Elucidating The Role of Tumor Educated Platelets In Promoting Epithelial To Mesenchymal Transition And Angiogenesis In Breast Cancer: Therapeutic Intervention By Aspirin

By

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Thesis submitted for the degree of

Doctor of Philosophy (Science)

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Certificate from the Supervisor

This is to certify that the thesis entitled ''Elucidating The Role of Tumor Educated Platelets In Promoting Epithelial To Mesenchymal Transition And Angiogenesis In Breast Cancer: Therapeutic Intervention By Aspirin'' submitted by Smt. Aishwarya Guha, who got her name registered on 06.06.2022 For the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of Dr. Saptak Banerjee and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

Saptak Banerjee 4/8/2025

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Acknowledgements

''Yoga-sthaḥ kuru karmāṇi saṅgaṁ tyaktvā dhanañjaya siddhy-asiddhyoḥ samo bhūtvā samatvaṁ yoga uchyate'' – Bhagvad Gita

The 'Para Brahma' Shri Krishna advices Arjuna to perform his actions, having abandoned attachment, having become equal in failure and success, for such equanimity is meant by Yoga and thus, at the end one will emerge victorious. As I wrap up-this non-linear Ph.D. journey, I would like to sincerely express my gratitude to each and every soul without whom this journey would have been impossible to conquer. Just like no battle is won single handed, similarly, the present thesis would not have had its shape without the efforts and contributions of these incredibly gifted people.

For the past few years, 37 Shayama Prasad Mukherjee Road, Kolkata, has been my safe haven. When I first walked in through the gates of our historically glorious Chittaranjan National Cancer Institute, CNCI, as a fresh post-graduate with no practical research experience, I was welcomed wholeheartedly at my second abode 'Department of Immunoregulation and Immunodiagnostics' by my supervisor Dr. Saptak Banerjee. Beyond technical knowledge, the most important lesson that I've acquired from my guide is the art of remaining calm and composed under pressure. Despite of having limited resources, Sir continued to be resolute and gladly offered assistance to those in need. His ceaseless work and sincere commitment towards science have served as a constant source of motivation for the lab's many post-graduate interns as well as the research scholars. His student-friendly demeanour is yet another really admirable trait. His experienced guidance has assisted me become a methodical researcher from a naïve, passionate fresher. I shall remain indebted to him for introducing me to the world of stem cells especially cancer stem cells. He has provided me the freedom to conduct experiments individually without any fear of failure. To him, failure is the first step towards success. In addition, he has provided me with an invaluable training to distinguish between healthy cells and microbe contaminated cells. His passion towards microscopy has enriched our manuscript with beautiful scientific illustrations that significantly reinforce our hypothesis. Besides, he was indispensable for fine tuning my presentation abilities and always encouraged us to present and discuss our research findings with the pioneers of the research field. It was only due to his support that I was able to present and discuss our research findings in prestigious international conferences. Words are not sufficient to express my gratitude towards him. I am fortunate enough to be part of his team and pursue my Ph.D. under his guidance.

Speaking of our department, the next person that springs to my mind is Baral Sir, Dr. Rathindranath Baral. Pursuing a Ph.D. is not merely an academic endeavour but a transformative journey of the mind and self. In this odyssey of knowledge, Sir, has been more than a guide an intellectual lighthouse in the vast ocean of inquiry. With patience akin to that of a philosopher and wisdom that transcends the boundaries of disciplines he has not only imparted knowledge but instilled in me the art of questioning, the courage to embrace uncertainty and the resilience to persist in the face of complexity. True mentorship, I have come to realize, is not just about answers but about shaping the questions that define one's scholarly path. For his unwavering support, insightful critique and belief in my pursuit, I am deeply indebted. In the words of Aristotle, "The roots of education are bitter, but the fruit is sweet." This journey has been arduous, yet it has been sweetened by the presence of a mentor whose guidance has been both rigorous and compassionate. For the patient guidance, for the challenges posed, for the encouragement in moments of doubt and for the relentless pursuit of truth that you have instilled in me, I am profoundly grateful. Your mentorship has been not just a guiding light but a philosophical anchor in my journey.

Next, I would like to express my deepest and sincerest gratitude to Dr. Anamika Bose, our very own Anamika di, whose unwavering support, encouragement and expertise have been instrumental in shaping my academic journey. The completion of this thesis would not have been possible without her invaluable guidance, patience and dedication. I am profoundly grateful for your insightful mentorship and the immense knowledge you have shared with me. Your ability to critically assess my work, challenge my ideas and push me to think beyond conventional boundaries has significantly strengthened my research. Your constructive criticism, meticulous attention to detail and high standards have played a crucial role in refining my analytical skills and enhancing the quality of this thesis. Beyond academics, your encouragement and reassurance during challenging times have been a source of great motivation, reminding me of the importance of perseverance and intellectual curiosity. Your ability to provide clarity and direction, especially during moments of uncertainty, has been incredibly reassuring. Your patience, kindness and willingness to engage in thought-provoking discussions have broadened my perspective and enriched my understanding of the subject matter. Your enthusiasm for research and commitment towards fostering independent thinking has been truly inspiring and I am grateful for the confidence you have instilled in me throughout this journey.

It is my privilege to have all three such scientific minds as my mentors during this incredible journey. Their complementary expertise and collaborative guidance have provided me with a well-rounded and enriching academic experience. Their dedication to my progress, willingness to invest their time and unwavering belief in my abilities has made a significant impact on my personal and professional development. The knowledge and skills I have gained under their supervision will continue to guide me in my future academic and professional endeavours.

Research is a collaborative work and we couldn't have pursued our study concept without the crucial assistance of our surgical collaborators. Their expertise, dedication and unwavering support have been fundamental in shaping the outcomes of this work. First and foremost, I extend my sincere appreciation to Dr. Neyaz Alam, whose exceptional surgical skills, clinical insights and commitment to patient care have greatly contributed to this study. His willingness to share the post-surgical patient samples along with his vast knowledge and expertise in the field of oncology has provided me with invaluable perspectives, enriching both the scientific and clinical aspects of this research. I am also immensely grateful to Dr. Smarajit Pal, Department of Clinical Biochemistry for providing me access to the Haematology Analyzer, Dr. Srabanti Hajra and her entire team of Department of Pathology, at CNCI, Hazra Campus for their collaborative spirit and commitment to excellence. Their support in facilitating clinical data collection, coordinating patient involvement and ensuring the highest ethical standards in surgical procedures has been critical to the success of this project. The seamless cooperation between clinicians, researchers and supporting staffs has reinforced the importance of multidisciplinary collaboration in scientific discovery. Additionally, I extend my heartfelt benediction to the surgical residents, nurses, phlebotomists and all the technical staffs who played a significant role in assisting with various aspects of this study. Their tireless efforts, patience and professionalism in handling research protocols, patient management and data acquisition along with providing access to patient records have significantly contributed to the integrity and reliability of the findings. This research stands as a testament to the power of teamwork and the collective pursuit of scientific and clinical excellence.

I would further like to acknowledge the brave and selfless cancer patients who willingly donated their tumor and blood samples and laid the foundation stone of this research. Despite the physical and emotional challenges of their journey, they chose to share a part of their-selves for the advancement of science and the betterment of future patients. This selfless

decision reflects an incredible spirit of resilience, compassion and hope. These contributions are not merely data points in a study but the first step towards discoveries that may one day change lives of many. Their legacy lives on in every finding, every new insight and every step forward in the fight against cancer. This work is dedicated to these valiant fighters with profound respect, admiration and gratitude.

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provided me with the ideal space to think critically, explore new ideas and push the boundaries of my research.

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Her guidance was fundamental in ensuring the success of my experiments and deepening my understanding of the techniques involved. Sukanya Di has been a wonderful companion and a source of joy throughout my time in the lab. We not only shared countless learning moments but also bonded over our shared love for food, making her not just a mentor but a fantastic foodie partner. Thank you, Sukanya Di, for your unwavering support, for being such a patient teacher and for the warmth and camaraderie you've brought into my research experience.

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and agarose gel electrophoresis. Her willingness to help, despite her busy schedule, greatly facilitated my learning and progress. Additionally, her constructive feedback and thoughtful insights on my manuscript were pivotal in refining my work. I truly appreciate her guidance and encouragement during my time in the lab.

The upcoming person in the list is Dr. Tapasi Das. Tapasi Di, whose expertise in proteomics has significantly contributed to the understanding of NLGP-Neem leaf glycoprotein, a natural anti-cancer compound derived from *Azadirachta indica*, has been a key source of knowledge throughout my work. Having worked on NLGP for over a decade, our lab has greatly benefited from her insights into its proteomic aspects. I feel fortunate to have had the opportunity to learn and refine techniques such as 2D gel electrophoresis and column chromatography under her mentorship. Her generosity in sharing her knowledge and expertise has greatly enriched my research and I am truly grateful for her constant encouragement and support.

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I would like to dedicate a special thank you to Jasmine, my first friend in the lab. From the very moment we joined the lab together, we embarked on this journey as a team, learning, experimenting and growing side by side. We started with a single vial of frozen cells, unsure of what lay ahead, but together we navigated the challenges of mastering cell culture techniques, each milestone marking our progress. Through every experiment, every set back and every success, we were each other’s support system. Our silly fights and reunions, the laughter, the tears and the shared moments of frustration will always be cherished memories. Jasmine has been an unwavering pillar of strength since day one, offering me encouragement, understanding and a sense of sisterhood that I will cherish forever. As we prepare to part ways, I reflect on how much this journey and Jasmine's friendship, have meant to me. Her presence in my life has made these years not just about the research but also about the invaluable bond we have formed. You will always hold a special place in my heart and I cannot express how much your support has meant to me. Thank you, my second author, for

being there through every step of this journey, for the countless memories and for being a friend I will treasure forever.

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inspiration. Your boundless love, patience and nurturing spirit have been a constant source of comfort during times of uncertainty. From my earliest years, you instilled in me the values of kindness, perseverance and the importance of education. Your wisdom, whether expressed through a gentle word or a quiet gesture, has guided me through many of life's challenges. In the moments when I doubted my own abilities, you reminded me of my strengths and the importance of believing in me. I am forever grateful for the sacrifices you made to provide me with opportunities and for the warmth and unconditional love you continue to give me. To my father, your dedication and commitment to hard work have been instrumental in shaping my character. You taught me the importance of discipline, responsibility and the value of striving for excellence in everything I do. Your unwavering belief in my potential, especially during times when I faced setbacks, provided me with the strength to keep going. Your steadfast support and encouragement have been crucial in helping me maintain focus and motivation, even when the road ahead seemed daunting. I am forever indebted to you for showing me the power of resilience and determination. Together, you have both shown me the significance of a strong work ethic, the importance of family and the value of pursuing one's dreams, no matter how difficult the journey may be. This thesis represents not only my hard work but also the sacrifices and love you both invested in me over the years. I dedicate this work to you, as it would not have been possible without your enduring belief in me and your constant presence in my life. Thank you for everything, for always being there with open arms and for giving me the foundation on which I have built my academic and personal life.

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encouraged me to pursue a career in academia, reminding me that true fulfilment lies in the pursuit of knowledge and in making a meaningful contribution to society through education. His belief in the value of intellectual growth and his steady support helped me stay focused on my academic aspirations, even when the lure of the corporate world seemed more tempting. I am deeply thankful to my uncle for his invaluable guidance, for being a constant source of wisdom and for encouraging me to follow my heart. His example has shaped not just my professional decisions, but also my outlook on life. This thesis is as much a result of his encouragement and mentorship as it is of my own efforts and I will forever be grateful for his influence in helping me chart my academic path.

As I reflect on this journey, I would like to take a moment to acknowledge my own perseverance, resilience and dedication that have brought me to this point. The path to completing a PhD is not easy and there were moments when the road ahead seemed uncertain and filled with obstacles. Yet, through it all, I remained committed to the pursuit of knowledge; often pushing myself beyond what I thought was possible. I am proud of the determination I showed in the face of challenges, the late nights spent grappling with complex ideas and the persistence that kept me moving forward even when things felt overwhelming. This journey has been one of immense growth, not just intellectually but personally, as I have learned to overcome setbacks, embrace failure as a learning opportunity and continue working toward my goals with unwavering resolve. Completing this thesis is not just a culmination of years of research and hard work, but a reflection of my own ability to adapt, learn and evolve. I have developed not only as a scholar but as a person and for this growth, I am deeply grateful. This acknowledgment is a reminder to myself that the efforts and sacrifices I made throughout this journey were worth it. The long hours, the moments of self-doubt and the relentless pursuit of excellence have all led to this achievement and I stand proud of the person I have become along the way.

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This thesis is far more than a scholarly endeavour it is a sacred pilgrimage undertaken under their watchful gaze. In moments when confusion clouded my path, it was their divine wisdom

that brought stillness and clarity. When fear and doubt crept into my heart, it was their grace that instilled courage, resilience and inner strength. And in those silent, solitary hours when shadows loomed large and despair threatened to overwhelm, it was their unwavering presence that became the light that guided me forward. Every obstacle overcome, every insight uncovered and every word penned in these pages carries the subtle imprint of their blessings. Their divine energy has flowed through this work like an invisible current, shaping not only the outcome but also the journey itself. To them, I offer this humble fruit of my effort not as an achievement, but as an offering. May this work serve as a testament to their boundless compassion and may it always remind me of the sacred force that walks with those who walk with faith.

Aishwarya Guha

Abbreviations

A

AB	Antibody
ABC Protein	ATP binding cassette protein
ALDH1	Aldehyde dehydrogenase 1
AML	Acute myeloid leukemia
Ag	Antigen
APC	Antigen Presenting Cell
AEC	3-amino-9-ethylcarbazole
ANOVA	Analysis of Variance

B

BD	Becton Dickinson
BCSC	Breast cancer stem cell
BCRP	Breast cancer resistant protein
BSA	Bovine Serum Albumin

C

^{o}C	Centigrade
CCR	CC Chemokine Receptor
cDNA	Complementary DNA

CAF	Cancer Associated Fibroblast
CSC	Cancer stem cell
CXCL	C-X-C Chemokine Ligand
CXCR	C-X-C Chemokine Receptor
CML	Chronic myeloid leukemia
COX	Cyclooxygenase
CTCF	Corrected Total Cell Fluorescence Intensity
CCL	CC Chemokine Ligand

D

DAPI	4′,6-Diamidino-2-phenylindole
DEPC	Diethyl Pyro-carbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate

E

EC	Ehrlich's Carcinoma
ECM	Extracellular Matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
F	
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FC	Flow-Cytometry
FITC	Fluorescein Isothiocynate
G	
GAPDH	Glyceraldehyde-3-P-dehydrogenase
GLOBOCON	Global Cancer Database
H	
HE	Hematoxylin Eosin
HR	Hours
HRP	Horse radish peroxidase
I	
IL	Interleukin
ICI	Immune checkpoint inhibitors
IFN	Interferon
IHC	Immunohistochemistry
J	
JAG1	Jagged 1

JAK	Janus kinase
K	
KLF4	Kruppel-like factor 4
M	
mTOR	Mammalian target of rapamycin
mAb	Monoclonal Antibody
MDR	Multidrug Resistance
MDSC	Myeloid-derived Suppressor Cell
MET	Mesenchymal to Epithelial transition
MFI	Mean Fluorescence Intensity
N	
NK	Natural Killer
NT	No-treatment
P	
PBS	Phosphate Buffer Saline
PBMC	Peripheral Blood Mononuclear cell
P-selectin	Platelet Selectin
PSGL1	Platelet Selectin Glycoprotein Ligand 1
PCR	Polymerase Chain Reaction
PGP	P-glycoprotein 1
PAGE	Poly Acrylamide Gel Electrophoresis

R

RP	Resting platelet
r	Recombinant
RPMI	Rosewell Park Memorial Institute Medium

S

SDS	Sodium Dodecyl Sulphate
STAT	Signal transducers and activators of transcription
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SNAIL	Snail Family Transcriptional Repressor
SLUG	Zinc finger protein SNAI2
SEM	Scanning Electron Microscope
SD	Standard Deviation
SEM	Standard Error of Mean
SOX2	SRY-box transcription factor 2
siRNA	Small interfering RNA

T

TEP	Tumor educated platelet
T cell	T lymphocyte
TBE	Tris Borate EDTA
TBS	Tris Borate SDS
TBST	Tris Borate SDS Tris Borate SDS Tween20

TGF	Transforming Growth Factor
TME	Tumor Microenvironment
TNBC	Triple Negative Breast Cancer
TWIST	Twist Family BHLH Transcription Factor
TCGA	The Cancer Genome Atlas

V

VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
Vim	Vimentin

W

WT	Wild type

Table of Contents

Abstract

Background: Despite existing reports highlighting role of platelets in tumorigenesis, its impact on breast cancer stem cells (BCSCs) remain underexplored. Our first ever report on murine and human system, accentuate that, tumor educated platelets (TEPs) of luminal-A and TNBC subtypes are distinct from healthy counterparts, collaborating with BCSCs to generate sub-variants that elevate tumor aggressiveness. While the pro-tumorigenic functions of platelets have been increasingly recognized, the role of platelet-poor plasma (PPP) remains underexplored in cancer biology. This study identifies PPP as a biologically active fraction of platelets with inhibitory effects on BCSC traits, including self-renewal and drug resistance.

Methods: Impact of TEPs on BCSCs was evaluated from primary breast tumor and blood samples of luminal-A/TNBC patients along with EC/4T1 murine breast tumor models and MCF-7/MDA-MB-231 cell lines. For downstream assays, TEPs were co-cultured with breast tumor samples or cell lines, followed by magnetic sorting of $CD44^{+}CD24^{-}$ BCSCs. TEP induced alterations of BCSCs were evaluated from 3D tumorsphere, colony formation, transwell migration, scratch-wound healing, matrigel invasion, *in-vitro* tube formation assays. Fluorescence-confocal microscopy, RT-PCR, flow-cytometry, western-blotting were utilized to decipher the role of genes and protein involved in stemness, metastasis along with the transcription factors in the downstream signaling cascade, followed by verifications by RNAi. Further, the influence of PPP on BCSCs was elucidated by co-culturing PPP with magnetically sorted $CD44^{+}CD24^{-}$ BCSCs of MCF-7 and MDA-MB-231. Using the 3D tumorsphere assay, colony formation assay and scratch-wound healing assay, PPP-induced changes to BCSCs were assessed. RT-PCR, flow-cytometry and ELISA were performed to investigate the changes in the expression of genes and proteins regulating stemness and drug resistance.

Results: TEPs have elevated expression of P-selectin and interacts with BCSCs via P-selectin and PSGL1 on BCSCs surface. Treatment with aspirin had restorative impact on P-selectin level, converting TEPs from active to resting platelet (RP) state. Under TEPs influence, BCSCs were tumorigenic, clonogenic, multidrug resistant, invasive with numerous invadopodia and remained skewed towards mesenchymal phenotype. Administration of RP or aspirin treated TEPs reduced TEP associated BCSC virulence both *in-vivo* and *in-vitro*. P-selectin-PSGL1 interaction resulted in binding of WNT to FRIZZLED followed by stabilization and nuclear translocation of β-Catenin. Nuclear β-Catenin promoted stemness-EMT-metastasis, along with stimulation of autocrine VEGF-VEGFR2 cascade. Inhibition of WNT and VEGFR2 by RNAi confirmed the critical role of this axis in regulating TEP's influence on BCSCs. In stark contrast, treatment with PPP resulted in a marked reduction in

stemness markers *oct-4, sox-2* and marginally in *nanog*. Also, a prominent decrease in sphere-formation and migratory efficiency and sensitization towards chemotherapeutic agents, by downregulating the expression of ABC transporter genes like *abcb1* and *abcc1*was noted. This suggested an inhibitory effect on the cancer stem cell phenotype by PPP.

Conclusion: These insights into TEP-BCSC interplay, acknowledges TEPs, as-well-as unveils novel receptor-ligand signalling cascade, which could be a beneficial therapeutic strategy to target cancer metastasis. Aspirin treatment of TEPs markedly impairs their pro-tumorigenic functions in breast cancer. Aspirin irreversibly inhibits platelet cyclooxygenase (COX-1), preventing thromboxane A_2 production and platelet activation, which abrogates surface P-selectin upregulation and the secretion of metastasis-promoting factors. As a result, aspirin-treated TEPs lose their ability to induce EMT and stem-like traits in breast cancer cells. Mechanistically, aspirin disrupted the adhesive P-selectin/PSGL-1 interaction between TEPs and BCSCs effectively dismantling the protective TEP~CSC interfaces. Contrary to the supportive role of TEPs in tumor aggressiveness, PPP treatment led to a consistent and significant downregulation of stemness-associated genes, reduced mammosphere-forming ability and increased sensitivity to chemotherapeutic agents. These findings underscore the therapeutic relevance of PPP as a naturally occurring, platelet-depleted plasma fraction that inherently lacks tumor-promoting influence. The ability of PPP to suppress stem-like traits and overcome drug resistance in BCSCs points to a novel and underexplored avenue for therapeutic intervention. Strategically developing plasma-based therapies that neutralize or deplete platelet-derived tumor-supportive factors could represent a non-toxic, adjunctive strategy to sensitize tumors to conventional therapies and curb metastatic spread. In summary, this thesis not only reinforces the critical role of TEPs in breast cancer progression but also introduces platelet-poor plasma as a promising, tumor-suppressive biological medium. Further investigation into the molecular mechanisms underlying PPP's inhibitory effects may yield new biomarkers and therapeutic targets for effectively disrupting the cancer stem cell niche and improving long-term outcomes in breast cancer patients.

General Introduction

Introduction

1.1 Brief history of cancer

Cell division is an intricately controlled phenomenon. However, when this process becomes differentially regulated, it results in the formation of an aberrant mass of cells called 'tumor' [1]. When the tumor cells detach from their primary site and begin to invade the surrounding tissues they are termed as malignant or cancerous tumors [2]. Cancerous tumors are characterized by an accelerated growth of abnormal cells, which extend beyond their normal boundaries. These cells can then intravasate into the other areas of the body and progress to different organs by a process known as metastasis [3].

According to the cancer atlas, origin of this deadly disease dates back to Jurassic period as evidenced from the presence of cancer cells in dinosaur fossils discovered in 2003 [4]. In 1932, earliest known hominid tumor was found in Homo erectus or Australopithecus, by Louis Leaky [5]. The oldest description of cancer was discovered in Egypt which dates back to 3000 B.C. and is called the 'Edwin Smith Papyrus' [6]. It describes 8 cases of tumors or ulcers of breast removed by cauterization with a tool called fire drill and further declare there is no cure of this disease. 'The father of medicine' Hippocrates used the terms 'carcinos' and 'carcinoma' to decipher to 'non-ulcerous 'and 'ulcerous' tumors respectively due to its resemblance to the finger like projections from crab [7]. Later Roman physician Celsus translated this Greek word to 'cancer' the Latin phrase for 'carcinoma' [8]. Further, Galen, another Greek physician, used the term 'oncos'-the Greek terminology for swelling, to describe to 'tumors' [9].

1.2 Theories of origin of the disease

Of the several theories regarding the origin of cancer, humoral theory of Hippocrates was widely accepted for a very long period. He hypothesized; the origin of this disease is due to excess accumulation of black bile. An imbalance in the four major body humours (blood, phlegm, yellow bile and black bile) can lead to the onset of any disease. Accumulation of black bile in any region of the body can lead to the development of tumors [10].

