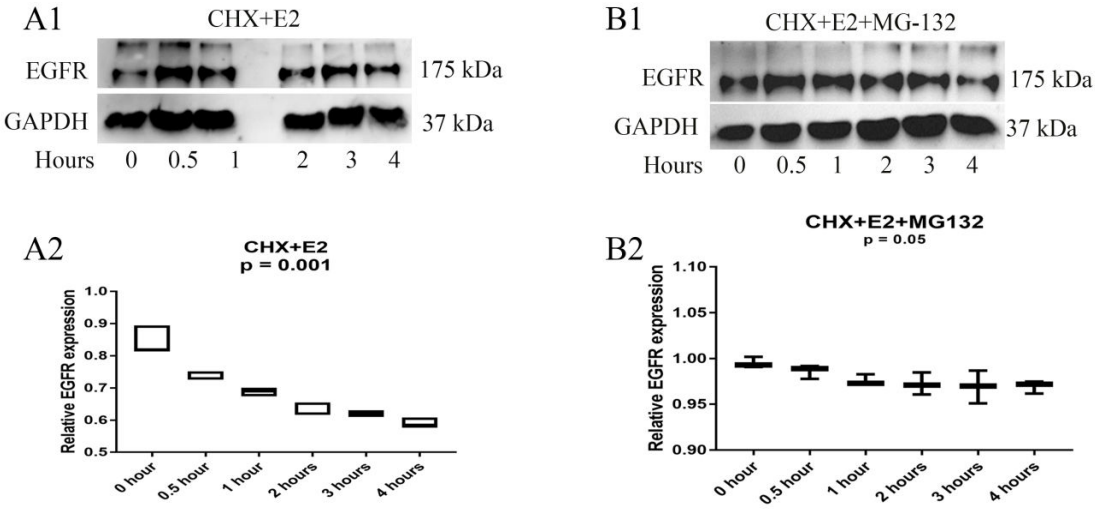


Fig. (3). A1. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1,2, 3 and 4 hours. (Representative blot) **A2**. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1, 2, 3 and 4 hours. Statistical analysis was performed by one way ANOVA (p=0.001). B1. Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide +MG-132 at 0, 0.5, 1,2, 3 and 4 hours. (Representative blot) **A2**. Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide + MG-132at 0, 0.5, 1,2, 3 and 4 hours. Experiment was done thrice in triplicates. Statistical analysis was performed by one way ANOVA (p=0.05).

it can be interpreted that 17β-estradiol promoted migration and inhibition of the MDA-MB-231 cells (Fig. **4**).

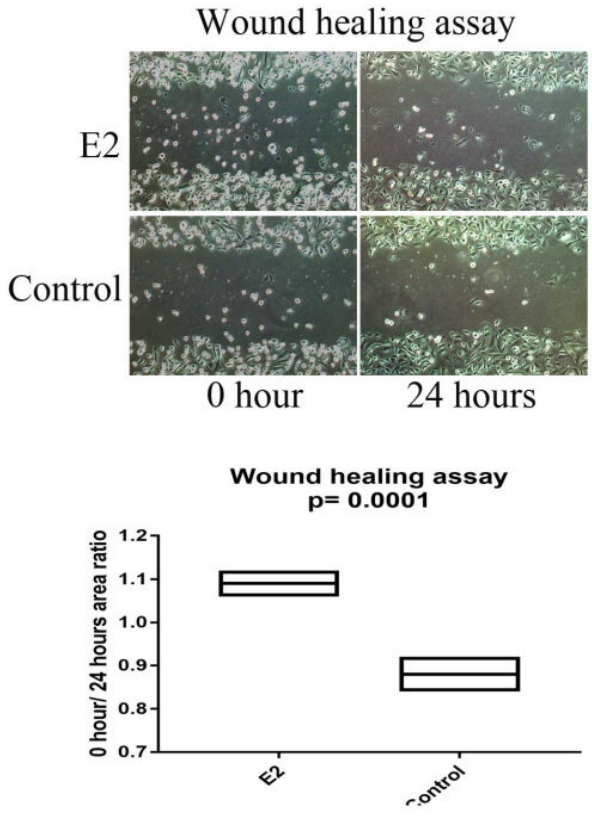


Fig. (4). Effect of 100 nM 17β-estradiol on MDA-MB-231cellline with 100 nM of E2 on wound healing. Experiment was done thrice in triplicates. Statistical analysis was performed using unpaired t test (p=0.0001).