The humoral theory was replaced by 'lymph theory' of Stahl and Hoffman in 1695. They believed that cancers are composed of fermenting and degenerating lymph which vary in density, acidity and alkalinity [11]. Further, German

pathologist Johannes Muller in 1838, discarded these theories and proposed that cancers originate from blastema, which are the budding elements between normal tissues [12]. It was not until the 19th century that the modern era of cancer research really began, with the efforts of eminent scientists like Rudolf Virchow. Virchow was the student of Johannes Muller and he for the first time identified cancer to be a disease of the cells and concluded that all cells including cancer cells arise from other cells. He hypothesized that cancers arise from the activation of dormant cells present in mature tissue. These dormant cells later came to be known as the cancer stem cells [13].

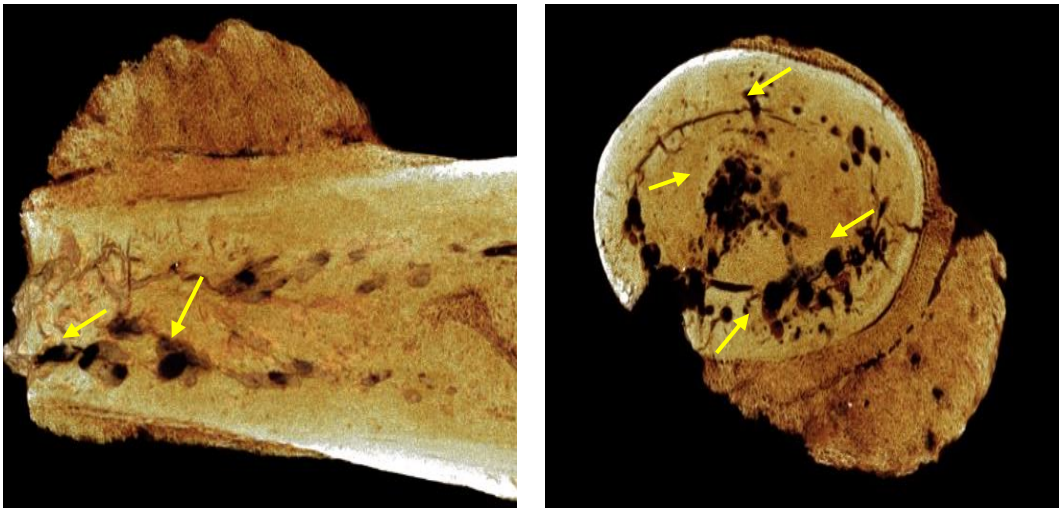

Figure 1- Fossilized human foot – Earliest evidence of cancer in fossilized human foot showing signs of osteosarcoma. The fossil has been discovered in a cave in South Africa. The toe belonged to one of the early hominins, either *Homo ergaster* or *Paranthropus robustus* that existed approximately 1.7 million years ago. ***Ref: Patrick S. Randolph-Quinneyet.al. Earliest hominin cancer: 1.7-million-year-old osteosarcoma from Swartkrans Cave, South Africa. South African Journal of Science***.

1.3 Types of cancer

Based upon their tissue of origin cancers can be broadly classified in to the following 4 types

1. Carcinoma

Carcinoma is cancers of the epithelial tissue lining and constitutes majority of the cancer types. Epithelial tissue lines most of the organs like oesophagus, stomach lining, lining of the lungs etc. Approximately 80-90% of all malignancies are carcinomas like breast, prostate, lungs, liver, kidney [14] etc. Carcinoma can be classified into the following types

i. ***In-situ carcinoma*** - In this type of carcinoma, the cancer remains restricted to its primary site of origin.

ii. ***Invasive carcinoma*** **-** Here the cancer cells invade the nearby surrounding tissue.

iii. ***Metastatic carcinoma*** **-** Tumor cells in this type spread to distant parts of the body [15].

Most common sub-types of carcinomas

a. ***Adenocarcinoma*** **-** Carcinoma of the glandular epithelial cells. These cells secrete fluids like mucus and digestive juices. Most prostate cancers, breast cancers, colorectal cancers and pancreatic cancers are adenocarcinomas [16].

b. ***Basal cell carcinoma*** **-** In this type, onset of cancer is usually at the basal cells, which are the deepest layer of skin cells.

c. ***Transitional cell carcinoma*** **-** Transitional cells are the ones line the epithelium of stretchable organs like bladder. Onset of the disease in these is referred to as transitional cell carcinoma.

d. ***Squamous cell carcinoma*** **-** These cells are flat and covers the surface of skin, lining of the throat, oesophagus etc. Unregulated division of such cells led to squamous cell carcinoma [17].

2. Sarcoma

Uncontrolled division of cells which constitutes the connective tissues of the body like bones, cartilages, muscles, tendons ligaments is defined as sarcoma. Sarcomas are of relatively less common than carcinoma and are most likely to affect children. Broadly they can be classified into the following two types [18]

i. ***Soft tissue sarcoma*** **-** Sarcoma of the soft tissues of the body like blood vessels or muscles

ii. ***Bone sarcoma*** **- It** is a rare type and forms in the bones.

Additionally, sarcoma can be sub-categorized into angiosarcoma, chondrosarcoma, clear cell sarcoma, dermato-fibrosarcoma-protuberans, epithelioid sarcoma, Ewing sarcoma, myeloid sarcoma, osteosarcoma, Kaposi sarcoma and synovial sarcoma [19].

3. Lymphoma

When immature lymphocytes start proliferating uncontrollably it can lead to the development of tumors in the lymph nodes or the lymphatic system as a whole which has been termed as lymphoma [20]. It is broadly of two types Non-Hodgkin and Hodgkin lymphoma.

i. ***Non-Hodgkin Lymphoma*** **-** It is the most common type of lymphoma and is more likely to develop in older patients but can occur at any age. It is further classified into a. B cell (85%-90%) b. T cell lymphomas (10%-15%) with the former being more common in occurrence [21].

ii. ***Hodgkin Lymphoma*** **-** Hodgkin lymphoma accounts for only 10% of the total cases. It is diagnosed by screening

patients for the presence of mutated cells called Reed Sternberg (RS) cells which are hallmark cells of Hodgkin lymphoma. These cells are abnormal lymphocytes with more than one prominent eosinophilic nucleus and sometimes a clear halo around the nucleolus. They originate from mature B cells and very rarely from T cells. The presence of RS cells is essential for diagnosing Hodgkin lymphoma. The number of RS cells increases as the disease progresses [22].

4. Leukaemia

Leukaemia is the cancer of body's blood forming cells including the bone marrow. Unlike the other tumors, leukaemia does not develop into solid masses and are therefore not detected by X-rays or CT-scans [23]. Hematopoietic stem cells develop into either myeloid or lymphoid progenitor cells, which ultimately give rise to all the major blood cells like RBC, WBC and platelets. However, abnormal multiplication in any one of these developing cells can overcrowd the normal cells leading to the onset of the disease. Early detection of the disease can significantly increase the chances of complete cure. Generally, chemotherapy, radiation therapy, stem cell transplant along with immunotherapy [24]. Leukaemia can be classified into the following major types [25]

i. ***Acute Leukaemia*** **-** Based upon the speed of progression acute leukaemia has been defined by the rapid multiplication of the leukemic cells leading rapid advancement within a very short span of time.

ii. ***Chronic Leukaemia*** **-** In comparison to acute type, in the chronic case, patients can remain with unnoticeable symptoms for years. It is more common in adults than children.

iii. ***Myelogenous and lymphocytic Leukaemia*** **-** According to the type of cell of origin leukaemia can be either myelogenous or lymphocytic. Myelogenous is the type in which the onset of the disease occurs in myeloid cells whereas the latter is characterized by the onset in lymphoid cells.

Apart from the basic classification, leukaemia has been divided into the following four types [26]

a. ***Acute Myelogenous Leukaemia – AML***
Most common in adults above 65years but can also affect children.

b. ***Acute Lymphocytic Leukaemia - ALL***

Most common targets are children, teens and adults upto 40years age.

c. ***Chronic Myelogenous Leukaemia – CML***
More common in older adults above 65 years. It rarely occurs in children and adults below 40.

d. Chronic Lymphocytic Leukaemia – CLL

Affect adults over 70 years. It is extremely rare in children.

1.4 Chronicle of cancer treatment

Surgery

In ancient times, cancer was treated with heat, herbal medicines and chemicals. Moreover, earliest physicians and surgeons believed that after surgical removal of tumor, there is every possibility of its relapse. The ardent reliance that cancer is a non-curable disease persisted for a very long period of time [27]. Three surgeons Bilroth in Germany, Handley in London and Halsted in Baltimore in 1894, worked to design for the very first 'cancer surgery'. They proposed removal of the whole tumor along with the lymph nodes in the region where the tumor was located [28]. Since then, this method of surgery became widely popularized [28].

Surgery is the first line of treatment for numerous malignancies like breast, ovarian, cervical, colorectal, oral, head and neck carcinoma etc. For breast cancer, surgery is the primary treatment for all stages except for stage V, also called as metastatic breast cancer [29]. However, for ovarian, cervical and oral cancers, surgery can only be performed for stage I, upto stage IIA and stages I and II respectively [30]. Whereas, in colorectal cancer surgery can be executed from stages 0-III [31]. For head and neck carcinoma, only early-stage cancers can be surgically treated [32]. For advanced stages, surgery followed by radiation or chemotherapy is the most commonly followed treatment regimen.

Radiation therapy

Wilhelm Conrad Roentgen, a German physics professor presented the world with a new kind of ray called the 'X-ray'. Within months of its discovered x-rays were used for diagnosis and soon radiation was used to treat cancer. Radium was the first radioactive element to be used for the purpose [33]. The major breakthrough of radiotherapy was achieved in France from the pioneering research conducted by Marie Curie [33]. Administration of daily doses of radiation for several weeks improved patient's survivability [34]. Radiation therapy is the first line of treatment for early skin, prostate, cervical and non-small cell lung carcinoma [35].

Chemotherapy

The importance of chemotherapy first came into light during the World War II. Soldiers exposed to mustard gas developed lymphoma, which could be treated with nitrogen mustard, an alkylating agent that killed cancer cells by damaging their DNA [36][37]. Since then numerous chemotherapeutic drugs are widely used for cancer treatment.

Hormone therapy

Thomas Beatson, during the 19th century observed that in rabbits, upon removal of ovaries, the breast stopped producing milk. He believed that activity of breasts was controlled by the ovaries. With this idea, he performed oophorectomy in advanced breast cancer and found that it resulted in improvement of patients [38]. He discovered that factor(s) from the ovaries were controlling development of breast tumor [38] [39]. This factor later came to be known as estrogen and this remarkable finding laid the foundation of modern hormone therapy [39]. Since then, numerous hormone blockers have been in used for cancer treatment [39].

The table below summarizes the most commonly used drugs for hormone therapy in breast cancer.

Classes of Drugs	Action	Examples
SERMs	Bind to estrogen receptors in breast cancer cells, block effects of estrogen, starving cancer cells, block or selectively inhibit estrogen receptors in breast cells	Tamoxifen, Evista (Raloxifene),Fareston (Toremifene)
Aromatase Inhibitors	Prevent production of estrogen in adrenal glands, lower the amount of estrogen in post-menopausal women	Aromasin (Exemestane),Femara (Letrozole), Arimidex (Anastrozole), Megace (Megestrol)
Biological Response Modifiers	Bind with certain proteins on breast cancer cells preventing their growth	Herceptin (Trastuzumab)
Other Hormonal Therapies	Block and breakdown estrogen receptors. Treats breast cancer that are dependent on estrogen	Zoladex (Goserelin acetate),Faslodex (Fulvestrant)

Table 1-Commonly used drugs for hormonal therapy in breast cancer treatment. *Ref: Barh D. Biomarkers, critical disease pathways, drug targets and alternative medicine in male breast cancer.*

Immunotherapy

William Bradley Coley, the father of immunotherapy, first attempted to treat bone cancer in 1891 with the aid of immune system. Coley used bacteria to purposely infect a patient who had numerous advanced tumors, including one in his throat that prevented him from eating. This mixture basically was an amalgamation containing toxins filtered from killed *Streptococcus pyogenes* and *Serratia marcescens.* This mixture later was popularized as Coley's toxins or Coley's vaccine [40]. The patient surprisingly recovered after this and soon he resumed normal life. Coley's work

leads the basis for treating cancers using immunotherapy [41]. Immunotherapy is a promising treatment option for advanced stage lung cancer, breast cancer, prostate cancer, head and neck carcinoma etc [42]. The table below enlists the most commonly used immunotherapeutic drugs for different subtypes and stages of breast cancer treatment along with their clinical trial number.

Immune Checkpoint Inhibitors					
Study Name/NCT Identifier	**Drug(s)/Novel Agent(s)**	**Study Phase**	**N**	**Primary Endpoint**	**Disease Setting**
TONIC NCT02499367	Nivolumab	II	84	PFS	TNBC, ≥2nd-line Metastatic
4147523 NCT02395627	Pembroluzimab	II	58	ORR	Postmenopausal ER+, ≥2nd-line Metastatic
Vaccine, Small Molecule Inhibitors & Others					
Study Name/Identifier	**Drug(s)/Novel Agent(s)**	**Study Phase**	**N**	**Primary Endpoint**	**Disease Setting**
11-202 NCT01570036	NeuVax	II	300	DFS	HER2+, Adjuvant
OSU 13117 NCT01964924	Trametinib + GSK2141795	II	41	ORR	TNBC, ≥2nd-line Metastatic
NYU 11-00598 NCT01421017	Imiquimod	I/II	55	ORR	≥2nd-line; + skin lesion, advance/metastatic

TKI with Anti-Angiogenic Properties					
Study Name/NCT Identifier	**Drug(s)/Novel Agent(s)**	**Study Phase**	**N**	**Primary Endpoint**	**Study Outcome**
SCRI BRE 122 NCT00887575	Neoadjuvant sunitinib + paclitaxel/carboplatin	I/II	54	pCR	Combo not recommended
ZACFAST NCT00752986	Fulvestrant +/− vandetanib	II	41	EFS	Terminated
NSABP FB-6 NCT00849472	Neoadjuvant AC ➔ +/− pazopanib	II	101	pCR	Increased toxicity; combo not recommended
A4061010 NCT00076024	Docetaxel +/− Axitinib	I/II	174	TTP	Not significant
RESILIENCE NCT01234337	Capecitabine +/− sorafenib	III	519	PFS	No advantage
Monoclonal Antibody					
Study Name/NCT Identifier	**Drug(s)/Novel Agent(s)**	**Study Phase**	**N**	**Primary Endpoint**	**Study Outcome**
Rose/TRIO-12 NCT00703326	Docetaxel +/− ramucirumab	III	1144	PFS	No OS advantage

Table 2-Immuno-therapeutic agents in breast cancer treatment. ***Ref: Friend S, Royce M. The changing landscape of breast cancer: how biology drives therapy. Medicines.***

1.5 History of breast cancer

Around 3500 years ago, ancient Egyptians described breast tumors in two distinct papyri 'The Edwin Smith Surgical Papyrus' and 'Ebers Papyrus' which considered the disease to be non-curable. According to the papyri non-curable tumors are 'cool to touch', bulging and spread all over the breast [43]. During the

17th and 18th centuries numerous myths about the occurrence of breast cancer were prevalent such as physical injury to breasts, viral contagion, lymph blockage, curdled milk left in the ducts etc [44]. It was only during the mid-20th century that scientists began to understand the genetics of breast carcinoma [45].

Further, early remedies for the disease were aimed towards providing temporary relieve than a permanent cure. Castor oil, opium, arsenic was commonly used for treatment along with underlying muscles [46]. It was not until 1894 that Dr. William S. Halstead, the professor of surgery at John Hopkins performed the first radical mastectomy, whereby along with the breast tissue, pectoralis major muscle was removed to prevent recurrence of the disease [47]. This practice of treatment was prevalent until the late 19th and early 20th centuries. Dr. Patey and Dr. Handley from London thereafter modified the existing protocol for radical mastectomy to preserve the pectoralis major muscle [48]. Post-surgery radiation and improved chemotherapy were used to eradicate the existing tumor cells [49].

Figure 2 –Earliest depiction of breast cancer treatment- Cauterization using fire drill was widely used as first line of treatment against the disease. ***Ref: Mastectomy, attributed to a Dutch artist, 17th century,WellcmeImages.***

Anatomy of human breast

Each human breast is divided into roughly 15–20 lobes. Each lobe is composed of smaller units called lobules that join to form milk-producing bulbs [50] [51]. Ducts are tiny tubes that line the lobe, lobules and bulbs. The ducts travel from the nipple to the areola, a central region of the skin [50] [51]. There are no muscles in the breasts and there is fat between the lobules and ducts [50] [51]. But beneath each breast are the inter-costal muscles that protect the ribs [52]. Additionally, each breast has lymph nodes that are grouped under the arm, above the collarbone [53].

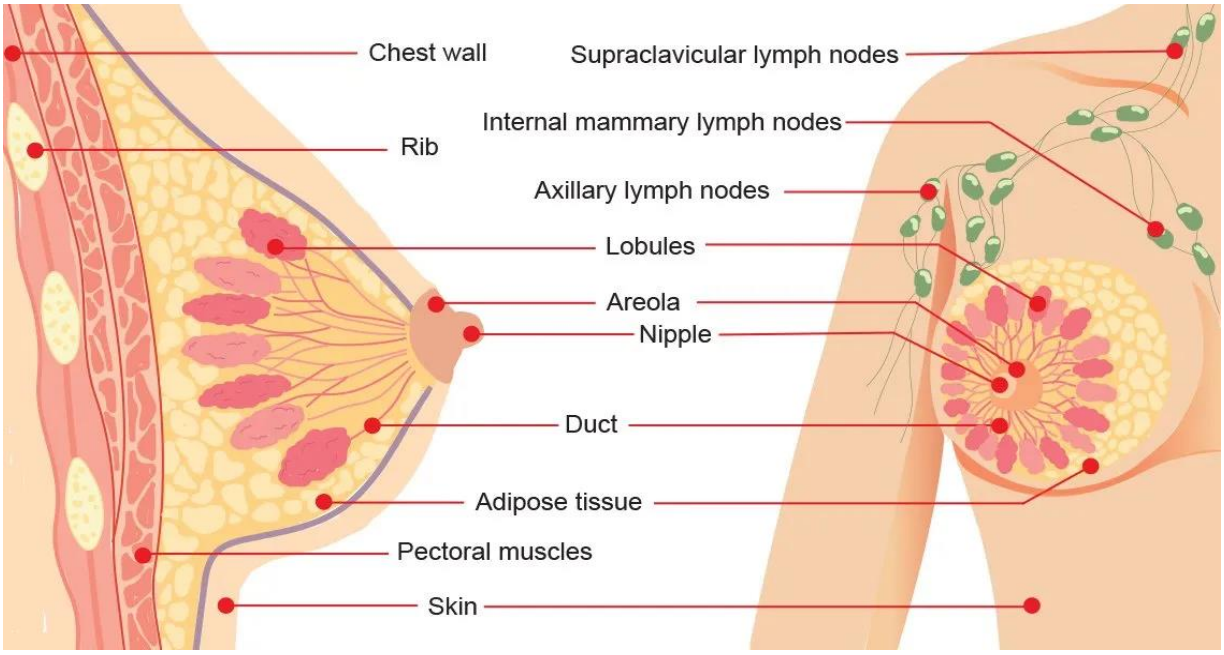

Figure 3- Anatomy of human breast. Each breast has numerous lobes subdivided into lobules which end into milk producing bulbs; all of these are connected by ducts. The space between ducts and lobules is fat filled. The ducts lead to the nipples and lymph nodes are present under the arm. ***Ref:Bazira PJet.al. Anatomy and physiology of the breast. Surgery (Oxford).***

1.6 Breast cancer statistics

WHO defines breast cancer to be disease of the breast characterized by uncontrollable division of abnormal cells leading to the development of tumor [54]. Globally breast cancer accounts for 12.5 of annual cancers cases making it the most common malignancy [54]. WHO reports of March 2024, mentioned breast cancer caused 6,70,000 deaths worldwide in 2022. Although, typical of females, 0.5-1% males have also been diagnosed with breast cancer [54].

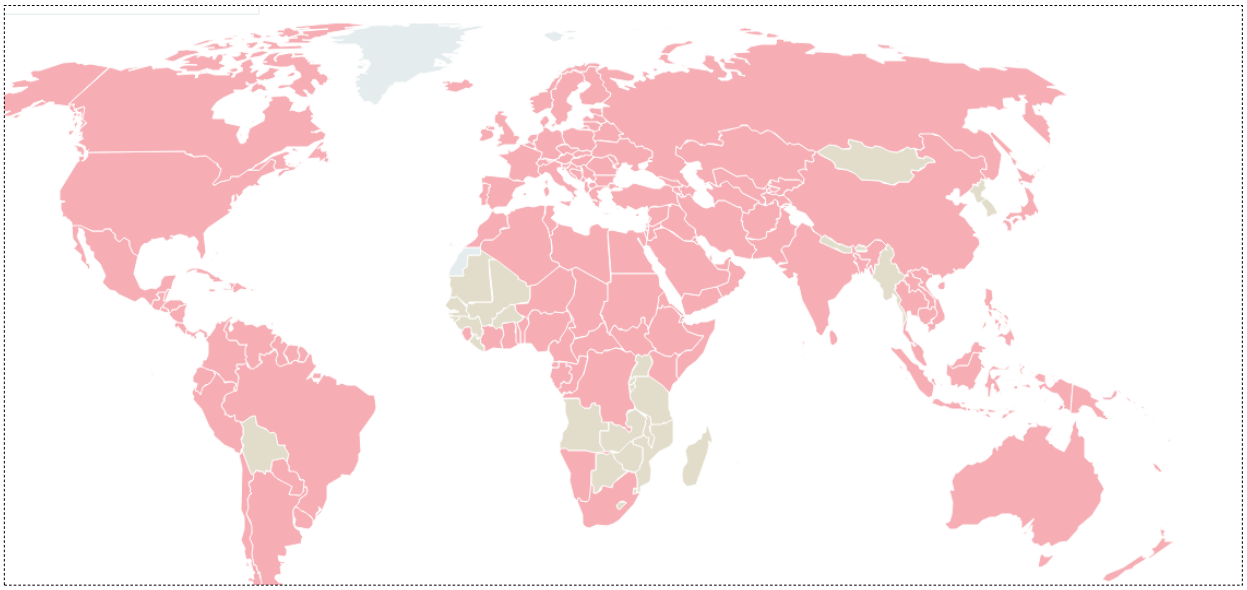

Figure 4: The cancer atlas – Depiction of prevalence of breast cancer worldwide. ***Ref:Abdul Manap AS et.al. Mapping the function of MicroRNAs as a critical regulator of tumor-immune cell communication in breast cancer and potential treatment strategies. Frontiers in Cell and Developmental Biology.***

A recent SURVCAN-3(Cancer Survival in Countries in Transition) study conducted in 2023, reported that the 3-year median survival of breast cancer throughout was 84%, whereas in India it was 68% [55]. Incidence of the disease is on rise in both rural and urban India [55]. At the state level, Kerala depicted highest incidence of the disease followed by Tamil Nadu, Telangana, Karnataka and New Delhi compared to the eastern and north-eastern states. If the current trend continues, then by 2025, disability adjusted life years (DALYS) is expected to reach 5.6 million [55]. In every 4 minute an Indian woman is diagnosed with breast cancer, of which, 50% of the cases are stage 3 and 4 [55]. The average % of patients in different stages of the disease in India, is: Stage I: 1–8%, Stage II: 23–58%, Stage III: 29–52%, Stage IV: 6–24% [55].

1.6.1 Cancer statistics in West Bengal

The ICMR report on 'Profile of Cancer and Related Factors in West Bengal-2021' mentions that the average annual number of cases and incidence rates per 100,000 of all ages is 4835 [56].

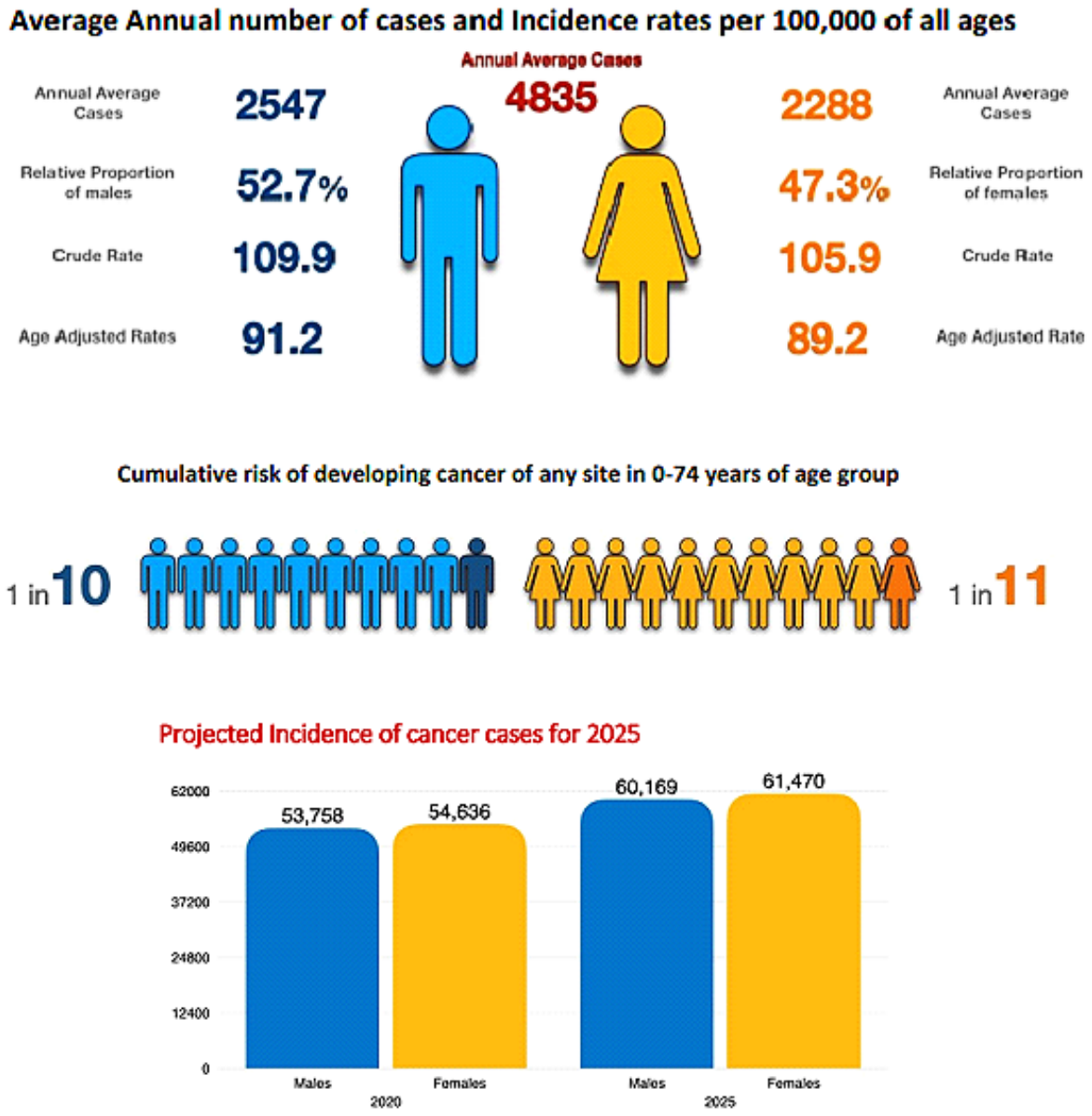

Figure 5: Cancer statistics of West Bengal –Annual average number of cases and incidence rates of West Bengal along with the cumulative risk of cancer occurrence. Projected incidence rate of cancer in both males and females in 2025.***Ref: Profile of Cancer and Related Factors-West Bengal, ICMR-National Centre for Disease Informatics and Research 2021.***

Amongst females, breast cancer is leading the list with an approximate relative proportion of 24.8% [56]. Of the diagnosed cases, majority of them, around 79% were localized only. Whereas 17% were loco regional and 3% exhibited distant metastasis [56].

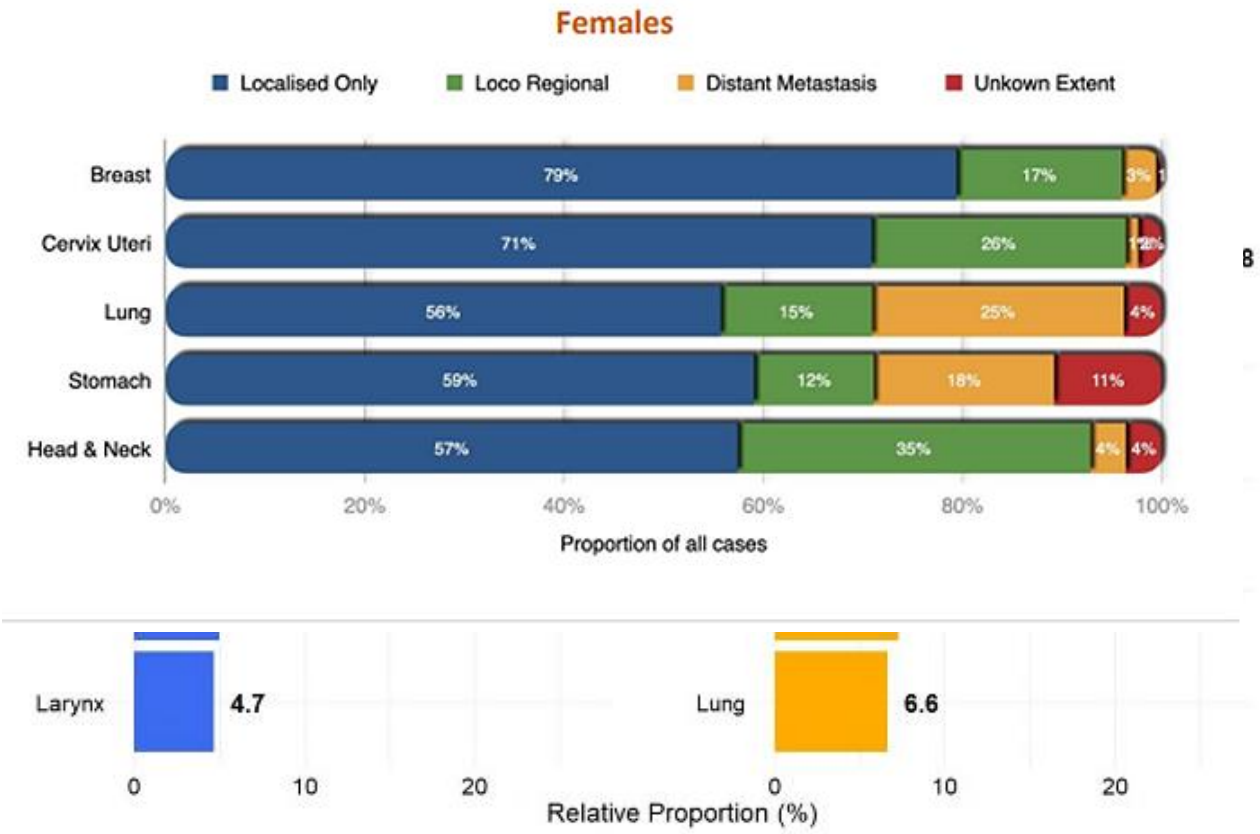

Figure 6: Breast cancer statistics of West Bengal –Bar chart depicting the relative proportion of five most common types of cancer in both males and females. Relative extent of metastasis in the diagnosed breast cancer cases. ***Ref: Profile of Cancer and Related Factors-West Bengal, ICMR-National Centre for Disease Informatics and Research 2021.***

1.7 Classification of breast cancer

Breast cancer is either non-invasive, which is restricted to the epithelial cellular components(in-situ) or invasive/infiltrative carcinoma characterized by invasion or infiltration of neoplastic cells into the stroma [57]. Both of these types can further be sub-classified into ductal or lobular type depending on the site of origin of the tumor.

a. Ductal carcinoma in-situ (DCIS)- Here, the abnormally proliferating epithelial cells lining the milk ducts remain restricted to their site of origin and do not invade the surrounding breast tissue [57].

b. Invasive ductal carcinoma (IDC) –In this type, abnormal cells growing in the lining of the milk ducts begin to invade into the surrounding breast region and further break into the lymph nodes or blood vessels to be carried to the other organs, resulting in metastatic breast cancer [57].

c. Lobular carcinoma in-situ (LCIS) – It is an uncommon condition in which cells with altered features begin to develop in

the lobules of the milk gland and do not spread beyond it [57].

d. Invasive lobular carcinoma (IDC) – The neoplastic cells spread beyond lobules of the milk gland and begin to invade into the surrounding tissue [57].

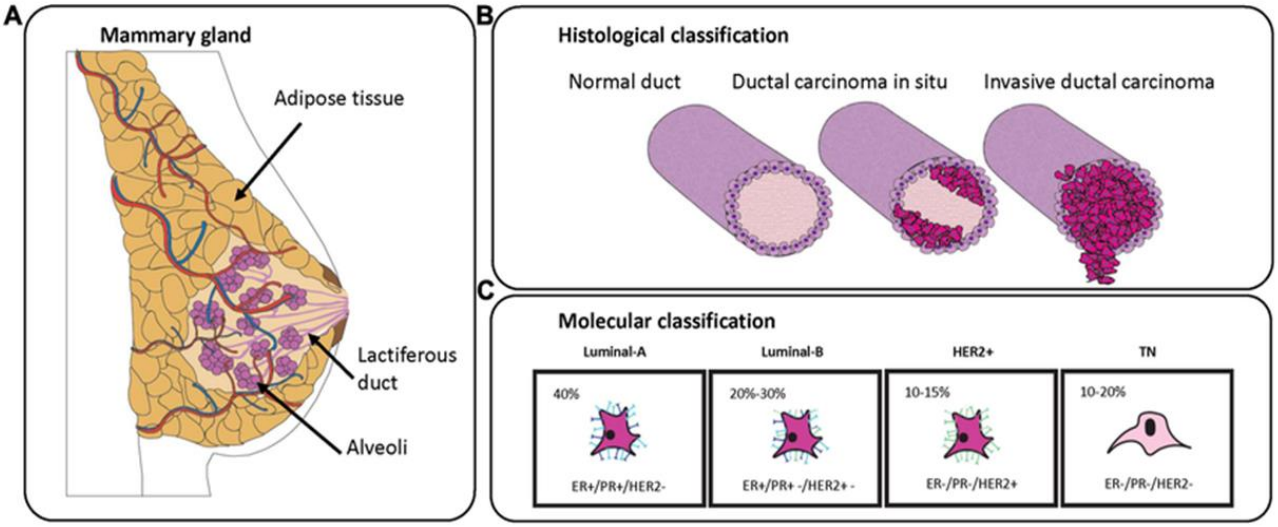

Figure 7: Histological classification of breast cancer – Breast cancer can be classified into either non-invasive or invasive carcinoma, based upon whether they remain restricted to their site of origin or infiltrate into the surrounding stroma. Each of these subtypes can further be sub-categorized into ductal and lobular carcinoma in-situ and ductal and lobular carcinoma invasive types. ***Ref: Gajdosova V et.al. Electrochemical nano biosensors for detection of breast cancer biomarkers. Sensors.***

1.7.1 Molecular classification of breast cancer

Breast cancer can be divided into four primarily molecular sub-types based upon the presence of hormone receptors and proteins involved in cancer [58].

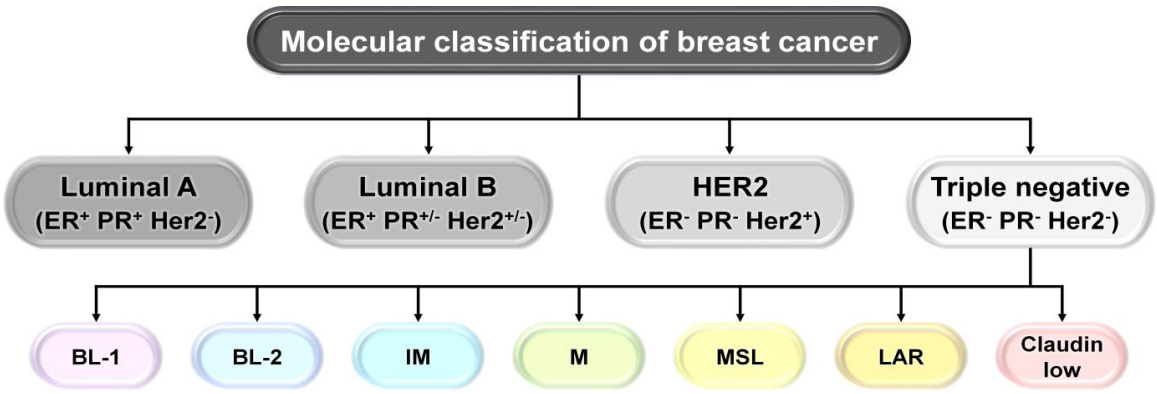

Figure 8:Molecular classification of breast cancer: Schematic representation of the molecular classification of breast cancer: based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR)and human epidermal growth factor receptor 2 (HER2), human breast carcinoma has been categorized into four different types: luminal-A (ER+, PR+, HER2−), luminal B (ER+, PR+/− , HER2+/−), HER2+and triple negative breast cancer (TNBC) (ER− , PR− , HER2−). TNBCs are further subdivided into transcriptome-based subtypes: basal cell-like type 1 (BL-1), basal cell-like type 2 (BL-2), immune-modulatory (IM), mesenchymal-like (M), mesenchymal stem cell-like (MSL), luminal-Androgen receptor (LAR)and claudin low. ***Ref: Guha et.al. Cancer stem cell–immune cell crosstalk in breast tumor microenvironment: a determinant of therapeutic facet. Frontiers in Immunology***.

Luminal-A and luminal B subtypes, due to the presence of hormone receptors, respond to anti-estrogen (aromatase inhibitors like anastrozole, letrozole and exemestane) or anti-progesterone (anti-progestins like mifepristone) therapies and thus have better prognosis than the remaining two sub-types [58] [59] [60]. TNBC, on the other hand, due to the absence of hormone receptors, is difficult to target and is considered as the most aggressive subtype of breast cancer [58] [59] [60]. Also, Luminal-A has low proliferative capacity with the highest rate of incidence followed by luminal B, HER2 and TNBC [58] [59] [60].

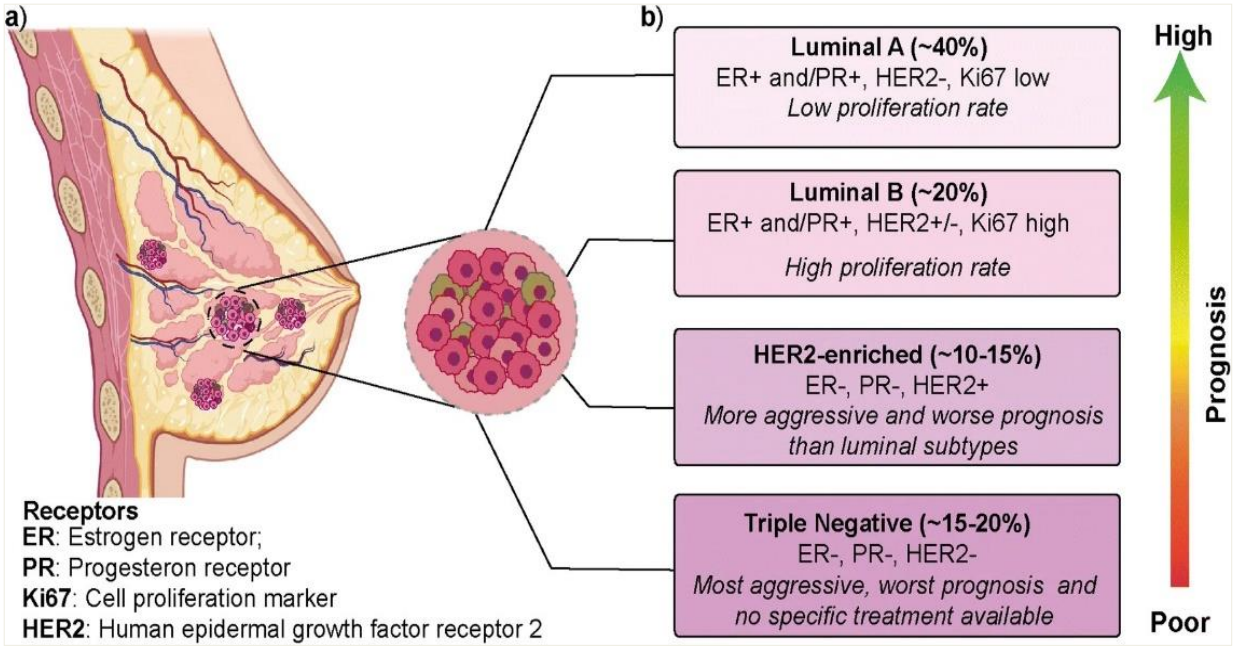

Figure 9: Incidence rate and proliferative capacities of breast cancer sub-types: Amongst all the sub-types, luminal-A has the highest rate of incidence and low proliferative capacity and overall better prognosis than luminal B, $HER2^{+}$ and TNBC. ***Ref:Firatligil-Yildirir B et.al. Recent advances in lab-on-a-chip systems for breast cancer metastasis research. Nanoscale Advances.***

1.8 Breast tumor microenvironment

Tumor microenvironment (TME) can be visualized as an ecosystem that surrounds the tumor. It includes immune cells, extracellular matrix, blood cells and various other cells like endothelial cells, pericytes, cancer associated fibroblasts etc [61] [62]. A constant interaction between the tumor cells and its TME influence each other in a positive or negative way [61]. Although, the arrangement of various cell types inside the TME may vary depending on the specific tumor, the microenvironments of several cancer types typically share similarities [63]. For instance, many cancers like glioblastoma, head and neck squamous cell carcinoma, medulloblastoma have a particularly fibrotic, or stiff, microenvironment [63] [64]. As a result, drugs have a harder penetrating time within the tumor and reaching its core. Many other cancer forms like renal cell carcinoma, breast tumors, angiosarcoma often have a more vascular, or packed with blood vessels, milieu around them [63] [65]. It might be simpler for medications to reach those cancer cells in certain situations [63] [65].

The breast TME consists of tumor cells, breast cancer stem cells (BCSCs), immune cells like macrophages, T-cells, dendritic cells, activated platelets etc., stromal cells and can be considered at 3 levels i) local (intra-tumoral), regional (in the breast) and distant (metastatic) levels [66] [67]. Along with it, the TME encompasses various components of the extracellular matrix (ECM), soluble factors like cytokines (IL1β, IL2, IL6, IL8, IL10, IL12, TNFα, IFNγ etc.) hormones etc. and physical properties which includes optimum pH and oxygen level for sustainable growth of the tumor cells [66] [67].

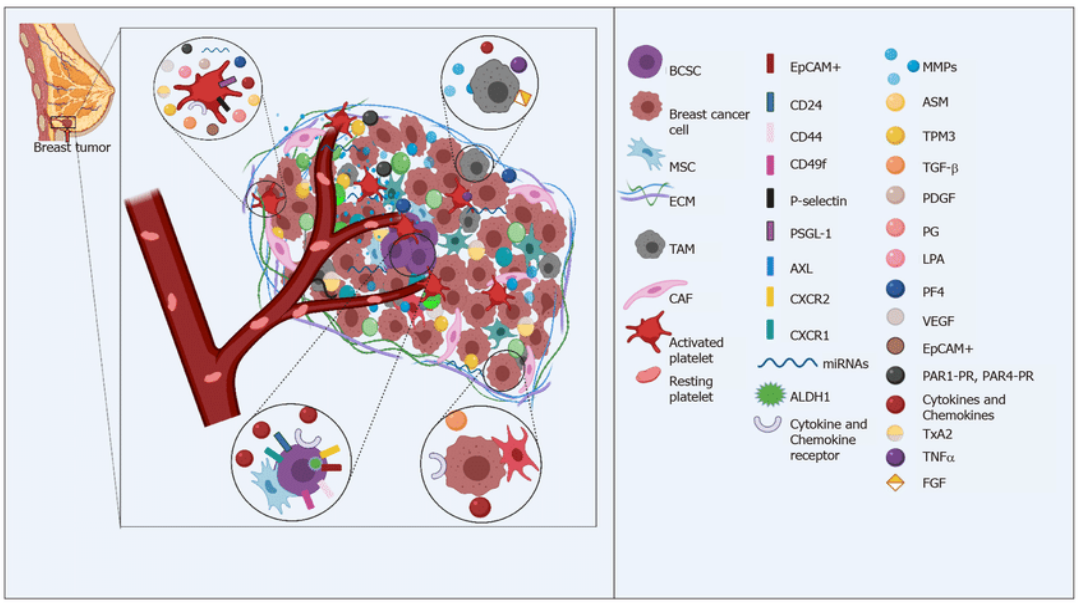

Figure 10: Components of the breast TME: The breast TME encompasses numerous cellular and extracellular components. Crosstalk between them contributes to the optimal conditions for tumor growth. ***Ref: Mendoza-Almanza G et.al. Role of platelets and breast cancer stem cells in metastasis. World journal of stem cells.***

1.9 Hallmarks of cancer: 'New Dimensions'

In the year 2000, Hanahan and Weinberg proposed the six hallmarks of cancer that provided a logical framework for understanding the remarkable diversity of neoplastic cells [68]. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis [68].