4. DISCUSSION

In this study, we observed that there was a reduced expression of EGFR in MDA-MB-231 cell lines compared to MCF-7 cell lines upon treatment with 17β-estradiol. This indicates that estrogen behaves differently with ER+ and TNBC cell lines. There was a reduced expression of EGFR in MDA-MB-231 cell lines upon treatment with β-estradiol and Cycloheximide when compared to cell lines treated with Cycloheximide alone. This further confirms that estrogen certainly degrades EGFR. We also observed that there was no reduced expression of EGFR in MDA-MB-231 cells treated with 17β-estradiol, Cycloheximide and MG-132 compared to cells treated with 17β-estradiol and Cycloheximide. These observations suggest that β-estradiol degrades EGFR in MDA-MB-231Cells and degradation is mediated by ubiquitination.

There were few limitations of the study. We did not use other types of TNBC cell lines. We did not perform RT-PCR to estimate mRNA levels. We did not change the levels of 17β-estradiol to the exact concentration EGFR inhibited. We did no elucidate the effect of estrogen antagonist. We did not study downstream molecules of EGFR.

Although breast cancer subtypes are genetically linked, environmental factors play a key role. Oral estrogen (OCP) consumption in western countries has been high and in developing countries is low. Accordingly, incidence of ER+ breast cancer is high and low, respectively. But, TNBC incidences are low and high respectively. This has led us to think that more usage of oral estrogens may lead to less incidences of TNBC. There are evidences that ER+ tumours and TNBC behaves indifferently in presence of estrogen. In our study we too tried to demonstrate that

17β-estradiol can act indifferently in different phenotypic breast cancers *in vitro*. We demonstrated that 17β-estradiol augment the proliferation of MCF-7 cells by increasing the expression of EGFR whereas β-estradiol decreases the proliferation of MDA-MB-231 cells by decreasing the production of EGFR.

TNBCs typically occur in younger women And African American women as well as among some patients with BRCA1 gene defects [1, 2]. Population-based data show that African American women have a higher incidence of TNBC and present with more advanced stages than Caucasian women [26]. This cancer subtype also associates with adverse biological features, including high mitotic count and very aggressive behavior. Based on current data, estradiol regulates gene expression of EGFR and other several proteins by genomic and non-genomic inputs [27, 28]. Genomic signals involve the direct action of nuclear-localized ERα as an estradiol regulated transcription factor or co-regulator. By contrast, non-genomic signaling involves extra nuclear events mediated by extra nuclear ERs often in cooperation with co activator or adaptor proteins [29]. Thus, estrogens promote the progression of ER+ breast cancers through predominant ERα. In TNBC, second type of estrogen receptor, termed estrogen receptor-beta (ERβ) is present. ERα and ERβ have reciprocal actions. Studies have demonstrated that ERβ1 inhibits epithelial mesenchymal transition (EMT) and invasion in basal-like breast cancer cells when they grow esther *in vitro* or *in vivo* in zebrafish. EMT is also because of hypoxia known in cancer development [30]. Thus, activation of ERβ in TNBC probably reduces the expression of EGFR [31]. EGFR expression can also be degraded by the activation of non-genomic pathways. In our study, we demonstrated that β-estradiol indeed causes degradation, also we tried to analyse how degradation occurs. There are various steps involved in degradation of EGFR. Upon activation, EGFR is tyrosine-phosphorylated, and subsequently recruits Cbl, an E3 ubiquitin ligase, and Grb2, an adaptor protein, for assembly of the ubiquitination complex, and interacts with Eps15 and AP-2, two endocytic adaptor proteins, to form clathrin-coated endocytic vesicles [32- 35]. The endocytic vesicles or endosomes containing ubiquitinated EGFR are recognized by the ubiquitin-binding protein Hrs and transported to multi-vesicular bodies (MVBs) [36, 37]. Finally, the MVBs fuse with lysosomes to complete the degradation of EGFR. We hypothesized that estradiol inhibits ubiquitination. To test our hypothesis, we treated MDA-MB-231 cells with cycloheximide and estradiol and observed it for degradation of EGFR. There was a 1.52 fold significant decrease in the expression of EGFR. Subsequently, to test another hypothesis that degradation of EGFR occurs due to facilitation of ubiquitination, we treated MDA-MB-231 cells with cycloheximide, estradiol and MG-132 where MG-132 effectively blocks the proteolytic activity of the 26S proteasome complex reduces the degradation of ubiquitin-conjugated proteins. We observed that there was a statistically insignificant 0.7 fold decrease in EGFR expression. This indicates that degradation of EGFR occurs upon treatment with 17β-estradiol, where degradation is mainly mediated by ubiquitination. We also tested our hypothesis on wound healing test. We treated MBA-MB-231 cells with or without estradiol; we observed that without estradiol, cell migration was very dynamic, a ratio of 0 hour to 24 hour gap was 1.09 after 24 hrs and motility of the MDA-MB-231 cells was inhibited and ratio of 0 hour to 24 hour gap was 0.88 with estradiol. This observation also proves that esrogen delays the proliferation of MDA-MB-231 cells.

EGFR is one of the receptors most commonly associated with human tumors and has been shown to correlate with the progression of many tumor types, including breast tumors [38-40]. Most often associated with aspects of tumor growth (*i.e*., proliferation, apoptosis, and cell survival), little emphasis has been placed on the effects of EGF on breast cancer cell migration. The complex process of cell migration is a critical component of many normal and patho-physiological processes, and its central role in the progression of tumors from a noninvasive to an invasive and metastatic phenotype is well known [41]. Epidermal growth factor receptor (EGFR) levels predict a poor outcome in human breast cancer and are most commonly associated with proliferative effects of epidermal growth factor. In this study, we tried to demonstrate the effect of estrogen with EGFR expression in different subtypes of breast cancers, mainly ER+ and TNBC tumours, whether they respond differently. We found that estradiol degrades EGFR in MDA-MB-231 and we could also demonstrate that degradation occurring through ubiquitination. The underlying mechanism of degradation appears related to the sorting of internalized EGFRs to either recycling or degradation [9]. Sorting EGFR requires conjugation of multiple ubiquitins, which mark the receptor for degradation [10]. Therefore, our study demonstrated that EGFR is degraded by ubiquitination.

Several lines of evidence support the possibility that EGFR plays a the role of a driver in a large fraction of TNBC. For example, EGFR gene amplification is commonly identified in metaplastic breast carcinoma, a basal-like fraction of tumors [42]. Likewise, gene expression signatures correlated TNBC with modules comprising EGF-like ligands, EGFR, and several downstream effectors [43]. Although TNBC clinical trials using EGFR inhibitors, including cetuximab, reported a lack of clinical benefit [44], our study offers an alternative strategy by degrading EGFR by estrogen. Experiments that used TNBC line indicated that down regulation of EGFR through ubiquitination can retard motility and proliferation of TNBC cell line.

By adding estogen, we detected EGFR degradation and degradation occurs through ubiquitination in MDA-MB-231 cellline. Our *in vitro* study require confirmation in animal

Models. Assuming confirmation *in vivo*, this study may help in understanding alternative pathway where degradation of EGFR by estrogen or other specific molecule receptors can be a targeted in the treatment

of TNBC. In the future, estrogen like molecules may be used as adjunct in the treatment of TNBC.

CONCLUSION

We concluded that β-estradiol degrades EGFR in MDA-MB-231 cells and this degradation occurs by ubiquitination. This study may help in understanding alternative pathways where degradation of EGFR by estrogen or other specific molecule receptors can be targeted in the treatment of TNBC.