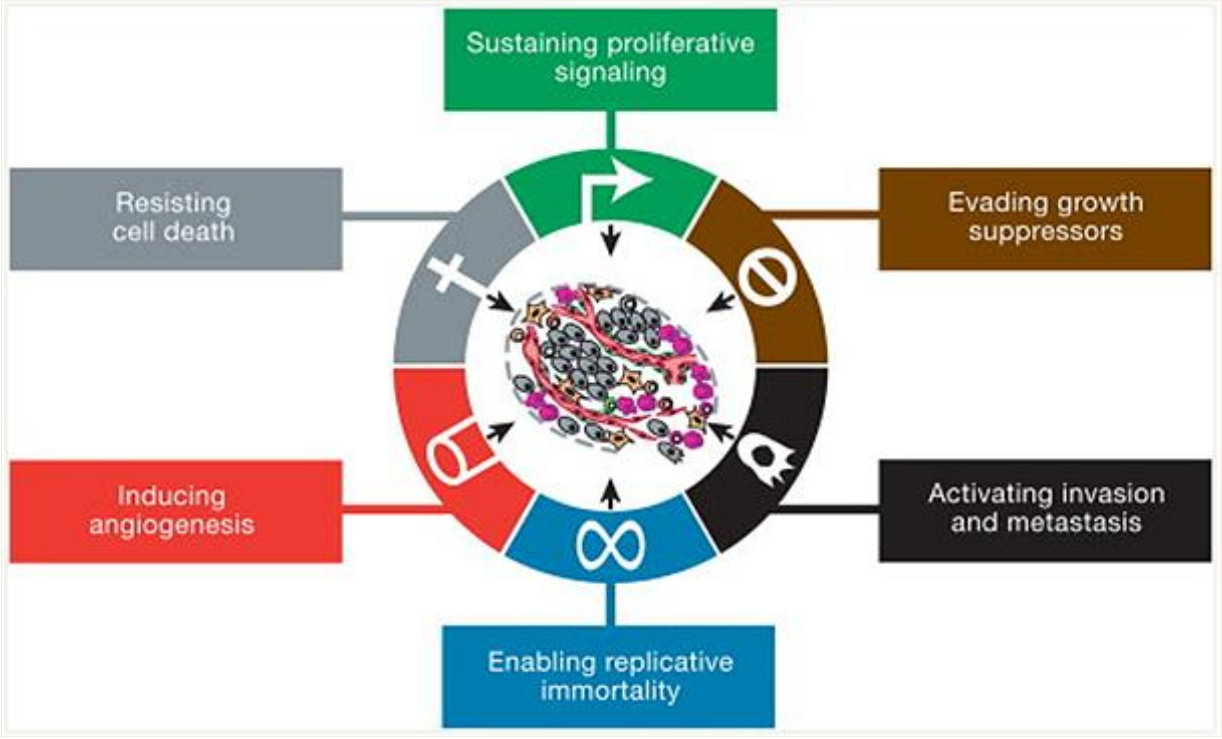

Figure 11: Hallmarks of cancer: The six hallmarks of cancer proposed by Hanahan and Weinberg. ***Ref: Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell.***

In the year 2011, Weinberg and Hanahan, incorporated two emerging concepts to the existing six, owing to the progress made over the years in knowledge of hallmarks of cancer and thus 'The Next Generation'

of the hallmarks came into existence [69]. These two emerging factors were

1. Reprogramming energy metabolism- This concept deals with the metabolic switch of cancer cells to aerobic glycolysis even in the presence of oxygen. As this process is much faster and produces several intermediates which in turn are used by malignant cells to produce proteins, DNA and lipids that further supports their proliferation [69].

2. Evading immune destruction–The immune system acts as a barrier to tumorigenesis. Tumor cells employ different mechanisms to evade the destructive effect of various immune cells like the helper and cytotoxic T cells, NK cells etc. Studies on various murine and human tumor models have reported that cancers with high infiltration of immune cells had better prognosis [69] [70].

Ten years later, in 2021, Hanahan further expanded these characteristic features and included the two emerging factors into the core hallmarks of cancer. Also, he proposed an additional emerging hallmark which was 'phenotypic plasticity and disrupted differentiation' to the existing list [69] [71]. The new list also highlighted the importance of non-mutational epigenetic reprogramming and microbiome and acknowledged them as 'enabling characteristics of cancer'. This new version also stressed on the functional importance of senescent cells within the TME [69] [71].

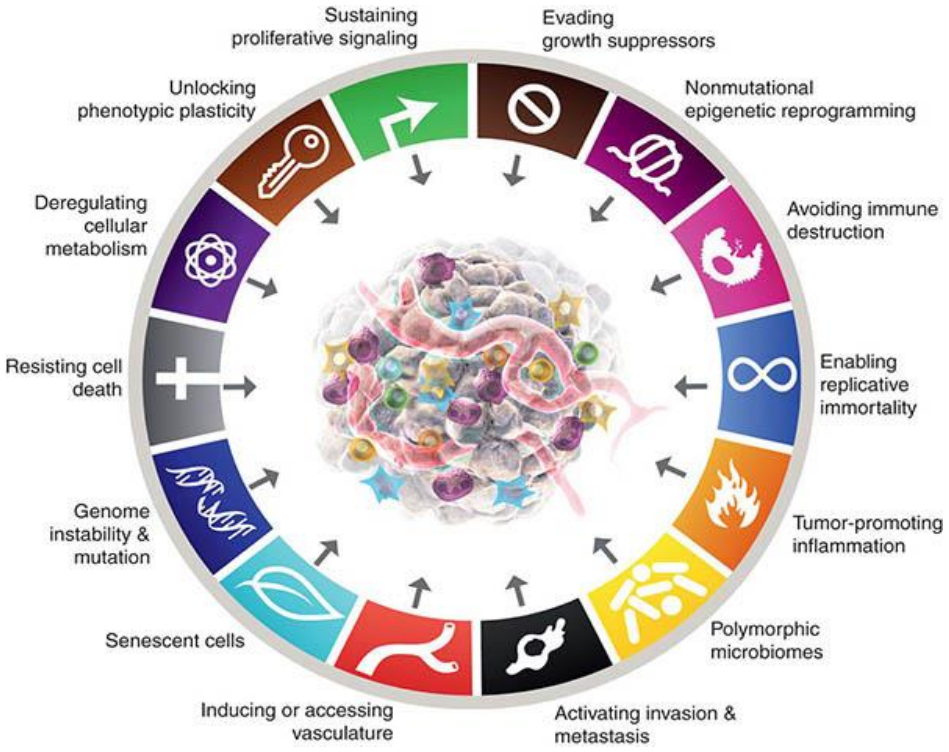

Figure 12: Hallmarks of cancer: New dimensions, proposed by Hanahan and Weinberg in 2022.
Ref: Hanahan D. Hallmarks of cancer: new dimensions. Cancer discovery

1.10 Epithelial to Mesenchymal Transition: EMT

Amongst this list, a very distinctive feature of cancer cells is activation of invasion and metastasis. Over 90% of cancer related mortalities across the globe is due to metastasis [72]. Tumor cells, prior to this process, undergo a series of changes which collectively is termed as EMT or epithelial to mesenchymal transition or EMT. During this event, epithelial cells lose their characteristic features of cell adhesion, polarity and they gain migratory and invasive capacities like the mesenchymal cells that facilitate their voyaging through the blood stream [73]. Upon reaching their target site, they undergo the reverse process of EMT to generate epithelial cells which divides further to form the secondary tumor bulk [74]. This process is known mesenchymal to epithelial transition or MET [74].

EMT has been classified into 3 different biological sub-types.

- Type 1 EMT – It occurs during normal embryogenesis and organ formation and is not involved with any fibrosis or invasive phenotype. Further, the primary mesenchyme so formed can undergo MET to generate the secondary epithelia [75].
- Type 2 EMT – This second type is associated with wound healing and fibrosis. It also promotes the generation of fibroblast and other associated cells required for tissue regeneration following injury [75].
- Type 3 EMT – Neoplastic cells undergo the 3rd type of EMT leading to invasion and metastasis which ultimately contributes in disease advancement [75] [76].

EMT is driven by several proteins and factors, such as, SNAIL, SLUG, cadherins, TWIST, KLF-4, NF-I, TBX-2, SIX, b-Myb, COX-2, ARF-6, FOXA-2, GATA-3, SMAR-1, ZEB and bHLH [77] [78]. Two main important transcription factors related to EMT are SNAIL1 (corresponding to SNAI1 gene) and SLUG (corresponding to SNAI2 gene). SNAIL-1 regulates E-CADHERIN expression by binding to E-box motifs located on the E-CADHERIN promoter [77] [78]. Consistent expression of SNAIL leads to complete inhibition of E-CADHERIN and triggers EMT [77] [78] and facilitates metastatic outspread of tumor cells.

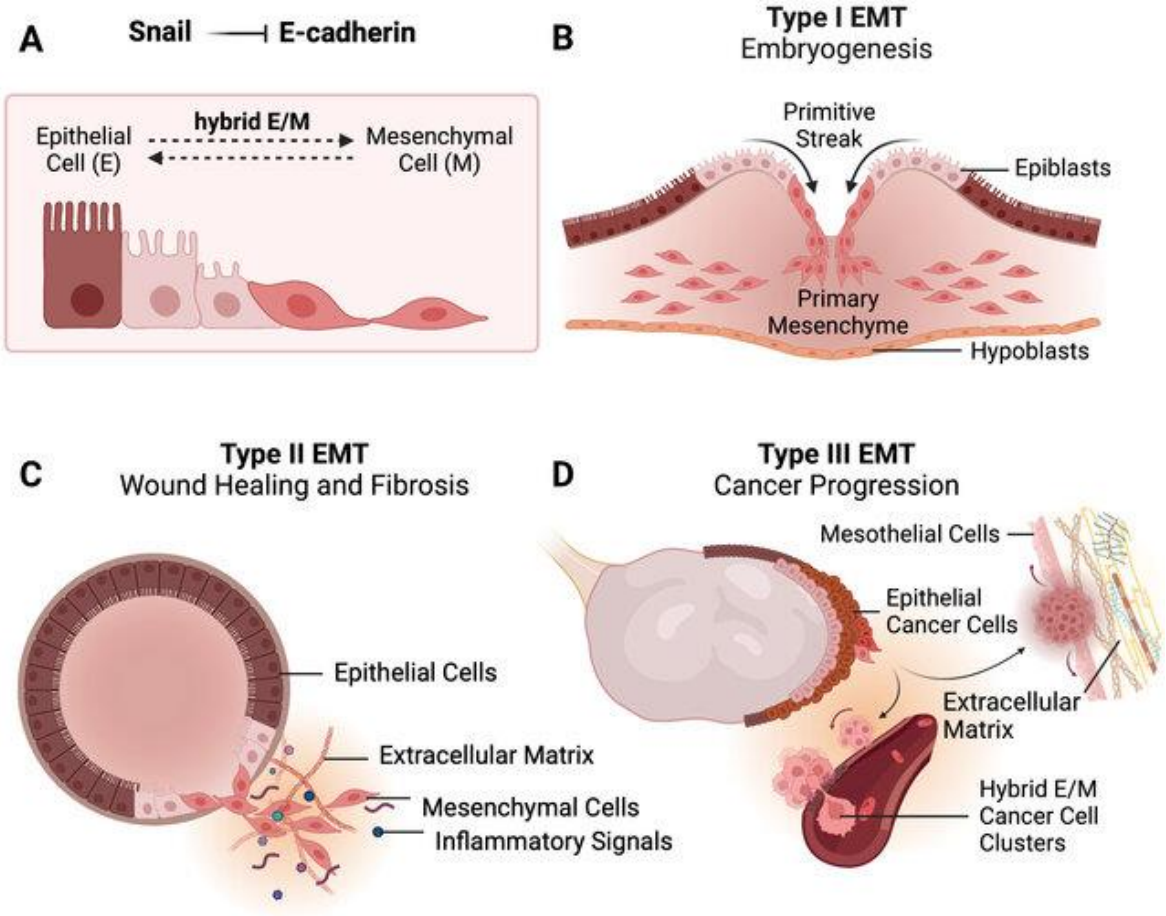

Figure 13: Types of EMT: SNAIL is an important transcription factor that suppress E-CADHERIN expression leading to EMT. EMT can be categorized into types I and II that occurs during normal embryogenesis and wound healing. Type III leads to neoplastic transformation of epithelial cells leading to cancer progression. ***Ref: Suzuki T et.al. Role of epithelial-mesenchymal transition factor SNAI1 and its targets in ovarian cancer aggressiveness. Journal of cancer metastasis and treatment.***

1.10.1 EMT – The key driver of metastasis

The importance of EMT in inducing invasion and dissemination of cancer first came into light after the phenomenal studies conducted by Batlle et.al in 2000, describing the suppression of E-CADHERIN by SNAIL, thereby leading to induction of metastasis [79]. For many years, EMT was only perceived as a temporary transitioning state that bestows the tumor cells with mesenchymal like properties that promote migratory ability of these cells [80]. The process of metastasis is intricate and multifaceted, involving significant phases that limit the rate of growth. It is well recognized that metastasis consists of distinct steps in

which tumor cells (i) detach and migrate away from the primary tumor site, (ii) invade neighbouring tissue and penetrate through basement membrane, (iii) enter the blood or lymphatic vessels, (iv) survive the condition of anoikis while they are detached from the tumor mass and in circulation, (v) exit the blood or lymphatic vessels at a distant organ, (vi) form micro-metastatic nodule, (vii) adapt and reprogram the surrounding stroma and form macro-metastasis [81]. The tumor cells capacity to spread and colonise distant areas in the face of anti-cancer therapies is a critical setback. Extensive research on EMT revealed that, properties of cancer cells beyond invasion like stemness, immunosuppression, drug resistance are all associated with EMT [82]. Acquisition of stem cell like properties via EMT allows the metastasizing tumor cells to survive the action of anti-cancer drugs due to the high expression of the drug efflux pumps making them therapy resistant [83].

The diversity of EMT is quite extensive and different EMT types are linked to different degrees of plasticity and metastatic potential. Based on their capability of inducing invasion and metastasis the following types of EMT has been observed

Partial EMT/Hybrid-1: The cells here are in transition between the epithelial [E] and mesenchymal [M] state and are the most plastic population and have high expressions of both E and M markers. They generally are found in collective invasion regions and have high metastatic colonization ability [84].

Intermediate EMT/Hybrid-2: Cells in this state do exhibit reduction of prominent 'E' markers and express the 'M'markers but does not reach an extreme 'M' state. It endows the cells with stemness, invasive and metastatic abilities [85].

Extreme EMT/M-state: The classical 'M' state markers are expressed by the cells. They are highly invasive and chemo-resistant but have reduced plasticity in reactivating proliferative states [86].

Amoeboid EMT/ Beyond M-state: The cells exit the EMT trans-differentiation process and transform into undifferentiated ameboid-like cells. This state is characterized by high mechano-plasticity, migration and metastasis ability.

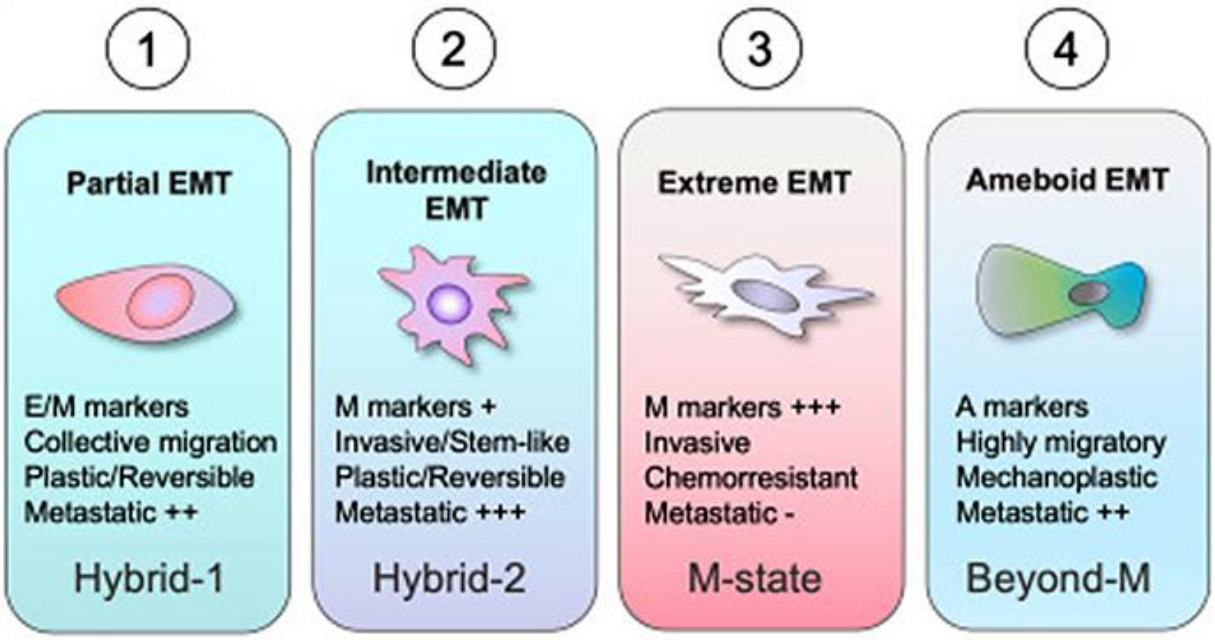

Figure 14: Spectrum of EMT: EMT is a highly plastic phenomenon and based on their capability of inducing invasion it is sub-classified into partial EMT, intermediate EMT, extreme EMT and Ameboid EMT. 1. Partial EMT- mixture of epithelial and mesenchymal like cells with moderate metastatic ability. 2. Intermediate EMT – Majority of mesenchymal cells with invasive, stem like properties and high metastatic capacity. 3. Extreme EMT – cells with highest expression of mesenchymal markers. 4. Amoeboid EMT – High migratory and mechano-plastic abilities. ***Ref: Celià-Terrassa T et.al. How important is EMT for cancer metastasis. PLoS biology.***

1.10.2 Transcription factors and proteins in EMT

The major transcription factors and proteins with prominent role in EMT are E-CADHERIN, VIMENTIN, SNAIL, SLUG, ZEB, TWIST

1. E-CADHERIN: A transmembrane glycoprotein E-CADHERIN forms adherens junction between epithelial cells**.** The E-CADHERIN gene (CDH1) is located on chromosome 16q22.1 and is around 100 kb long. The gene is composed of 16 exons that ranges between 115 to 2245 bp, with a total of 15 introns between them [89]. E-CADHERIN expression begins from 2-celled embryonic stage and is essential for adhesion between the blastomeres. Aberrant E-CADHERIN expression can lead to failure of polarization, compaction and trophectoderm formation by the embryos.

In cancer, loss of E-CADHERIN contributes in the progression of majority of solid malignancies including breast tumor [90]. Further, selective loss of E-CADHERIN leads to de-differentiation and invasiveness of tumor cells. Highly aggressive cancers like oesophagus,

stomach and ovary, exhibit reduced E-CADHERIN expression [91]. Downregulation of E-CADHERIN has been shown *in vitro* to be linked to the acquisition of the mesenchymal phenotype, which is linked to invasive behaviour and the loss of the epithelial phenotype [92]. Additionally, when E-CADHERIN is continuously generated, partial or complete reversal of the phenomenon has been observed.

With respect to breast carcinoma, E-CADHERIN has been described as a potent tumor suppressor and a relationship between tissue E-CADHERIN level and hormone receptor expression has been demonstrated. Mostly, low E-CADHERIN status has been observed in estrogen receptor negative breast tumors. In addition to other indicators of an adverse prognosis, such as an elevated tumor size, a higher histological grade, the development of distant metastasis and ER receptor negative tumors, decreased or impaired E-CADHERIN expression is linked to a shorter disease-free interval and overall survival [93]. Moreover, in the two main histological subtypes of breast cancer, distinct ways of modulating E-CADHERIN expression have been identified. Infiltrative lobular breast carcinomas (ILC) are, for the most part (85%), entirely E-CADHERIN-negative, whereas infiltrating ductal breast cancers typically exhibit either no E-CADHERIN expression at all or merely a heterogeneously reduced expression [94].

The fact that infiltrative lobular breast cancer has a high rate of complete and irreversible E-CADHERIN inactivation, suggests that E-CADHERIN functions as a true tumor suppressor in this particular histological subgroup of sporadic breast cancers. This is corroborated by the discovery that E-CADHERIN is already inactivated in early non-invasive LCIS, which runs counter to a concept that limits E-CADHERIN to acting as an invasion suppressor [95]. In ductal breast tumors, a heterogeneous decrease of E-CADHERIN expression is typically seen in the transcriptional level, this negative regulation appears to be reversible, permitting re-expression in the secondary metastatic tumor location, favouring MET [96].

2. VIMENTIN: The VIM gene encodes the structural protein VIMENTIN in humans. Its name is derived from the Latin vimentum, which means a variety of pliable rods. VIMENTIN is a type III intermediate filament (IF) protein, which is expressed on mesenchymal cells [97]. Structurally, the VIMENTIN monomer includes the non-helical amino (head) and carboxyl tail that caps the central α-helical

domain. It is believed that two monomers are co-translationally expressed in a manner that promotes their contact to create a coiled-coil dimer, the fundamental subunit of VIMENTIN assembly [98]. Metastatic tumors exhibit high levels of VIMENTIN, which is associated with poor patient outcomes [99]. In this context, VIMENTIN is a prominent marker for EMT or epithelial to mesenchymal transition. As it has been previously mentioned that EMT is broadly of three types, VIMENTIN plays a predominant role in both type I and type II EMTs along with the cancer promoting type III [100]. VIMENTIN expression in breast tumors has been demonstrated in numerous studies to be a significant prognostic factor [101]. VIMENTIN-positive cells are linked to drug resistance, low PR, low ER, greater invasiveness, high grade tumors and enhanced proliferation of tumor cells [101]. Furthermore, clinical studies conducted on various grades of breast carcinoma revealed that VIMENTIN expression was upregulated majorly in grade 3 breast carcinoma. The findings supported the EMT theory, which holds that low-grade tumors maintain their ability to adhere because they are enriched with E-CADHERIN and related proteins. The cells transit into mesenchymal state as their grade increases, losing their ability to adhere due to more generation of mesenchymal proteins like VIMENTIN [102].

3. SNAIL and SLUG: Both SNAIL and SLUG have prominent role in downregulating the expression of E-CADHERIN by binding to two proximal E2-boxes of E-CADHERIN promoters. In breast cancer SNAIL and E-CADHERIN expressions have been reported to be inversely correlated [103]. Along with E-CADHERIN SNAIL also represses the expression of genes encoding tight junction proteins like claudins and occludins [104]. Developmental epithelial-mesenchymal transition has been explicitly linked to members of the SNAIL family of zinc finger proteins, which implicate cadherin regulation [105]. In a number of altered cell lines, SNAIL and SLUG have also been shown to have a negative correlation with the expression levels of E-CADHERIN.

Previous studies on breast cancer have reported that SNAIL or SLUG expression can be correlated with poor outcome and lymph node metastasis [106]. According to Vincent-Salomon and Thiery, the development of IDC is associated with changes in the expression of adhesion-related factors that are implicated in SNAIL/SLUG-induced EMT, such as decreased E-CADHERIN and increased N-CADHERIN expression [107].

Furthermore, overexpression of SNAIL was substantially linked to higher tumor stage, while overexpression of SLUG was linked to a greater tumor grade and negative ER status [108]. Studies from primary breast cancer cells revealed that when these cells were transduced with SNAIL and grafted in mice subcutaneously along with the in-activation of Her2/neu pathway, rapid recurrence of the tumor was observed [109]. This indicated towards the possible involvement of SNAIL in recurrence of tumor *in-vivo* [109]. Thus, in an attempt to properly classify ductal carcinomas and eventually develop suitable treatment strategies, it may be possible to manipulate the expression of SNAIL and SLUG, which appear to reflect different tumor invasion modalities [110].

4. TWIST: The basic helix-loop-helix protein TWIST triggers EMT fundamentally by regulating E-CADHERIN to N-CADHERIN ratio [111]. TWIST does not directly supress E-CADHERIN but enhances the expression of N-CADHERIN to a level that exerts a dominant effect on breast cancer cells [112]. TWIST also plays a predominant role in inducing morphological changes associated with EMT and also elevates cancer stem like traits in tumor cells [112]. It is also involved in other tumor promoting effects like chemical resistance to metastasis and invasiveness associated with common chemotherapy [113]. TWIST expression is correlated to an increase in the expression of other mesenchymal markers, such as fibronectin, VIMENTIN, αSMA, along with N-CADHERIN [113].

In approximately 54% of breast cancer patients, TWIST expression can be correlated positively with tumor size, Ki67 levels and Her2 expression [114]. Overexpression of TWIST can lead to extensive alterations in the morphology of these cells, along with significant impact on the proliferation, migratory/invasive capacity and expression of biomarkers linked to EMT. Several studies have reported that TWIST can significantly expand the population of breast cancer stem cells as evidenced by an increase in CD44+/CD24-and ALDH+ cells, increased exclusion of Hoechst 33342 and Rhodamine 123 dyes due to increased expression of ABCC1 transporters and the initiation of tumor formation from low-cell inoculums [115]. This capacity to generate tumors from small inoculums unequivocally demonstrates that TWIST is a key factor influencing the phenotype of breast cancer stem cells. Moreover, cells from the xenograft tumors produced by the $CD44^{+}/CD24^{-}$ subpopulation had higher levels of TWIST expression in their nuclei

than cells from the $CD44^+/CD24^+$ subpopulation [116]. Accordingly, it can be concluded that TWIST can generate breast cancer stem cells with different levels of carcinogenicity, which may be similar to that observed in breast cancer stem cells. Drug resistance to anticancer medications that target microtubules, such as vincristine and taxol, has been linked to overexpression of TWIST. This is important since vincristine's primary drug efflux pump is ABCC1 (MRP1) [117]. Numerous research reports have validated that these TWIST overexpressing cells have upregulated ABCC1 which promote chemoresistance and establishment of stem cell like phenotype in these cells [117].

5. ZEB: Zinc finger E-box binding homeobox1 promotes tumor invasion and metastasis by inducing EMT in carcinoma cells. ZEB stimulated tumor cells with mesenchymal traits and promotes multidrug resistance, proliferation and metastasis indicating the importance of ZEB induced EMT in cancer development [119]. Unlike TWIST, ZEB binds directly to the E-CADHERIN promoter and functions as transcriptional repressor. At the same time, it also promotes the expression of VIMENTIN and N-CADHERIN [118]. Significant studies have also proved the importance of ZEB in therapy resistance including chemotherapy and radiation therapy thereby contributing in disease advancement. In primary breast cancer, elevated ZEB1 expression promotes EMT by inhibiting the production of epithelial marker E-CADHERIN. Thus, the transformed tumor cells with high ZEB1 lose their epithelial characteristics to adopt a mesenchymal phenotype. On the other hand, when metastatic breast cancer develops in a distant region, mesenchymal-epithelial transition (MET) process occur with lower ZEB1 level in order to restore the epithelial characteristics and eliminate the mesenchymal/motile phenotype [120].In a mouse xenograft model of breast cancer, overexpression of ZEB1 was linked to increased metastatic potential, indicating a function for ZEB1 in invasion and metastasis of human tumors [121].Moreover, by controlling the levels of several inflammatory cytokines, including interleukin 6/8 (IL-6/8), ZEB1 helps to establish the tumor microenvironment and promote development of basal like breast tumors [122]. Fu et al. recently demonstrated that increased ECM remodelling, immune cell infiltration and angiogenesis were linked to ZEB1 activation and high expression in the stroma. [123].

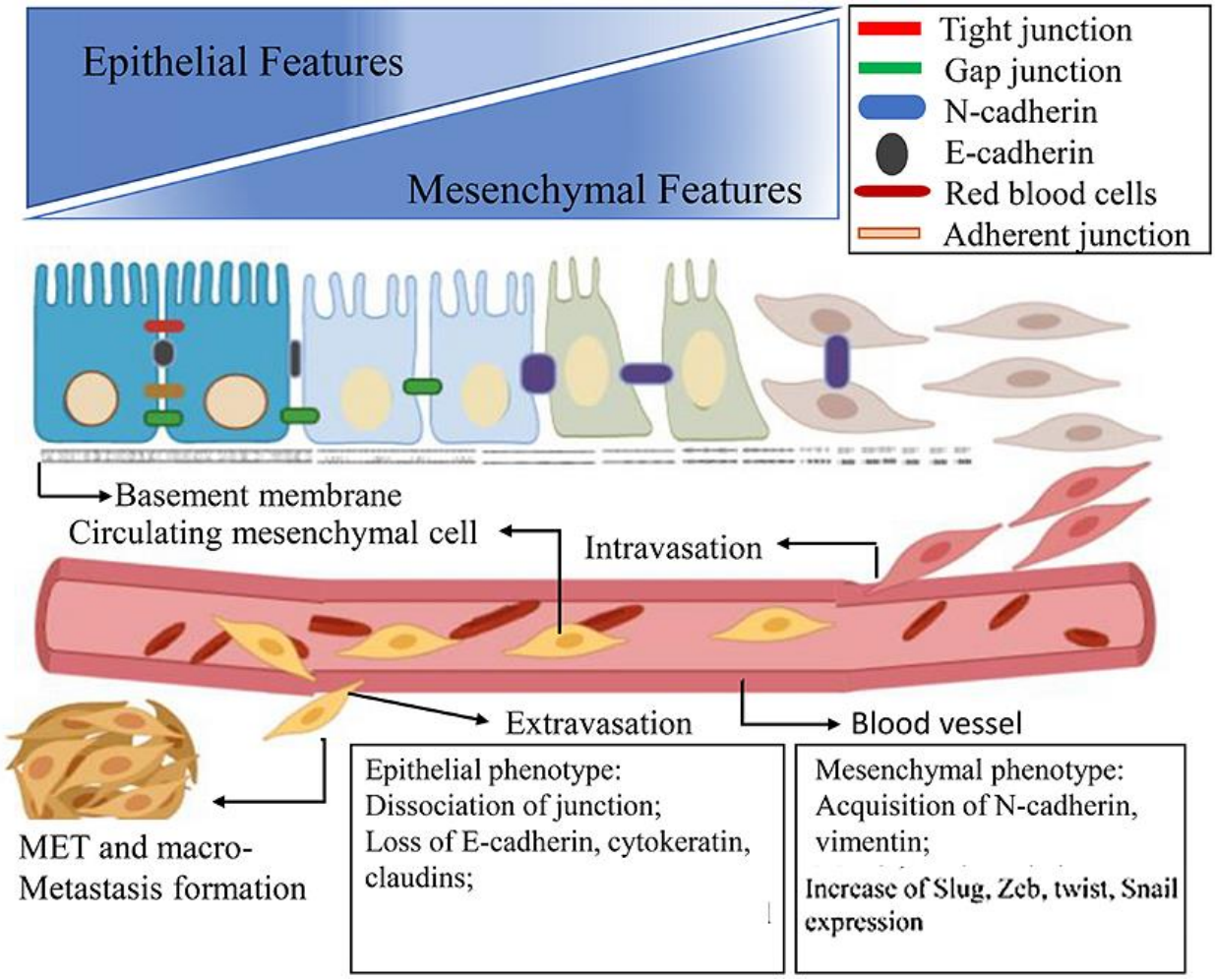

Figure 15: Role of transcription factors in EMT: Transcription factors like SNAIL, S, TWIST, ZEB promotes the loss of proteins involved in cell-to-cell contact like E-CADHERIN, cytokeratin, claudins and gain of mesenchymal markers like VIMENTIN and N-CADHERIN, thereby promoting EMT. ***Ref: Din ZU et.al. Crosstalk between lipid metabolism and EMT: emerging mechanisms and cancer therapy. Molecular and Cellular Biochemistry.***

1.10.3 Signalling cascade in EMT

EMT is regulated by several signalling cascades, including TGFβ, Notch, WNT, Hedgehog, TNFα and RTKs. All these pathways are the convergence on the transcriptional factors SNAIL, SLUG, TWIST, ZEB.

1. WNT pathway: The Wnt cascade is regulated by canonical or non-canonical signalling. Canonical signalling is associated with β-catenin-dependent expression. It has been reported that β-catenin accumulation in nucleus is linked to a poor prognosis in cancer [124]. Conversely, non-canonical Wnt signalling does not result in β-catenin expression in the nucleus and is β-catenin-independent. By inhibiting GSK-3β function, Wnt can stabilise the amounts of SNAIL and β-catenin, causing EMT and cancer spread.

The elevation of SNAI1 expression activates Wnt/β-catenin. According to certain research, changes in the Wnt pathway and elevated β-catenin expression are key factors in the development of breast cancer [125]. Mutational alterations like amplification, methylation, deletion along with post-transcriptional and post-translational modifications at gene and protein levels respectively, results in modification of the various components of the Wnt cascade in breast cancer cells. Further, in TNBC and basal like breast cancer, high levels of majority of the canonical and non-canonical Wnt receptors have been reported [126].

Additionally, Wnt signalling plays a pivotal part in classification of breast cancer. The two most prevalent histological subtypes of breast cancer, accounting for 70–75% and 10–14% of cases, respectively, are invasive ductal carcinoma no-special-type (IDC-NST) and invasive lobular carcinoma (ILC). It has been reported that there is a substantial correlation between histological type and β-Catenin expression. While ILCs lack nuclear expression (0%) and membranous expression (14.7%), the majority of IDCs exhibit a regular pattern of β-Catenin expression, with nuclear expression (12.5%) and membranous expression (80.6%) [127].

It has also been reported from clinical studies that, β-Catenin protein level increases as the tumor progresses from histological grade I to grade III, with grade I exhibiting the lowest level while grade III has the highest expression of this protein. Thus, from these observations, β-Catenin can be considered as a prognostic marker of advanced stage breast carcinoma. Accordingly, diagnostic as well as therapeutic strategies directed towards β-Catenin shall prove beneficial to the patients. Studies conducted by Brabletz et al. on high clinical grade adenocarcinoma revealed that 89 % of the patients had diffused cytoplasmic expression of β-Catenin. Whereas, in the nucleus the level of β-Catenin protein was extremely prominent. He postulated that nuclear β-Catenin might play an integral role in EMT by supressing E-CADHERIN that ultimately results in loss of cellular polarity and adhesion. This in turn facilitates detachment of tumor cells and their migration through the blood vessels or the lymph nodes, accompanied by a boost in the expression of invasion related genes. Several other studies have also confirmed the importance of β-Catenin in EMT. Apart from regulating E-CADHERIN, it also stimulates the expression of VIMENTIN and in this way, it regulates the crucial process of metastasis [128].

2. **VEGF pathway:** Vascular endothelial growth factor (VEGF) constitutes a family of structurally and functionally related proteins that includes VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor. These proteins are biologically active in their homo-dimeric or heterodimeric forms and bind to tyrosine kinase (TK) receptors expressed on the cell surface, such as VEGFR1, VEGFR2 and VEGFR3 [129]. According to available research, VEGF acts in both normal and malignant cells in a variety of ways and an important contribution of which involves development of breast cancer. At the transcript level, breast tumor cells have been shown to exhibit high VEGF expression compared to normal or benign breast cancer samples. Approximately, 72–98% of breast cancer is positive for VEGF by immunohistochemistry (IHC). VEGF expression in breast cancer has been correlated with size, histologic grade, estrogen receptor (ER) negativity, progesterone receptor (PR) negativity, human epidermal growth factor receptor-2 (HER2) over-expression and lymph node metastasis [129]. It was also interesting to note that, tumors with higher VEGFR2 expression had higher expression of EMT markers, such as TWIST1 and VIMENTIN, whereas the same tumors had lower expression of E-CADHERIN. This suggests that VEGFR2 may be a potential mediator of EMT in breast cancer [129]. In addition to its widely recognised function in angiogenesis, VEGF is essential for the maintenance of stem cells, as evidenced by its significance for stem cells in haemopoietic, endothelial, muscular, cardiac, neuronal and adipose tissues. Recent reports have elaborated the pivotal role of VEGF in regulating self-renewal of cancer stem cells in lungs, brain and breast tumors.

Studies conducted by Zhao et al. on TNBC breast cancer subtype revealed that VEGF trigged VEGFR2, increases mammosphere and ALDH1 activity in TNBC cell lines as well as primary breast tumor samples. Further, via EMT it stimulates the production of cells with stem cell like properties. VEGFR2 in the downstream recruit JAK2/STAT3 which in-turn stimulates the expression of Myc and SOX2 [130].

3. TGFβ pathway: In EMT, TGFβis triggered by both Smad and non-Smad molecules. In Smad dependent pathway, binding of TGFβ to TGFβRII activates TGFβRI, which then induces the formation of Smad2/3complex [131]. This complex then complexes with Smad4 and together they regulate the expression of EMT related genes.

In non-Smad pathway TGF-β triggers the AKT/PI3K, Ras /Raf/ MEK/ERK and

Wnt/β-catenin signalling pathways. Both Smad and non-Smad pathways work together to regulate SNAIL/SLUG/ TWIST/ ZEB and thus control EMT.TGF-β crosstalk with other signalling pathways, including Notch, Wnt/β-catenin, nuclear factor (NF)κB and RTKs, induces EMT and plays critical roles in maintaining the mesenchymal phenotype of invasive/metastatic tumor cells [131].

4. Notch pathway: It has been found that there are four Notch receptors (Notch1-4) and five ligands (Jagged1, 2and Delta-like1, 3, 4). TGF-β of the EMT programming is modulated by the Notch signalling, which also stimulates the NF-κB pathway. Numb mediates notch signalling. In human epithelial cells as well as breast cancer cells, numb is a negative regulator of EMT. It is discovered that the increase of EMT is connected to decreased Numb expression. Research has indicated a possible link between the high overexpression of Notch signalling and the generally low survival rate of individuals with breast cancer. In order to trigger EMT, notch signalling synchronises with other routes [132]. HEY1, a Notch target gene and Jagged 1, a Notch ligand, are coordinated by Notch signalling. By transcriptionally activating SNAIL or by using lysyl oxidase (LOX), the Notch signalling controls the expression of SNAIL. By activating hypoxia-inducible factor 1-α (HIF-1α), the Notch signalling upregulates LOX [132]. This, in turn, stabilises SNAIL and leads to an upregulation of EMT programming, which in turn triggers the invasion of cancer cells. Further information suggested that Jagged1-mediated activation of Notch IC through positive regulation of SLUG suppresses E-CADHERIN, inducing EMT in breast cancers [132].

5. Hedge-hog pathway: The hedgehog (Hh) pathway, which is linked to stem cell renewal, is another signalling system involved in breast cancer EMT. The Hh pathway is involved in tissue homeostasis, stem cell renewal and embryonic development. Three glioma-associated oncogene (GLI) transcription factors GLI1, GLI2 and GLI3 are in charge of either inhibiting or activating the transcription of these components in the Hh pathway. There is evidence to show that the Hh pathways have a role in EMT in breast malignancies. Colavito et al. found that breast cancer cells undergoing EMT express GLI1 at a high level [133]. Moreover, it was demonstrated that the Hh pathway is connected to the characteristics of cancer stem cells and the interaction between NFkB and GLI1 [133]. Similar to canonical and non-canonical signalling in the Wnt pathways hedge-hog pathway can

also be classified in the same way. According to a study, drug resistance, invasion and EMT are all brought on by non-canonical GLI1 activation by hypoxia or other inflammatory cytokines in breast cancer cells [133].

6. TNF-α pathway: It is a crucial cytokine that is involved in inflammation, tumour progression and cellular homeostasis, among other functions. By inhibiting E-CADHERIN and activating MMP9, TNF-α stimulates angiogenesis, invasion and metastasis linked to EMT reprogramming [134]. TWIST-1 overexpression is linked to the induction of TNF-α in EMT. TNF-α overexpression has been linked to breast cancer cells' higher propensity to spread and invade other areas of the body. It has been demonstrated that TNF-α-induced EMT and cancer stemness features are regulated by increased TWIST-1 expression [134]. According to recent data, TWIST-1 expression encourages the spread of breast cancer cells in mice. Recent research has shown that prolonged exposure to TNFα activates NF-κB and IKKβ, which causes the transcriptional repressor TWIST1 and EMT as well as cancer stemness properties [134].

7. RTK pathway: The contribution of several RTK to the EMT of breast cancer cells has been identified. RTKs are activated by fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and epidermal growth factor (EGF). HGF signals are connected to tumour metastasis and epithelial differentiation through the downregulation of E-CADHERIN [135] [136]. Additionally, linked to the HGF pathway is the transcription factor SNAIL, which triggers EMT. The activation of RTK itself may not be sufficient to elicit EMT. The contribution of other pathways, including TGF-β, Wnt, Notch, NF-κB and ERK/MAPK pathways may lead to the EMT establishment [137] [138].

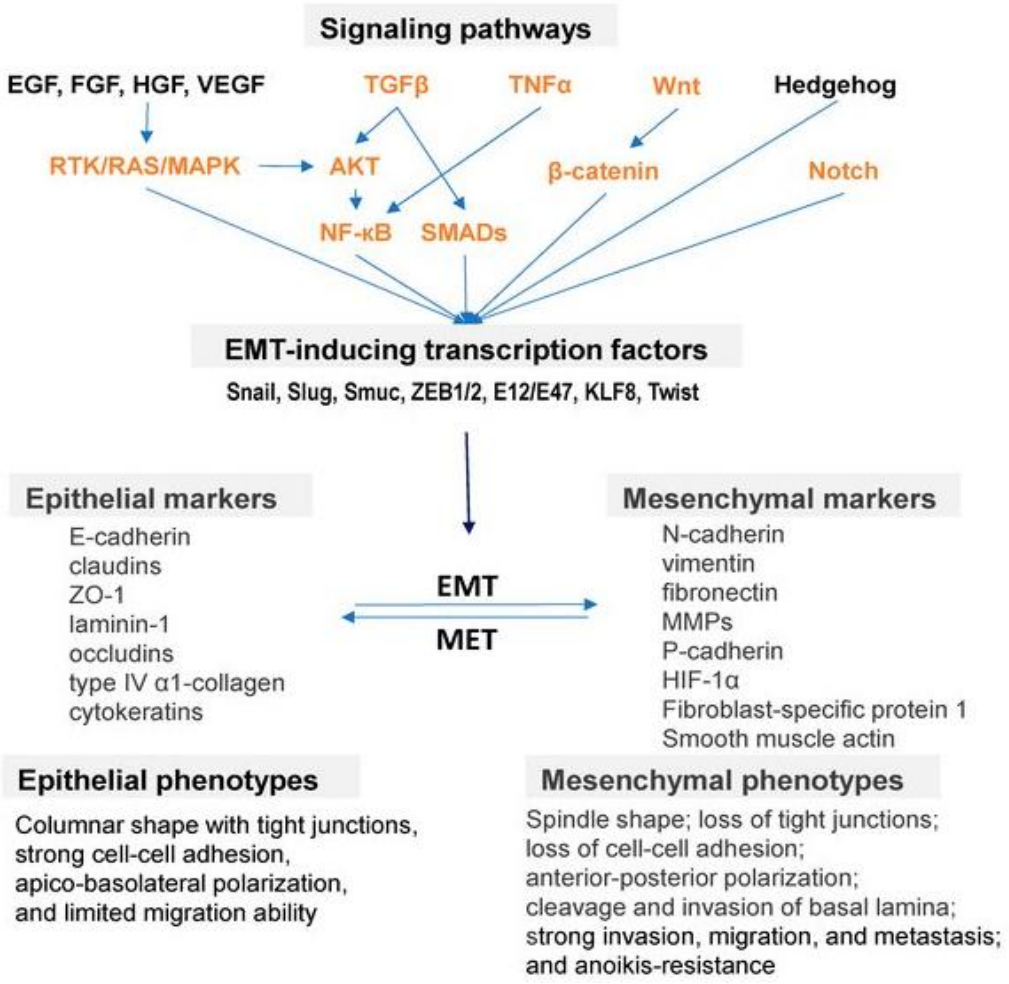

Figure 16: Signalling cascades of EMT: EMT is controlled by several signalling pathways like including WNT, Notch, TGFβ, Hedgehog, TNFα and RTKs. Components of these pathways function individually and in crosstalk with each other to regulate the expression of transcription factors and gene involved in EMT. ***Ref: Yang C et.al. The Multifaceted Roles of MicroRNA-181 in Stem Cell Differentiation and Cancer Stem Cell Plasticity. Cells.***

1.11 MET – Mesenchymal to Epithelial Transition

The epithelial–mesenchymal transition (EMT) and the mesenchymal–epithelial transition (MET) are acknowledged as key events for the dissemination of carcinomas. Cancer metastasis is a sequential series of events. The histological similarities between primary and metastatic tumors are a current subject of interest and it has been suggested that MET at metastasis sites plays a role in the process of metastatic tumor formation. As a result, mounting experimental evidence suggests that the epithelial phenotype is crucial for the development of metastatic tumours. Interestingly, E-CADHERIN-positive metastatic foci were found when

the mesenchymal-like breast cancer cells (MDA-MB-231), in which E-CADHERIN expression is transcriptionally repressed by methylation of the E-CADHERIN promoter, were injected into the mammary fat pads of mice providing a more direct indication that these E-CADHERIN-expressing metastasis may originate from E-CADHERIN-negative cells [139] [140] [141]. These experimental results imply that after the EMT that permits escape, cancer cells should go through another MET in the secondary organ environment. This is because the EMT of carcinomas is essential for the initial escape by permitting individual cell movement and invasion only while MET helps in the establishment of secondary tumors [139] [140] [141].

1.12 EMT- MET- Cancer Stem Cells

Cancer stem cells (CSC) constitute a small minority of neoplastic cells within a tumor and are defined operationally by their ability to seed new tumors. They are also known as tumor-initiating cells as a result. Tumor-initiating cells, or CSCs, were initially identified by Lapidot and associates. Since then, CSCs have been found in a wide range of solid tumors, such as those of the breast, colon, endometrium, pancreatic, prostate, ovary and brain. CSCs demonstrated a high degree of tumorigenicity. Tens of thousands of cells with different phenotypes failed to create tumors, but in the work of Al-Hajj and colleagues, tumor cells displaying the $CD44^{+}/CD24^{-}$ cell surface marker profile were sufficient to establish tumors in mice.

CSCs are capable of proliferating in low adherence cell culture conditions in the presence of growth factors like epidermal growth factor and basic fibroblast growth factor to generate floating spheroids called tumorspheres or mammospheres. The number of spheroids represents the number of CSCs, while their size depicts their proliferative capacity. Clinically, CSCs are responsible for relapse because of their resistance to radiation, chemotherapy and molecular targeted therapy [142].

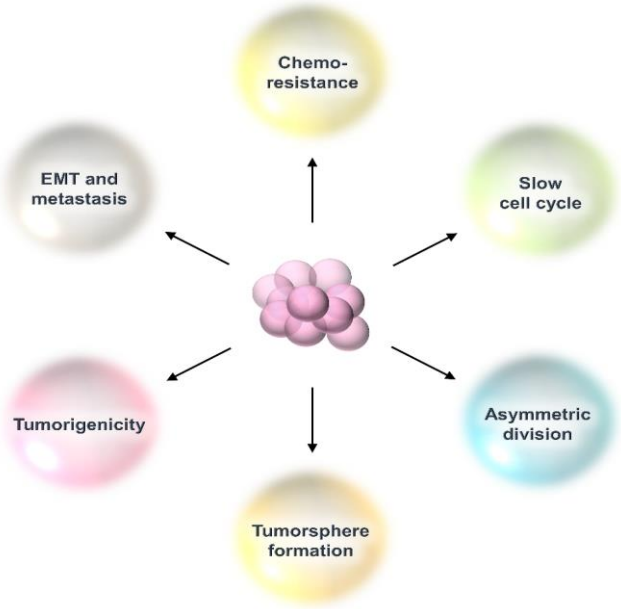

Figure 17: Properties of cancer stem cells: CSCs show characteristics resembling those of typical stem cells. They are dormant cells that divide slowly. They have the capacity for self-renewal, thereby maintaining their population of undifferentiated cells. They divide asymmetrically to create daughter cells that undergo differentiation. This kind of cell division allows them to maintain their own pool while also producing the bulk of the tumor. They are immortal cells because they can withstand chemotherapy or radiation treatment. Following that, these therapy-resistant cells display all of EMT's characteristics and a heightened ability for metastasis. They can grow in poor adherence cell culture plates under *in-vitro* conditions to form tumorspheres. ***Ref: Guha et.al. Cancer stem cell–immune cell crosstalk in breast tumor microenvironment: a determinant of therapeutic facet. Frontiers in Immunology***

Breast cancer continues to be the leading cause of cancer related mortalities amongst women globally, despite advances in diagnosis and treatment. This largely is due to the presence of breast CSCs, which like the other CSCs are treatment resistant, tumorigenic and metastatic. There are several theories regarding the origin of BCSCs. One theory supports that BCSCs arise from the dedifferentiation of non-stem cells (mammary epithelial cells). Genetic and epigenetic alterations as well as changes within the tumor microenvironment (TME) contribute to the dedifferentiation of non-stem cells to the BCSC phenotype. Another theory suggests the presence of multipotent mammary stem cells as well as unipotent luminal and basal progenitor cells within the mammary glands. Accumulation of mutation in these progenitor cells may give rise to BCSCs. Several bodies of evidence suggest that BCSCs can arise from normal stem cells by accumulating mutations [142].