LIST OF ABBREVIATIONS

AKT	=	Ak strain transforming murine thymoma viral oncogene
ANOVA	=	Analysis of variance
AP-2	=	Adaptor protein 2
Cbl-gene	=	Casitas B-lineage lymphoma
DMEM	=	Dulbecco's modified Eagle's medium
E2	=	17β-estradiol
EGFR	=	Epidermal growth factor receptor
Erb2	=	Erythroblastic oncogene B, also Her2
Erα	=	Estrogen receptorα
ERβ	=	Estrogen receptor β
Esp15	=	Epidermal growth factor receptor substrate 15
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
Grb2	=	Growth factor receptor-bound protein 2
HER2/neu	=	Human epidermal growth factor receptor 2
MAPK	=	Mitogen-activated protein kinase
MCF-7	=	Michigan Cancer Foundation-7,
MD-AMB-231	=	MD Anderson-Metastatic Breast-231
MG-132	=	Proteosome inhibitor
OCP	=	Oral contraceptive pill
PI3K	=	Phosphoinositide 3-kinases
PKA	=	protein kinase A
PR	=	Progesterone receptor
RTK	=	Receptor tyrosine kinases
TNBC	=	Triple negative breast cancer

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical committee approval was obtained from the institution (Ref:SDMIEC:0741:2016).

HUMAN AND ANIMAL RIGHTS

No human or animal was utilized in the research.

CONSENT FOR PUBLICATION

We declare that we have consent to publish this article.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

This study was not funded by funding agencies.

CONFLICT OF INTEREST

The authors confirm that this article's content has no conflict of interest.

ACKNOWLEDGEMENTS

We Acknowledge Central Research Laboratory, SDM College of Medical sciences & Hospital, Dharwad, India for providing infrastructure.

REFERENCES

[1] Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med 2010; 363(20): 1938-48. http://dx.doi.org/10.1056/NEJMra1001389 PMID: 21067385

[2] Morris GJ, Naidu S, Topham AK, *et al.* Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology, and End Results database. Cancer 2007; 110(4): 876-84. http://dx.doi.org/10.1002/cncr.22836 PMID: 17620276

[3] Bolla M, Chedin M, Souvignet C, Marron J, Arnould C, Chambaz E. Estimation of epidermal growth factor receptor in 177 breast cancers: correlation with prognostic factors. Breast Cancer Res Treat 1990; 16(2): 97-102. http://dx.doi.org/10.1007/BF01809293 PMID: 2176111

[4] Slamon DJ, Godolphin W, Jones LA, *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989; 244(4905): 707-12. http://dx.doi.org/10.1126/science.2470152 PMID: 2470152

[5] Rimawi MF, Shetty PB, Weiss HL, *et al.* Epidermal growth factor receptor expression in breast cancer association with biologic phenotype and clinical outcomes. Cancer 2010; 116(5): 1234-42. http://dx.doi.org/10.1002/cncr.24816 PMID: 20082448

[6] Nicholson RI, Hutcheson IR, Jones HE, *et al.* Growth factor signalling in endocrine and anti-growth factor resistant breast cancer. Rev Endocr Metab Disord 2007; 8(3): 241-53. http://dx.doi.org/10.1007/s11154-007-9033-5 PMID: 17486454

[7] DeSantis CE, Fedewa SA, Goding Sauer A, Kramer JL, Smith RA, Jemal A. Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. CA Cancer J Clin 2016; 66(1): 31-42. http://dx.doi.org/10.3322/caac.21320 PMID: 26513636

[8] Brewster AM, Chavez-MacGregor M, Brown P. Epidemiology, biology, and treatment of triple-negative breast cancer in women of African ancestry. Lancet Oncol 2014; 15(13): e625-34. http://dx.doi.org/10.1016/S1470-2045(14)70364-X PMID: 25456381

[9] Spangler JB, Neil JR, Abramovitch S, *et al.* Combination antibody treatment down-regulates epidermal growth factor

receptor by inhibiting endosomal recycling. Proc Natl Acad Sci USA 2010; 107(30): 13252-7. http://dx.doi.org/10.1073/pnas.0913476107 PMID: 20616078

[10] Goh LK, Huang F, Kim W, Gygi S, Sorkin A. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol 2010; 189(5): 871-83. http://dx.doi.org/10.1083/jcb.201001008 PMID: 20513767