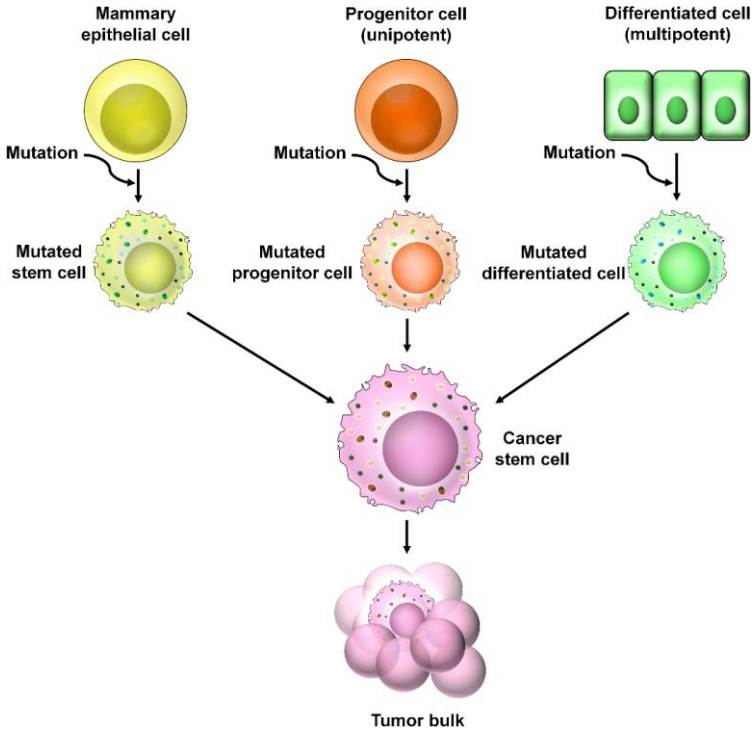

Figure 18: The origin of BCSCs. Numerous theories are prevalent regarding the origin of BCSCs. According to one theory, genetic and epigenetic alternations of non-stem cells within the TME cause the dedifferentiation of these cells into CSCs. A second theory suggests the presence of unipotent progenitor cells which accumulate mutations over time to give rise to CSCs. A third theory predicts that CSCs arise from multipotent mammary stem cells that have undergone mutational changes. ***Ref: Guha et.al. Cancer stem cell–immune cell crosstalk in breast tumor microenvironment: a determinant of therapeutic facet. Frontiers in Immunology***

BCSCs are characterized based on the expression of several markers derived from breast cancer cell lines, transgenic mouse models and patient-derived tumors. Among these, the most commonly used markers are $CD44^{+}/CD24^{-}$ and alcohol dehydrogenase 1 ($ALDH1^{+}$). ALDH belongs to the family of NAD(P)+-dependent enzymes which are involved in the detoxification of a wide variety of aldehydes to their corresponding carboxylic acids. It mainly functions in converting vitamin A (retinol) to retinoic acid. It maintains the characteristics of cancer stem cells including drug resistance, thereby contributing to disease relapse [142]. $CD44^{+}/CD24^{-}$ and $ALDH1^{+}$ represent two distinct subpopulations of BCSCs which are different from one another. These two states are highly dynamic and interchangeable. Their number varies among different subtypes of BC. Among all subtypes, luminal-A BC has the lowest proportion of BCSCs which

contributes to its best prognosis. Luminal B, however, has higher proportions than luminal-A but lesser than TNBC or $HER2^+$ breast cancers. The $HER2^+$BC is characterized by the presence of $ALDH1^+$ epithelial BCSCs leading to its poor prognosis. TNBC is the most heterogeneous subtype and is characterized by the presence of the highest population of BCSCs. Claudin-low TNBC has higher proportion of mesenchymal BCSCs ($CD44^+/CD24^-$), whereas the basal-like TNBC has higher proportions of $ALDH1^+$ epithelial BCSCs and also certain amounts of mesenchymal BCSCs [142].

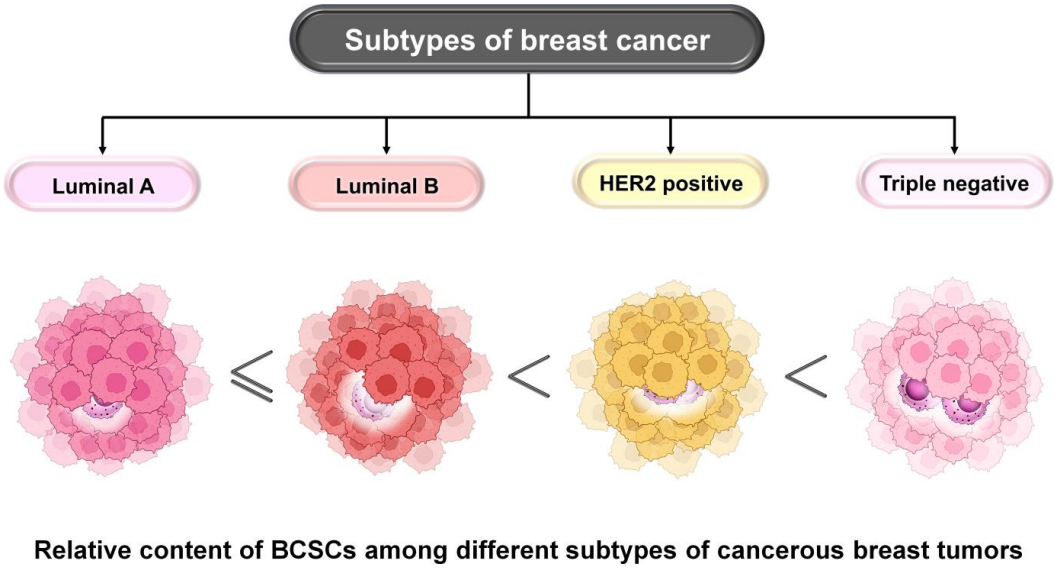

Figure 19: Relative content of BCSCs among different subtypes of BC. The proportion of BCSCs varies among different subtypes of BC and this correlates with their prognosis. Luminal-A has the lowest proportion followed by luminal B, $HER2^+$and TNBC subtypes of BC. ***Ref: Guha et.al. Cancer stem cell–immune cell crosstalk in breast tumor microenvironment: a determinant of therapeutic facet. Frontiers in Immunology***

Growing data suggests that CSCs have a role in metastasis development and colonisation. Apart from its role in CSC production, EMT could endow differentiated tumor cells with the capacity for self-renewal, hence facilitating the creation of secondary tumors consisting of heterogeneous cancer cells in remote locations. In addition to proliferating through symmetric divisions, CSCs can also self-renew by asymmetric cell division, which adds to the diversity of cancer cells. Adult stem cells may use similar biological mechanisms to facilitate tissue regeneration as a means of facilitating cancer spread through EMT.

When the EMT-produced migratory CSCs reach distant tissues, they might develop into secondary tumours that even show epithelial phenotype through MET when they reach distant areas. The creation of secondary metastatic nodules is also thought to be facilitated by MET, the opposite of EMT that is seen during embryonic development. Mani and colleagues, provided what appears to be the first evidence that EMT causes the emergence of breast cancer cells with stem cell-like traits. They showed that when differentiated HMLE (human mammary epithelial cells) cells were exposed to TGF-β1 or SNAIL or TWIST overexpression, EMT was induced, resulting in the acquisition of the $CD44^{+}/CD24^{-}$ stem cell profile. Another independent group supported this finding by demonstrating that TGFβ therapy increased the amount of stem cells in mammary epithelial cells as determined by their cell surface antigenic profiles, capacity to form mammospheres in culture and ductal outgrowths in xenotransplant tests. Furthermore, it was demonstrated that transfection with the two EMT inducers, SNAIL and SNAIL2, in ovarian cancer resulted in the de-repression of stemness genes, such as Nanog and KLF4 and up to five times a higher percentage of $CD44^{high}/CD117^{high}$ CSCs.

This provides more evidence that the induction of EMT in cancer cells that have undergone more differentiation can produce CSC-like cells [142] [143].

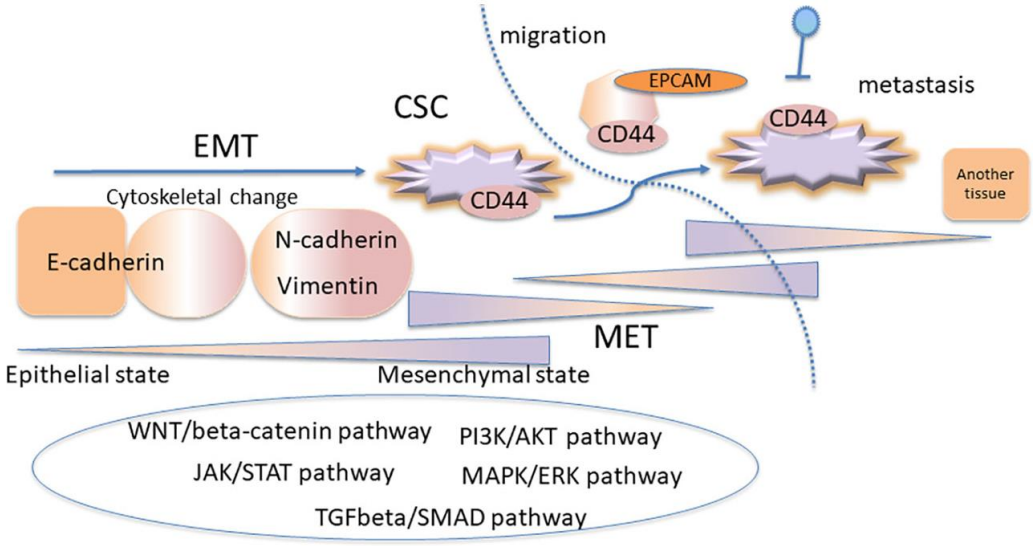

Figure 20: EMT-MET-CSC crosstalk in disease progression: EMT bestows tumor cells with mesenchymal like traits and at the same confers stem cell like features enabling them to effectively

generate secondary tumor by MET post invasion and metastasis. ***Ref: Filip S et.al. Distant metastasis in colorectal cancer patients do we have new predicting clinicopathological and molecular biomarkers? A comprehensive review. International journal of molecular sciences.***

1.13 Clinical significance of EMT and MET

EMT is the key mechanism through which cancer cells become invasive and migratory, allowing the cancer cells to spread. As a result of EMT process, epithelial cells exhibit enhanced motility, decreased intercellular adhesion and fibroblast-like characteristics. Tumor growth is correlated with the production of proteins specific to mesenchymal cells and the loss of epithelial markers and the EMT process releases single cells in conjunction with invasion. Numerous studies have noted a loss of epithelial traits together with an increase in mesenchymal markers in the invasive front of different malignancies, suggesting that EMT may play a role in the development of an invasive phenotype that results in metastasis. According to recent studies, EMT can result in the development of cancer cells that have traits similar to those of cancer stem cells, such as the ability to self-renew and start new tumors, escape from immune surveillance, increased resistance to apoptosis and diminished senescence. These traits also cause cancer cells to become resistant to therapy. For instance, it was discovered that the lung carcinoma cell line A549 and ovarian carcinoma cells both exhibited chemoresistance in response to TWIST or SNAIL-induced EMT [144] [145].

Additionally, pancreatic tumor cells resistant to gemcitabine showed phenotypic alterations linked to epithelial-mesenchymal transition and developed stem cell-like traits. In MCF-7 cells, EGFR-induced EMT has also been connected to tamoxifen resistance. Interestingly, EMT has also been demonstrated to induce drug resistance in pancreatic cells and that drug sensitivity was restored upon reversing EMT through ZEB-1 silencing [146] [147] [148].

It was discovered that endometrial cancer cells resistant to radiation had a mesenchymal phenotype, including reduced expression of E-CADHERIN, so supporting the link between therapy resistance and EMT. The development of radio-resistance and chemo-resistance in ovarian cancer cells is also linked to the upregulation of SNAIL and SLUG in these cells [149]. Moreover, it has been suggested that the EMT process is linked to resistance to targeted therapy, which may allow it to avoid dependence on this pathway by activating its downstream

targets. Furthermore, carcinoma cells capacity to endure the transition from primary tumors to sites of dissemination should be dependent on their inherent resistance to apoptosis.

These selective benefits might even make cancer cells immune to traditional treatments, allowing them to spread and survive for a long time at remote locations. It is possible that many patients relapse and their tumors grow resistant to additional therapies because of the presence of therapeutically resistant CSCs, possibly as a result of the EMT process. These results offer strong evidence in favour of MET's involvement in dissemination sites. The more differentiated epithelial cells that are often the focus of classical chemotherapy and endocrine therapy may produce a significant proportional increase in tumor cells with stem/progenitor characteristics.

1.14 Angiogenesis

Normal physiological functioning of the body including growth, wound healing, embryonic development etc. all are significantly impacted by blood vessels. Tumor cells require neovascularisation to transfer nutrients and eliminate metabolic waste, which is a crucial mechanism for tumor growth and spread [150] [151] [152]. The process of angiogenesis involves the formation of new blood vessels from pre-existing ones, enabling the delivery of nutrients and oxygen to the body's tissues. Mammalian cells are usually found within 100 to 200 μm of blood vessels, which is the oxygen diffusion limit. Neovascularisation is thus utmost necessary for tumor growth larger than 1-2 mm in diameter, according to several studies [150] [151] [152]. Judah Folkman first proposed the crucial function of angiogenesis in tumor progression in 1971. He characterised tumors as "hot and bloody.

Angiogenesis in tumor can occur in numerous ways. Sprouting angiogenesis is the most common type in which new branches of blood vessels arise from pre-existing ones and infiltrate tumor tissues via migration of endothelial cells at the tip of the branch along with the proliferation of stem cells. Intussusceptive angiogenesis results from splitting of the double lumen into two vessels that infiltrate into tumor tissues.

The third type of angiogenesis is vasculogenesis which involves recruitment and differentiation of progenitor endothelial cells to form new blood vessels [153].

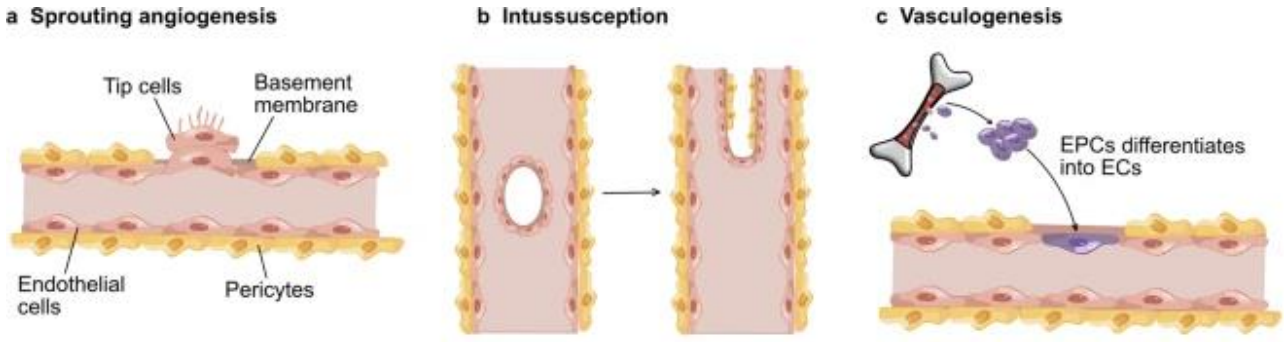

Figure 21: The major types of angiogenesis: Angiogenesis can occur in either of the three ways- Sprouting involves proliferation and migration of endothelial tip cells. Splitting and rearrangement of existing blood vessels into two occurs in intussusceptive angiogenesis. While vasculogenesis results from differentiation of progenitor endothelial cells. ***Ref: Liu ZL et.al. Angiogenic signaling pathways and anti-angiogenic therapy for cancer. Signal transduction and targeted therapy.***

The phrase "angiogenic switch" was first used in the late 1980s to describe a time-limited event during tumor progression where the ratio of pro-angiogenic (VEGF, bFGF, EFGF, PDGF, MMPs etc.) to anti-angiogenic factors (endostatin, thrombospondin, angiostatin, plasminogen activator inhibitor-1) shifts in favour of a pro-angiogenic outcome, leading to the progression of avascularized hyperplasia from dormant to outgrowing vascularised tumor and ultimately to malignant tumor [154] [155] [156].

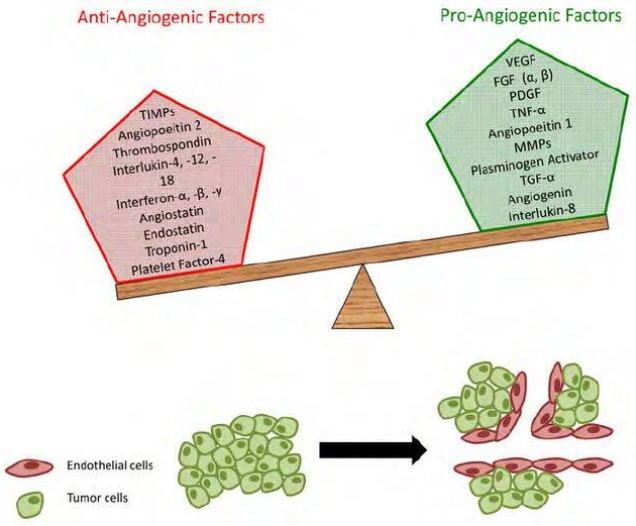

Figure 22: The angiogenic switch: The dynamic balance between pro and antiangiogenic factors regulates the process of angiogenesis. In cancer, this balance is lost and the shift towards pro-angiogenic molecules helps the rapidly proliferating tumor cells to survive by generating new blood vessels from the pre-existing ones. ***Ref: Burrell K, Zadeh G. Molecular mechanisms of tumor angiogenesis. Tumor Angiogenesis.***

In the context of breast cancer, angiogenesis is pivotal for both local tumor growth as well as distant metastasis. Additionally, clinic-pathological studies have confirmed that highly vascular fibrocystic lesions are associated with greater risk of breast cancer [157]. The rapidly proliferating tumor cells consume high oxygen and nutrients that can create an oxygen deficient milieu. This hypoxic microenvironment induces the expression of hypoxia inducing factor HIF1α. HIF1α promotes angiogenesis by transcriptional activation of downstream target molecules like VEGF, EphA1 etc. Further, HIF1α can induce EMT by promoting the expression of N-CADHERIN, VIMENTIN, SNAIL, SLUG and at the same time downregulate E-CADHERIN [158] [159].

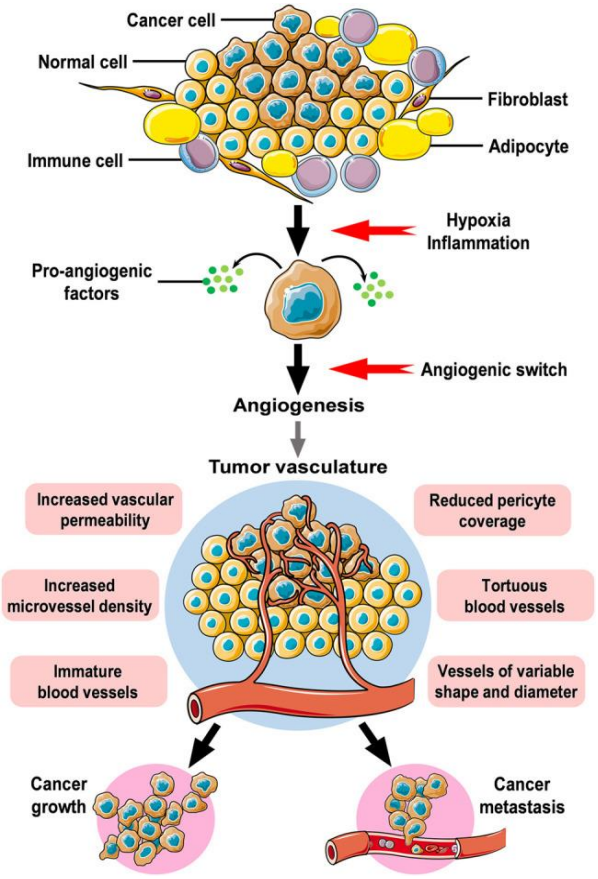

Figure 23: Importance of hypoxia in angiogenesis: The tumor cell remains surrounded by various stromal cells including fibroblasts, immune cells and adipocytes. Highly inflammatory microenvironment induces hypoxia, which in turn, stimulates the secretion of pro-angiogenic factors from the tumor cells. This disrupts the angiogenic balance leading to the formation of new blood vessels that supports the growth and metastasis of rapidly proliferating tumor cells. ***Ref: Ayoub NM et.al. Targeting angiogenesis in breast cancer: current evidence and future perspectives of novel anti-angiogenic approaches. Frontiers in pharmacology.***

Studies at both experimental and clinical levels have demonstrated the predominance of VEGF in breast cancer. In-fact, it has been reported that overexpression of VEGF occurs frequently prior to invasion of breast cancer cells. Correlation studies have revealed that serum VEGF levels corelates positively with advancement of breast cancer [160]. In addition to VEGF, invasive human breast cancer expresses a number of other pro-angiogenic factors, such as pleiotrophin, acidic and basic FGF, placental growth factor, TGF-β1and PDGF [161]. Along with angiogenesis, several other non-angiogenic pathways of vascularization has also been reported in breast cancer. Stessels et.al., 2004; Andonegui-Elguera et.al., 2020, described vascular mimicry as an important process in vasculogenesis of breast tumors. They postulated that vascular mimicry and co-option were associated with poor prognosis and metastasis of breast tumor cells. Angiogenesis inhibitors typically impede the expression or inhibit the function of pro-angiogenic factors released by tumor cells by targeting their receptors on endothelial cells.

Although the use of angiogenesis inhibitors has shown promising results in several solid tumor types, these medications have not been able to improve survival in breast cancer. For example, a randomized phase III trial on metastatic breast cancer patients showed that addition of bevacizumab to chemotherapeutic drug capecitabine in second-line therapy improved response rate compared to capecitabine treatment alone (Miller et.al. 2005). Functionally, bevacizumab selectively binds to circulating VEGF thereby hampering its binding to VEGF receptors. However, this combination neither improved progression-free survival – PFS; nor the overall survival - OS rates. Another monoclonal antibody, Ramucirumab directed towards VEGFR2 in phase II randomized trial showed similar results like bevacizumab and capecitabine combination therapy. The addition of ramucirumab to capecitabine in previously treated patients with locally advanced and metastatic breast cancer failed to improve PFS and OS compared to capecitabine therapy alone. Moreover, the frequency of adverse effects was increased in the combination group and included headache, anorexia, constipation, epistaxis and hypertension (Vahdat et.al. 2017).