[11] Crowder JJ, Geigges M, Gibson RT, *et al.* Rkr1/Ltn1 Ubiquitin Ligase-mediated Degradation of Translationally Stalled Endoplasmic Reticulum Proteins. J Biol Chem 2015; 290(30): 18454-66. http://dx.doi.org/10.1074/jbc.M115.663559 PMID: 26055716

[12] Tran JR, Brodsky JL. Assays to measure ER-associated degradation in yeast. Methods Mol Biol 2012; 832: 505-18. http://dx.doi.org/10.1007/978-1-61779-474-2_36 PMID: 22350909

[13] Kao SH, Wang WL, Chen CY, *et al.* GSK3 β controls epithelial-mesenchymal transition and tumor metastasis by CHIP-mediated degradation of Slug. Oncogene 2014; 33(24): 3172-82. http://dx.doi.org/10.1038/onc.2013.279 PMID: 23851495

[14] Hampton RY, Rine J. Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J Cell Biol 1994; 125(2): 299-312. http://dx.doi.org/10.1083/jcb.125.2.299 PMID: 8163547

[15] Hochstrasser M, Varshavsky A. In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. Cell 1990; 61(4): 697-708. http://dx.doi.org/10.1016/0092-8674(90)90481-S PMID: 2111732

[16] Hanna J, Leggett DS, Finley D. Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. Mol Cell Biol 2003; 23(24): 9251-61. http://dx.doi.org/10.1128/MCB.23.24.9251-9261.2003 PMID: 14645527

[17] Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002; 82(2): 373-428. http://dx.doi.org/10.1152/physrev.00027.2001 PMID: 11917093

[18] Hershko A. The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. Cell Death Differ 2005; 12(9): 1191-7. http://dx.doi.org/10.1038/sj.cdd.4401702 PMID: 16094395

[19] Ravid T, Hochstrasser M. Diversity of degradation signals in the ubiquitin-proteasome system. Nat Rev Mol Cell Biol 2008; 9(9): 679-90. http://dx.doi.org/10.1038/nrm2468 PMID: 18698327

[20] Gallastegui N, Groll M. The 26S proteasome: assembly and function of a destructive machine. Trends Biochem Sci 2010; 35(11): 634-42. http://dx.doi.org/10.1016/j.tibs.2010.05.005 PMID: 20541423

[21] Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem 1983; 258(13): 8206-14. http://dx.doi.org/10.1016/S0021-9258(20)82050-X PMID: 6305978

[22] Metzger MB, Pruneda JN, Klevit RE, Weissman AM. RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. Biochim Biophys Acta 2014; 1843(1): 47-60. http://dx.doi.org/10.1016/j.bbamcr.2013.05.026 PMID: 23747565

[23] Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem 2009; 78: 477-513. http://dx.doi.org/10.1146/annurev.biochem.78.081507.101607 PMID: 19489727

[24] Lander GC, Estrin E, Matyskiela ME, Bashore C, Nogales E, Martin A. Complete subunit architecture of the proteasome regulatory particle. Nature 2012; 482(7384): 186-91. http://dx.doi.org/10.1038/nature10774 PMID: 22237024

[25] Das S, Roth CP, Wasson LM, Vishwanatha JK. Signal transducer and activator of transcription-6 (STAT6) is a constitutively expressed survival factor in human prostate cancer. Prostate 2007; 67(14): 1550-64. http://dx.doi.org/10.1002/pros.20640 PMID: 17705178

[26] Amirikia KC, Mills P, Bush J, Newman LA. Higher population-based incidence rates of triple-negative breast cancer among young African-American women : Implications for breast cancer screening recommendations. Cancer 2011; 117(12): 2747-53. http://dx.doi.org/10.1002/cncr.25862 PMID: 21656753

[27] Pietras RJ, Márquez-Garbán DC. Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res 2007; 13(16): 4672-6. http://dx.doi.org/10.1158/1078-0432.CCR-07-1373 PMID: 17699844

[28] Hammes SR, Levin ER. Extranuclear steroid receptors: nature and actions. Endocr Rev 2007; 28(7): 726-41. http://dx.doi.org/10.1210/er.2007-0022 PMID: 17916740

[29] Yan Y, Li X, Blanchard A, *et al.* Expression of both estrogen receptor-beta 1 (ER- β 1) and its co-regulator steroid receptor RNA activator protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with estrogen receptor-alpha (ER- α)-negative early breast cancer (EBC). Ann Oncol 2013; 24(8): 1986-93. http://dx.doi.org/10.1093/annonc/mdt132 PMID: 23579816

[30] Pezzuto A, Carico E. Role of HIF-1 in Cancer Progression: Novel Insights. A Review. Curr Mol Med 2018; 18(6): 343-51. http://dx.doi.org/10.2174/1566524018666181109121849 PMID: 30411685

[31] Thomas C, Rajapaksa G, Nikolos F, *et al.* ERbeta1 represses basal breast cancer epithelial to mesenchymal transition by destabilizing EGFR. Breast Cancer Res 2012; 14(6): R148. http://dx.doi.org/10.1186/bcr3358 PMID: 23158001

[32] Waterman H, Levkowitz G, Alroy I, Yarden Y. The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. J Biol Chem 1999; 274(32): 22151-4. http://dx.doi.org/10.1074/jbc.274.32.22151 PMID: 10428778

[33] Jiang X, Huang F, Marusyk A, Sorkin A. Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Biol Cell 2003; 14(3): 858-70. http://dx.doi.org/10.1091/mbc.e02-08-0532 PMID: 12631709

[34] Confalonieri S, Salcini AE, Puri C, Tacchetti C, Di Fiore PP. Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. J Cell Biol 2000; 150(4): 905-12. http://dx.doi.org/10.1083/jcb.150.4.905 PMID: 10953014

[35] Ramaekers CH, Wouters BG. Regulatory functions of ubiquitin in diverse DNA damage responses. Curr Mol Med 2011; 11(2): 152-69. http://dx.doi.org/10.2174/156652411794859269 PMID: 21342128

[36] Lu Q, Hope LW, Brasch M, Reinhard C, Cohen SN. TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation. Proc Natl Acad Sci USA 2003; 100(13): 7626-31. http://dx.doi.org/10.1073/pnas.0932599100 PMID: 12802020

[37] Bache KG, Brech A, Mehlum A, Stenmark H. Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. J Cell Biol 2003; 162(3): 435-42. http://dx.doi.org/10.1083/jcb.200302131 PMID: 12900395

[38] Radinsky R, Risin S, Fan D, *et al.* Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. Clin Cancer Res 1995; 1(1): 19-31. PMID: 9815883

[39] Harris AL, Nicholson S, Sainsbury R, Wright C, Farndon J. Epidermal growth factor receptor and other oncogenes as prognostic markers. J Natl Cancer Inst Monogr 1992; •••(11): 181-7. PMID: 1627426

[40] Jain A, Jain SK. Advances in Tumor Targeted Liposomes. Curr Mol Med 2018; 18(1): 44-57.

http://dx.doi.org/10.2174/1566524018666180416101522 PMID: 29663884

[41] Price JT, Bonovich MT, Kohn EC. The biochemistry of cancer dissemination. Crit Rev Biochem Mol Biol 1997; 32(3): 175-253. http://dx.doi.org/10.3109/10409239709082573 PMID: 9239493

[42] Reis-Filho JS, Pinheiro C, Lambros MB, *et al.* EGFR amplification and lack of activating mutations in metaplastic breast carcinomas. J Pathol 2006; 209(4): 445-53. http://dx.doi.org/10.1002/path.2004 PMID: 16739104

[43] Hoadley KA, Weigman VJ, Fan C, *et al.* EGFR associated expression profiles vary with breast tumor subtype. BMC Genomics 2007; 8: 258. http://dx.doi.org/10.1186/1471-2164-8-258 PMID: 17663798

[44] Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? Cancer J 2010; 16(1): 23-32. http://dx.doi.org/10.1097/PPO.0b013e3181d24fc1 PMID: 20164687