To increase the effectiveness of therapeutically accessible anti-angiogenic medications, it is crucial to comprehend the vascular biology of breast cancer at various stages and molecular kinds. The appropriate use of angiogenesis inhibitors

might also be improved by a better comprehension of the adaptive and intrinsic resistance mechanisms. The effectiveness and longevity of anti-angiogenic therapy will be enhanced by additional research on the function that stromal cells in the tumour microenvironment play in inducing resistance to anti-angiogenic medications. Another extremely crucial factor is the cohort being studied. This is because it enables the identification of breast cancer patients who would most benefit from anti-angiogenic medications. The significance of non-VEGF/VEGFR signalling pathways in the vascularization of breast cancer should also be investigated further in order to identify therapeutic targets that may be used in clinical contexts.

1.15 Emerging roles of platelets in cancer biology

Blood contains many types of cells: white blood cells (monocytes, lymphocytes, neutrophils, eosinophils, basophils and macrophages), red blood cells (erythrocytes) and platelets.

Nearly one trillion blood platelets, which have an average life span of only 8 to 10 days, are in circulation in adult humans [162] [163]. One of the main roles of platelets is to act as the "adhesive-aids" of the circulation, aggregating to create a platelet clot, altering shape and secreting their granular contents in response to blood vessel damage. Additionally, platelets have ancillary functions in the regulation of innate immunity and angiogenesis.

About 100 billion new platelets must be created every day from bone marrow megakaryocytes in order to maintain platelet counts of 150–400 $x10^9$ platelets per litre of whole blood [164] [165] [166] [167].

1.16 Ultrastructure of platelets

Platelets are small (2–3µm), anucleated, disc-shaped fragments of which two-thirds are present in the general circulation with the remaining third reversibly sequestered in the spleen.

Several distinctive structural features of platelets include a defined plasma membrane, surface membrane invaginations that form the spectrin-based membrane skeleton, the actin-based cytoskeletal network, the peripheral band of microtubules, the open canalicular system (OCS), the dense tubular system (DTS), the closed-channel network of residual endoplasmic reticulum and various organelles, such as α-granules, dense-granules, peroxisomes, lysosomes and mitochondria [168] [169] [170] [171]. Platelet mitochondria play a crucial role in various platelet functions.

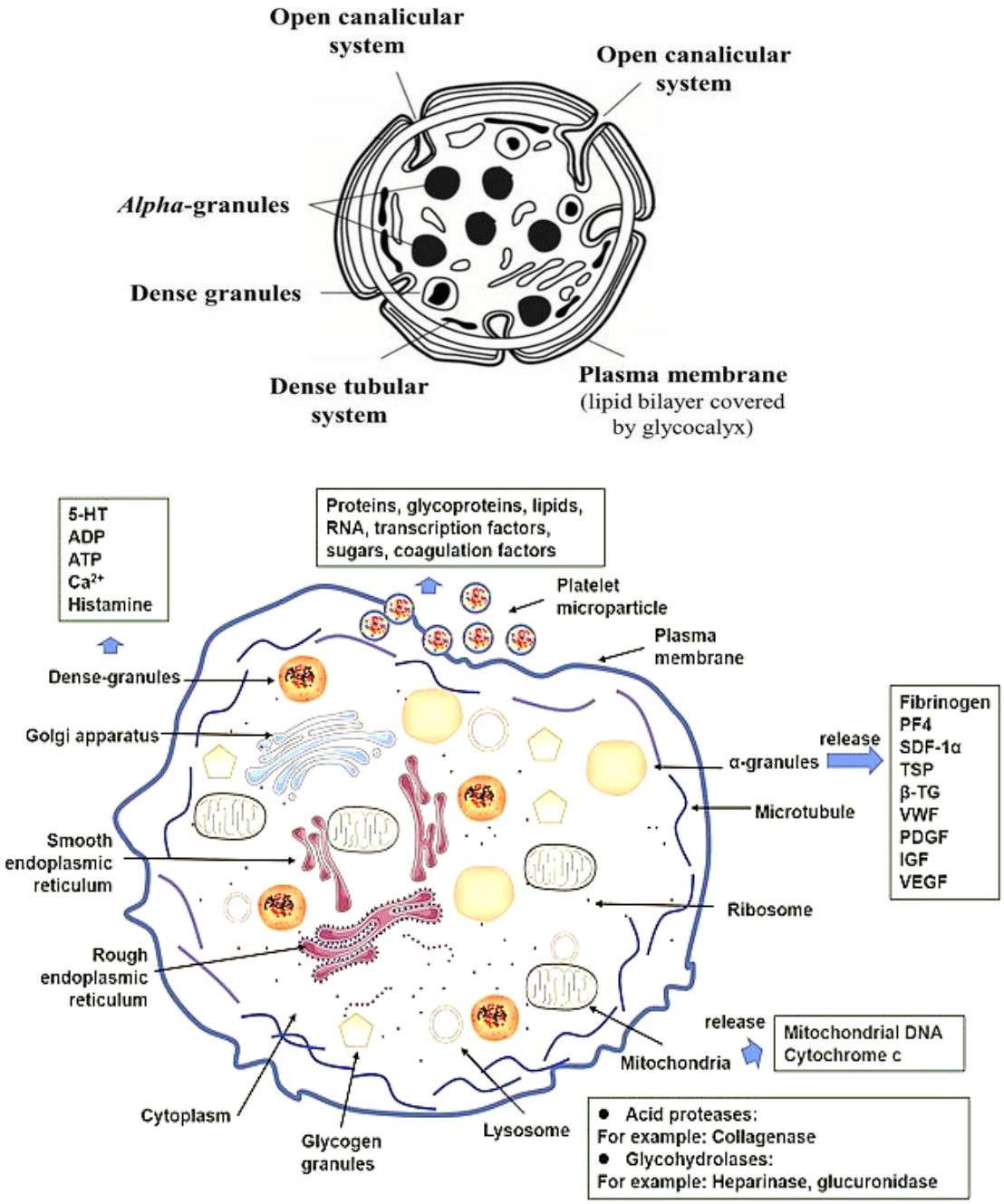

Figure 24: Ultrastructure of platelets: Platelets ultrastructure includes a defined plasma membrane, spectrin-based membrane skeleton, the peripheral band of microtubules, OCS, DTS, endoplasmic reticulum and various organelles, such as α-granules, dense-granules, peroxisomes, lysosomes and mitochondria. ***Ref: Ma Y et.al. Platelet mitochondria, a potent immune mediator in neurological diseases. Frontiers in Physiology.***

1.17 Biogenesis of platelets

Megakaryocytes are progenitor cells found in the bone marrow that generate platelets and release them into the bloodstream. Thousands of platelets are released from a single megakaryocyte during the complex series of remodelling activities required for megakaryocytes to produce platelets. Deviations from this procedure may lead to clinically serious illnesses. Platelet counts below 150,000/µl, known as thrombocytopenia, can result in insufficient clot formation and a higher risk of bleeding, whereas platelet counts above 600,000/µl, known as thrombocythemia, can elevate the risk of thrombotic events, such as myocardial infarction, stroke and peripheral ischemia [172]. Thrombopoietin (TPO), the primary regulator of thrombopoiesis, is currently the only known cytokine required for megakaryocytes to maintain a constant platelet mass. TPO is thought to act in conjunction with other factors, including IL-3, IL-6 and IL-11, although these cytokines are not essential for megakaryocyte maturation [172]. Megakaryocytes tailor their cytoplasm and membrane systems for platelet biogenesis. Before megakaryocyte has the capacity to release platelets, it enlarges considerably to an approximate diameter of 100 µm and fills with high concentrations of ribosomes that facilitate the production of platelet-specific proteins. Cellular enlargement is mediated by multiple rounds of endomitosis, a process that amplifies the DNA by as much as 64-fold [172] [173] [174]. TPO, which binds to the c-Mpl receptor, promotes megakaryocyte endomitosis. c-Mpl also known as thrombopoietin receptor belongs to the type I cytokine receptor family and was discovered as an oncogene of the murine myeloproliferative leukaemia virus. It is mostly expressed in the megakaryocyte lineage, which includes progenitor cells and platelets, as well as in haematopoietic tissues, including certain pluripotent haematopoietic stem cells. By coupling to its ligand, thrombopoietin (TPO), c-Mpl executes it signalling function of regulating platelet formation and differentiating megakaryocytes. Severe haematopoietic disorders are driven by aberrant TPO signalling. For instance, profound thrombocytopenia results from TPO or c-Mpl mutations that inactivate the signalling cascade. Whereas gain of functional mutation of either of them leads to thrombocythemia [172] [175]. During endomitosis, chromosomes replicate and the nuclear envelope breaks down. Although interconnected mitotic spindles assemble, the normal mitotic cycle is arrested during anaphase B. The spindles

fail to separate and both telophase and cytokinesis are bypassed. Nuclear envelope reformation results in a polyploid, multilobed nucleus with DNA contents ranging from 4N up to 128N. In addition to expansion of DNA, megakaryocytes experience significant maturation as internal membrane systems, granules and organelles are assembled in bulk during their development. In particular, there is the formation of an expansive and interconnected membranous network of cisternae and tubules, called the demarcation membrane system (DMS), which was originally thought to divide the megakaryocyte cytoplasm into small fields where individual platelets would assemble and subsequently release. DMS membranes have continuity with the plasma membrane and are now thought to function primarily as a membrane reservoir for the formation of proplatelets, the precursors of platelets. A dense tubular network and the open canalicular system, a channelled system for granule release, are also formed before the assembly of proplatelets begins. Specific proteins associated with platelets, such as vWF and fibrinogen receptors, are synthesized and sent to the megakaryocyte surface, while others are packaged into secretory granules with such factors as vWF, which is loaded into α-granules. Still other proteins, such as fibrinogen, are collected from plasma through endocytosis and/or pinocytosis by megakaryocytes and are selectively placed in platelet-specific granules. Also assembled during megakaryocyte maturation are mitochondria and dense granules, which, like α-granules, derive from Golgi complexes. Thus, as terminally differentiated megakaryocytes complete maturation, they are fully equipped with the elements and machinery required for the major task of platelet biogenesis [172] [173] [174].

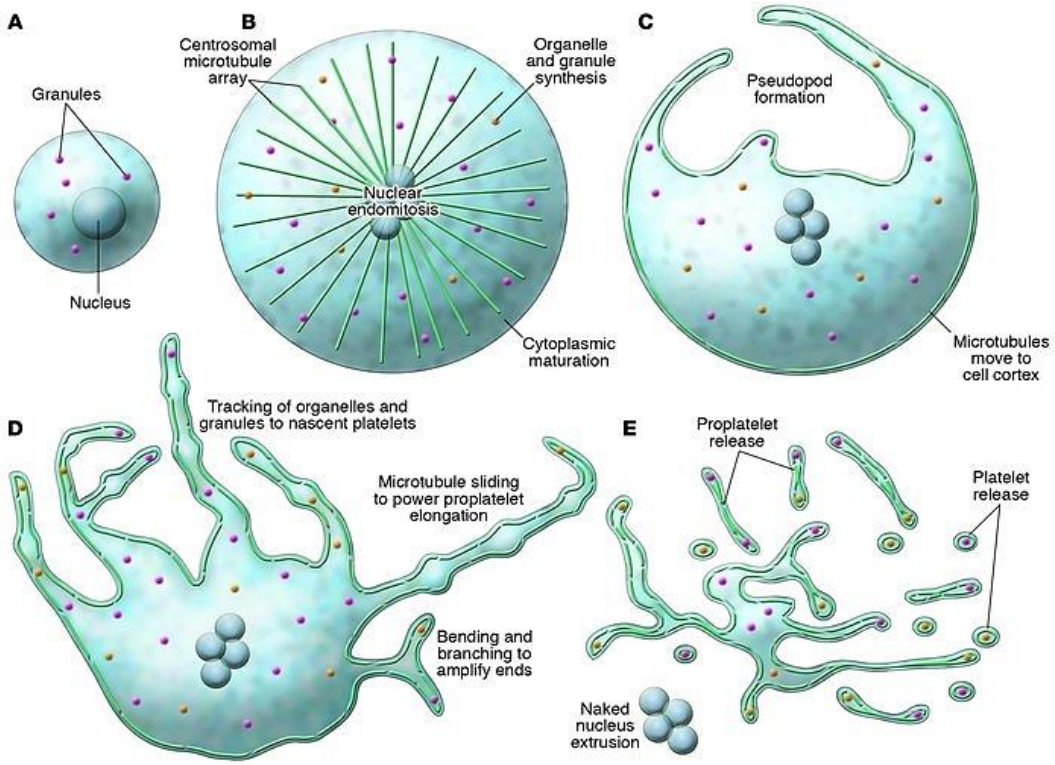

Figure 25: Biogenesis of platelets: As megakaryocytes transit from immature cells (A) to released platelets (E), a systematic series of events occurs. (B) The cells first undergo nuclear endomitosis, organelle synthesis and dramatic cytoplasmic maturation and expansion, while a microtubule array, emanating from centrosomes, is established. (C) Prior to the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. Proplatelet formation commences with the development of thick pseudopods. (D) Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends, where nascent platelets assemble. Proplatelet formation continues to expand throughout the cell while bending and branching amplify existing proplatelet ends. (E) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. The nucleus is eventually extruded from the mass of proplatelets and individual platelets are released from proplatelet ends. ***Ref: Patel SR et.al. The biogenesis of platelets from megakaryocyte proplatelets. The Journal of clinical investigation.***

1.18 Evolution of platelets – from haemostasis to cancer….

For an extensive period of time, the physiological role of platelets was restricted to thrombus formation and maintaining haemostasis in the body. Platelets were initially observed in 1865 by M.Schultz, however, Bizzozero in 1882, first characterized them. Following this, substantial work conducted by William Osler, James Wright and William Duke described the fundamental role of platelets in haemostasis. It took a century of research to decipher the importance of platelets in malignancy. In 1968, after 100 years of their discovery, Gasic et al., first

described the association between platelet number and metastatic cancer potential in the 19th century. Since then, numerous studies have been conducted to elaborate the functional role of platelets in cancer.

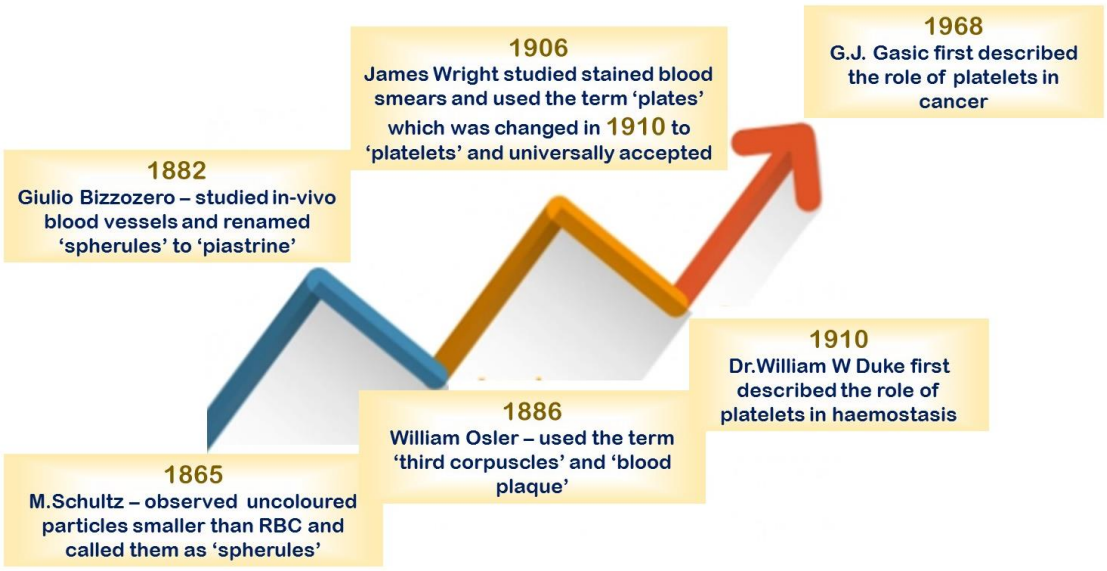

Figure 26: Timescale of functional evolution of platelets: Since their first discovery in 1865 extensive research work has been carried out by numerous physicians and scientists to elaborate their physiological function in haemostasis. After almost a century, the potential role of platelets in cancer was described by G.J. Gasic and team. Numerous have been conducted further to decipher in details the role of thrombocytes in malignant progression.

Elevated platelet count, which are often as high as 3–4 trillion in an individual cancer patient, has been associated with tumor spread and poor prognosis. Tumor cells can aggregate and activate platelets, leading to the initiation of a thrombus through the process known as tumor cell-induced platelet aggregation (TCIPA). TCIPA is correlated with tumor's propensity to produce thrombosis as well as their capacity for metastasis. Platelet-tumor cell interactions and the signalling pathways that these interactions can stimulate have been identified as fundamental determinants of cancer metastasis. Platelets protect the metastatic tumor cells from immunosurveillance process [176] [177].

One way is that P-Selectin molecule on the surface of activated platelets interacts with PSGL1 expressed by tumor cells. This allows platelets to completely enclose the tumor cells, shielding them from attack by the various immune cells like the NK cells in circulation. Further, as a result of this interaction, platelets become activated by

the tumor cells and are termed as ‘tumor educated platelets’ or ‘TEPs’ [178] [179]. TEPs differ significantly from normal counterpart producing numerous filopodial processes that facilitates sequestration and protection of the tumor cells. They are a repository of various biologically active molecules that influence tumor progression by modulating EMT, metastasis and angiogenesis [176] [180].

1.19 Platelets in EMT

The prerequisite of metastasis is that tumor cells must survive in the circulation. This is ensured by the dynamic crosstalk between platelets and tumor cells. Platelet-tumor cell interaction is also strongly associated to an array of events in the tumour metastasis cascade, including neoangiogenesis, adherence to vascular endothelium, EMT, trans-endothelial migration and immune surveillance [181] [182]. Upon their entry into the bloodstream, tumor cells initially engage with the peripheral platelets. Tumor cells exploit various mechanisms to induce platelet activation. They can release soluble platelet agonists which bind to specific platelet receptors. This adhesion activates receptors signalling pathways and eventually leads to conformational change of membrane integrin from low-affinity to high-affinity. The high-affinity integrins on activated platelets can form a firm adhesion with tumor cells, just like a "platelet cloak", which is believed to facilitate the survival and metastasis of tumor cells in circulation. Such tumor cell activated platelets are commonly termed as ‘tumor educated platelets’ or ‘TEPs’ [181].

Additionally, binding proteins (podoplanin, PDPN) that bind to platelet surface adhesion proteins (C-type lectin-like receptor 2, CLEC-2) are expressed by tumor cells that stimulate platelet activation. Intracellular granules rapidly merge with the plasma membrane upon platelet activation. Soluble proteins are discharged into the extracellular milieu, while membrane-bound proteins from α granules are exposed to the platelet surface. The initial metastatic niche is formed by tumor cells and activated platelets and the onset of EMT is mediated by a sequence of alterations in active platelets [182] [183] [184] [185] [186].

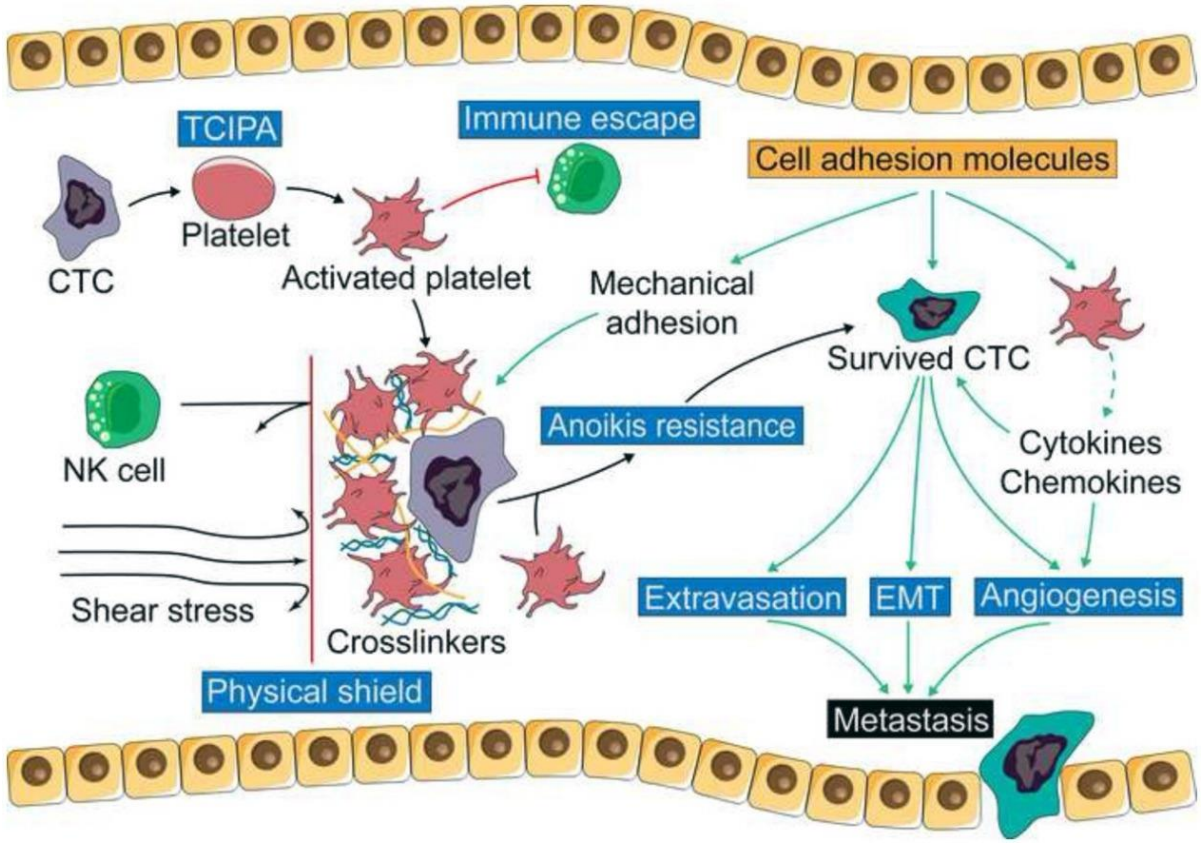

Figure 27: Interaction between platelets and tumor cells to promote EMT: Schematic illustration of the interactions between platelet and tumor cell in the circulation system to promote EMT. Various receptor ligand interactions between tumor cells and platelets converts the later from resting to activated state. Activated platelets protect the tumor cells from shear stress and promote EMT and metastasis. ***Ref: Liu Y et.al. Platelet-mediated tumor metastasis mechanism and the role of cell adhesion molecules. Critical reviews in oncology/hematology.***

Several cytokines and growth factors are released from TEPs trigger EMT by various signalling pathways that ultimately lead to the expression of EMT related transcription factors.

1. **Transforming growth factor β (TGFβ):** TGFβ1 secreted by TEPs can directly trigger EMT by via TGFβ/Smad pathway. Platelets are the main source of TGFβ1 in circulation. It has been reported that its TGFβ1 content is 40–100 times than that of other cells. Therefore, tumor cells wearing a "platelet cloak" can directly perceive platelet-derived TGFβ signal and thus response accordingly. TGFβ/Smad work to regulate SNAIL/SLUG/TWIST/ZEB and thus control EMT. Further, TGF-β crosstalk with other signalling pathways, like Notch, Wnt/β-catenin, NFκB and RTKs, induces EMT and plays critical roles in maintaining the mesenchymal phenotype

of invasive/metastatic tumor cells [187] [188].

2. **Platelet derived growth factor (PDGF):** PDGF is another critical EMT driver that contributes to cancer invasion. The PDGF family consists of four structurally related polypeptide chains that constitute five functional homo-or-heterodimers: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD. Platelet-derived PDGF can induce EMT by activating P38 /MAPK signal and up-regulating the expression of MMP2/MMP9. Moreover, PDGF-D released by tissue-resident stem cells is responsible for EMT in breast cancer cells and this effect can be neutralized by PDGF antibody. It should be noted that a variety of tumor cells can also express PDGFs and PDGFRs to function in an autocrine way. Interestingly, there is a cross-link between PDGF and TGFβ signal to promote EMT. Breast cancer cells in TGFβ-induced EMT state express an autocrine PDGF/PDGFR loop and continuous autocrine signal is involved in the maintenance of EMT state. Moreover, inhibition of PDGFR signal not only impaired EMT but also led to apoptosis [189] [190].

3. **Lysophosphatidic acid (LPA):** Platelets are a major source of LPA. Autotaxin (ATX) is a glycosylase that participates in modulating the level of LPA in plasma and has unique phospholipase D activity, which can catalyse a series of lysophospholipid precursors to generate LPA. Activated platelet release ATX and LPA and LPA levels are significantly elevated based on the further catalytic capacity of ATX. In addition to platelets, tumor and tumor stroma are also the major sources of ATX. Tumor cells in EMT state are accompanied by up-regulation of LPAR mRNA levels and increased responsiveness to LPA. Some tumor cells also produce LPA to promote invasion and platelet aggregation [191] [192].

4. **Platelet derived microparticles (PMPs):** PMPs are also released by activated platelets along with the above-mentioned molecules. While in blood circulation, PMPs promote EMT of tumor cells and enhance their adhesion abilities. The invasion ability of breast cancer cells incubated with PMPs has been reported to be significantly enhanced. It has also been observed that PMPs promote tumor metastasis and angiogenesis by stimulating cell proliferation, increasing expression of angiogenic factors and endothelial growth factors and enhancing adhesion to endothelial cells [193] [194].

1.20 Platelets in angiogenesis

For the metastatic spread of cancer tissue, growth of the vascular network is

important. The processes whereby new blood and lymphatic vessels form are called angiogenesis and lymph-angiogenesis, respectively. Both have an essential role in the formation of a new vascular network to supply nutrients, oxygen and immune cells and also to remove waste products. Angiogenesis is a multi-step process involving endothelial cells, including initiation, migration, tube formation and differentiation (maturation). The formation of novel blood vessels happens through several consecutive steps [195]:

1. Angiogenic factors production in ECs creates the wall of an existing small blood vessel (capillary) and the release of these factors.

2. Binding of these factors to ECs surface receptors.

3. ECs activation, secretion of enzymes [Matrix metalloproteinases (MMP)] that reduce and destroy the extracellular matrix (the surrounding tissue), invasion of the matrix, division, ECs proliferation.

4. Strings of new ECs organize into hollow tubes creating new networks of blood vessels.

The hypothesis that platelets are involved in the process of angiogenesis was raised almost 20 years ago by Pinedo et al. The presence of activated platelets was observed in the tumor vasculature in sarcoma patients. Platelets stimulate EC proliferation and tube formation in vitro and induce angiogenesis in vivo, which is dependent on platelet adherence to the differentiating ECs through their surface adhesion molecules. Activated platelets induce TF expression on ECs by interaction between platelet CD154 and CD40 present on ECs to induce coagulation. Ligation of CD40 stimulates expression of adhesion molecules, e.g., E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) that enhances adhesion of inflammatory cells to the ECs [196] [197] [198].

Platelet α-granules contain, as mentioned above, proangiogenic factors like VEGF, PDGF, TGFβ1, Serotonin, IGF1, bFGF, EGF etc as well as antiangiogenic factors TIMPs, PAI, TSP1, Endostatin, Angiostatin etc. Also, platelet progenitor cells synthesize and release VEGF, while platelets transport and, upon activation, secrete VEGF, which is the most important proangiogenic molecule. In breast cancer patient's inflammation promotes secretion of VEGF from platelets, which is the most important proangiogenic molecule. Moreover, the platelet function is altered in cancer patients, as platelets from women with early breast cancer released

significantly more VEGF upon thrombin or TF stimulation than platelets derived from healthy controls. In breast, colorectal, renal and ovarian cancer patients, the platelet count correlated significantly with serum VEGF content. Furthermore, the concentration of platelet-derived VEGF is a better predictor of tumor progression than serum VEGF levels [199] [200] [201] [202] [203].

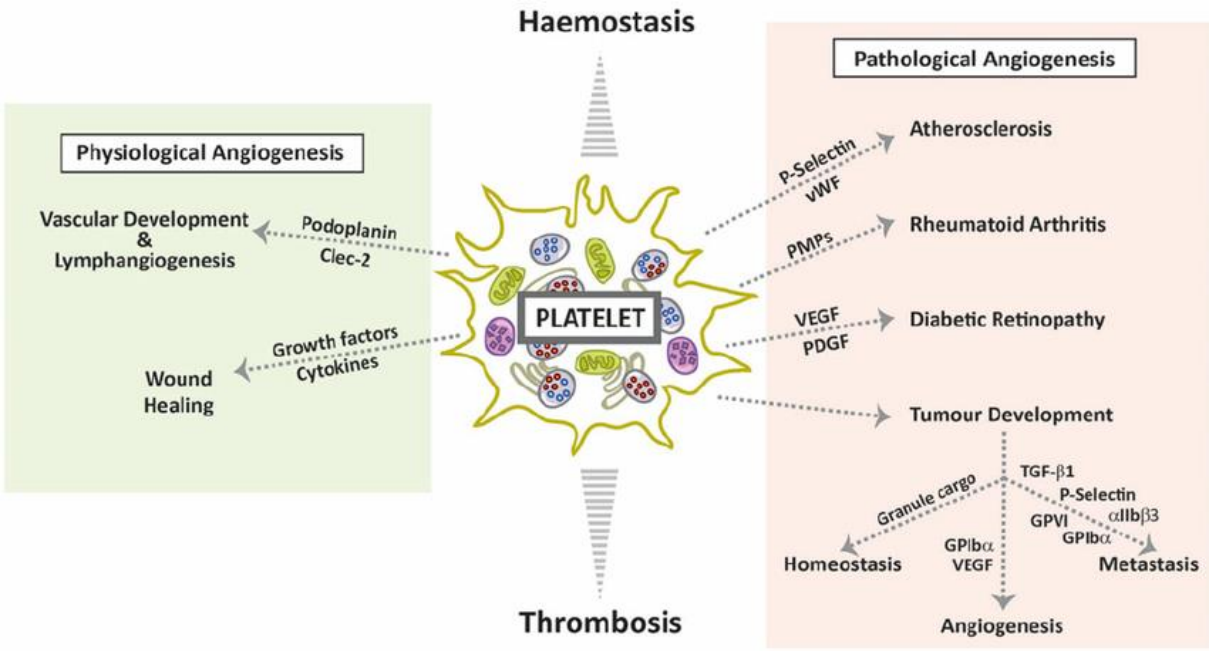

Figure 28: Role of platelets in angiogenesis: Platelets release various cytokines and growth factors which are essential in inducing angiogenesis. Angiogenesis ensures steady supply of oxygen and nutrients to the rapidly proliferating tumor cells. ***Ref: Walsh TG et.al. The functional role of platelets in the regulation of angiogenesis. Platelets.***

1.21 Link between EMT-Platelet-Cancer Stem Cells in malignancy

As previously described, cancer stem cells (CSCs) are a rare population of undifferentiated cells that reside within the tumor and exhibit properties similar to normal stem cells and are referred to as tumor-initiating cells. They possess the capacity for self-renewal, which refers to a cell's ability to divide endlessly in an undifferentiated condition. They replicate seldom or slowly and have infinite potential for proliferation. They can divide asymmetrically to produce daughter cells with the capacity to differentiate. Clinically, CSCs are responsible for treatment resistance and cancer relapse because of their relative resistance to

radiation, chemotherapy and molecular targeted therapy. These residual cells after therapy have all the hallmarks of epithelial to mesenchymal transition (EMT) with increased metastasis capacity. Also, CSCs are capable of proliferating in low adherence cell culture conditions in the presence of growth factors like epidermal growth factor and basic fibroblast growth factor to generate floating spheroids called tumorspheres. The number of spheroids represents the number of BCSCs, while their size depicts their proliferative capacity.

Recent studies on cancer stem cells have revealed that these cells are more efficient in activating platelets than the tumor cells themselves. In a study involving the mice breast cancer cell line 4T1, it was observed that spheroids obtained by growing 4T1 cells in serum-free media and low adherence condition were more efficient in activating platelets than the 4T1 cells themselves. These spheroids were more efficiently coated by platelets than by the 4T1 cells [204]. The interaction between these platelets and cancer stem cells causes the release of TGF-β1 from the α-granules of platelets. TGFβ1/Smad work to regulate SNAIL/SLUG/TWIST/ZEB and thus control EMT. Further, TGF-β crosstalk with other signalling pathways, like Notch, Wnt/β-Catenin, NFκB and RTKs, induces EMT and plays critical roles in maintaining the mesenchymal phenotype of invasive CSCs [205].

Another study on glioma patients demonstrated that glioma stem cells effectively activate platelets to promote glioblastoma tumorigenesis. Furthermore, platelets promoted the growth and stemness of glioma stem cells by elevating the expression of stemness markers OCT4 and NANOG. Also, platelets influenced CSCs were highly proliferative and produced tumorspheres which were greater in size than untreated glioma stem cells. Interesting, these stem cells have been shown to produce an important platelet activator – thrombin. Thrombin can potentially activate platelets causing them to undergo morphological changes and form aggregates. At the same time, it also causes degranulation of their granules. Such activated platelets function further to promote stemness of glioma stem cells along with *in-vivo* tumorigenesis. As a result, a novel bidirectional interaction occurs between platelets and glioma stem cells, whereby platelets increase the ability of these cells to self-renew whereas, glioma stem cells create and release thrombin to increase platelet activation. This relationship serves as a positive feedback loop for the tumorigenic effects of platelets on glioblastoma [206]. These

studies thus substantiate the importance of targeting platelets by anti-platelet medications like aspirin, statins along with various small molecules inhibitors that will function to disrupt the bridge between EMT and CSCs which in turn will benefit patients.

1.22 Anti -platelet therapies in cancer

A wide range of evidence suggest that platelets, one of the many haemostasis components, contribute significantly to the development of cancer by acting as immune cells and supplying surface and granular contents for various interactions. As a result, for many years, anti-platelet therapy's ability to treat cancer has been thoroughly studied. Anti-platelet medications have the potential to reduce tumour growth, enhance patient survival and prevent cancer. However, there are indications that antiplatelet medication may accelerate the formation of solid tumours, a condition known as "cancers follow bleeding." The debates surrounding antiplatelet medications warrant more research to determine the role of platelet-directed therapy if any in the overall framework of anticancer care [207] [208] [209].

In breast carcinoma, aspirin has been reported to significantly inhibit metastasis and is also pivotal in neoadjuvant chemotherapy (Wang & Huang, 2020). According to clinical trials, aspirin can prevent platelet aggregation, lessen the development of small tumor thrombi in blood vessels and lower the chance of metastasis. (Tao et.al., 2021). According to an article published in Nature India on 29th October, 2020 by Bhattacharya A. et. al. revealed that 'Aspirin, when used in combination with the anticancer drug doxorubicin, can help shrink drug-resistant invasive breast tumors by inhibiting the growth of cancer stem cells.' Aspirin treatment improved the anticancer therapeutic effect by inhibiting the breast cancer cells drug-resistance mechanism. The study demonstrated that Scaffold/matrix associated region-binding protein 1 (SMAR1) inhibits the production of a key protein that contributes to the development of drug-resistant breast cancer cells. OCT4 and SOX2 are two proteins that cancer stem cells use to inhibit SMAR1, which contributes to the unchecked proliferation of cancer cells. Aspirin treatment suppresses the expression and levels of OCT4 and SOX2. This, in turn, restores the activity of SMAR1, silencing the action of the drug-resistant protein and thus making the cancer cells sensitive to doxorubicin treatment [210].

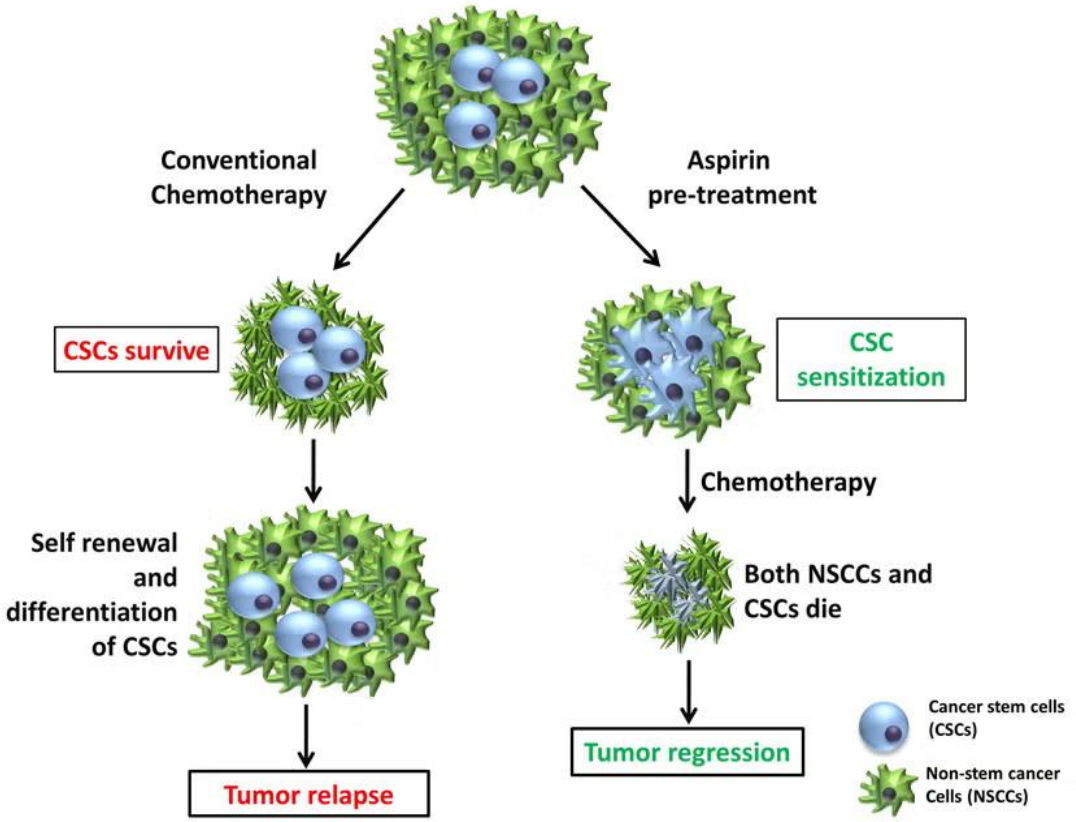

Figure 29: Aspirin therapy to target cancer stem cells: Pre-treatment with aspirin prior to chemotherapy sensitizes the non-targetable CSCs. This results in better response towards chemotherapeutic drugs like doxorubicin leading to apoptosis of both the CSCs and non-stem cancer cells. ***Ref: Bhattacharya A et.al. SMAR1 repression by pluripotency factors and consequent chemoresistance in breast cancer stem-like cells is reversed by aspirin. Science Signalling.***

Aspirin is also a majorly used anti-platelet agent. For over 50 years aspirin has been the choice of drug for cardiovascular treatment. However, the balance between risk and benefit remains a point of concern for majority of the physicians. Low-dose aspirin exerts anti-metastatic and anti-proliferative effects by targeting platelet cyclooxygenases (COX-1, COX-2) via acylation of serine 530, preventing platelet activation over the remaining lifetime of the affected platelets. Consistent with the potential role of platelets in various stages of cancer progression, inhibition of platelet reactivity by aspirin attenuates: platelet-mediated proliferation of tumor cells, adhesion of tumor cells to the endothelium, aggregation of platelets on tumor cells, invasion of tumor cells and translocation and EMT of tumor cells [211] [212] [213] [214] [215].

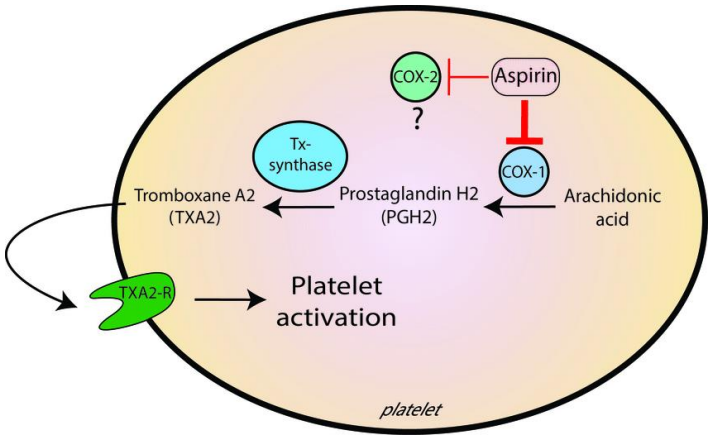

Figure 30: Mechanism of antiplatelet activity of aspirin: Aspirin blocks the activity of COX-1 and COX-2 that prevents the conversion of arachidonic acid to prostaglandin H2 and further to thromboxane A2 that prevents platelet activation. ***Ref: Russo I et.al. Platelets, diabetes and myocardial ischemia/reperfusion injury. Cardiovascular diabetology.***

The table below encapsulates the observations from various *in-vivo* and *in-vitro* studies that have demonstrated aspirin's role in cancer prevention.

***In vitro* and *in vivo* studies**	**Effect**
Zhao 2013 [216]	Platelet apoptosis *via* caspase-3 activation
Ding 2014 and Ding 2017, MM1.S and RPMI-8226 myeloma cell lines [217]	Activation of caspases, upregulation of Baxand downregulation of Bcl-2 and VEGF, Potentiation of inhibitory effect of bortezomib
Cook 2015, SK-OV-3 ovarian cancer cells [218]	Inhibition of epithelial-to-mesenchymal transition phenotype
Mitrugno 2017, SW480 colon andPANC-1 cancer cells [219]	Inhibition of oncoprotein c-MYC expression and reduced proliferative potential of cancer cells
Vad 2014, B16-F0 melanoma cells and skin B16-F0 melanoma tumor mouse model [220]	Increase in reactive oxygen species (ROS) formation, Inhibition of tumor growth
Guillem-Llobat 2016, HT29 human colon carcinoma cells [221]	Inhibition of epithelial-to-mesenchymal transition phenotype reduced metastases rate
Sitia 2012, Hepatocellular carcinoma [222]	Reduction of immune-mediated pathological effects in the liver and tumorigenesis
Ogawa 2014, Lewis lung carcinoma cells in mice model [223]	Reduction of mediastinal lymph node metastasis
Etulain 2013 [224]	Inhibition of pro-angiogenic activity of the platelets

Table 3: Therapeutic role of aspirin in cancer: Apart from functioning as an anti-thrombotic drug, aspirin has prominent anti-malignant activities that have been proved by several *in-vitro* and *in-vivo* studies in different types of cancer.

Along with aspirin, several other drugs are also widely used to target platelets in order to address tumor progression.

Receptor	Inhibitor, drug	In vitro effect	Animal models
ADP receptor P2Y12	SR 25989, cangrelor, clopidogrel, ticlopidine, ticagrelor	Inhibition of platelets aggregation, adhesion and complexes with tumor cells and suppression of angiogenesis	Reduction of lung, liver and bone marrow metastasis
GP IIb/IIIa	Tirofiban, eptifibatide, abciximab Rhodostomin A11 hGAG Inhibitor of PSI domain	Inhibition of platelets aggregation, adhesion, angiogenesis Reduction of TCIPA Inhibition of the adhesion, migration and invasion of tumor cells in bone extracellular matrice Platelet lysis within tumor environment Inhibition of platelet/tumor cells complexes Inhibition of platelet aggregation	Inhibition of metastasis on Lewis lung carcinoma mouse model
GP1b/IX/V, GPVI	Monovalent Fab fragments	Reduction of TCIPA	Inhibition of metastases
P-selectin	Heparin, dermatan sulfates	Inhibition of interaction between platelet and tumor cells	Inhibition of metastases
CD 151 [Huang 2016]	CD 151-directed antibody	Inhibition of angiogenesis	–
PAR-1, PAR-4	RWJ- -58,259, SCH-79797 or RWJ-56110 siRNA	Inhibition of interaction between platelet and tumor cells, inhibition of angiogenesis	Inhibition of tumor growth, weight, and metastasis
CLEC-2 and podoplanin	2A2B10, NZ-1	Inhibition of TCIPA	Inhibition of pulmonary metastasis

Table 4: Role of antiplatelet agents in cancer: List of several drugs and inhibitors directed towards various platelets receptors are widely used and *in-vitro* and *in-vivo* studies have shown promising results [225] [176] [226] [211] [227] [228] [229].

1.25 Role of platelet poor plasma in cancer

Platelet-poor plasma (PPP) is the plasma fraction of blood that remains after the removal of platelets through controlled centrifugation. While often considered a by-product in clinical and laboratory settings, PPP represents a biologically active fluid rich in soluble proteins, clotting factors, cytokines, chemokines, metabolites and extracellular vesicles. Unlike platelet-rich plasma (PRP), which is commonly used for its regenerative properties, PPP has traditionally received less attention. However, recent studies have highlighted its potential significance in various physiological and pathological contexts, including cancer [230].

In particular, the composition of PPP is known to be altered in cancer patients, reflecting systemic responses to tumor growth, inflammation and immune modulation. These changes suggest that PPP may play an active role in influencing tumor biology and may serve as a valuable source of biomarkers or therapeutic modulators. As such, exploring the role of PPP in breast cancer progression may provide novel insights into tumor-host interactions and uncover new avenues for diagnosis, prognosis and treatment [231] [232] [233]. From a diagnostic perspective, PPP offers a minimally invasive source for liquid biopsy, potentially allowing the detection of tumor-specific biomarkers such as circulating tumor DNA (ctDNA), microRNAs and exosome-associated proteins. These markers can provide early insights into tumor presence, subtypes and progression, even before clinical symptoms emerge. Clinically, this could facilitate early detection, risk stratification and real-time monitoring of treatment response or disease recurrence. Our primary objective in this study was to elucidate the differences between normal platelets and TEPs derived from breast cancer patients and to investigate how TEPs contribute to critical oncogenic processes such as stemness, EMT and metastatic dissemination. Through comprehensive profiling and functional assays, we sought to uncover the mechanisms by which TEPs support cancer progression, thereby identifying potential biomarkers or therapeutic targets. Building upon these findings, we hypothesized the second part of our investigation focused on an often-overlooked component of blood, the platelet-poor plasma (PPP). While platelets have been extensively studied for their role

in cancer, PPP, which remains after the removal of platelets, contains a complex mixture of soluble factors, cytokines and extracellular vesicles that may also influence tumor behaviour.

Emerging evidence suggests that PPP can modulate cancer cell phenotype, immune evasion and response to therapy. Recent studies have highlighted that the composition of PPP is significantly altered in cancer patients, reflecting systemic changes driven by tumor-host interactions. Such changes may render PPP a source of pro-tumorigenic signals; however, paradoxically, it may also harbour factors with potential anti-tumor or immune-modulatory properties that could be harnessed clinically [234] [235]. Therapeutically, PPP may influence cancer biology by modulating the tumor microenvironment, immune cell activity and intercellular communication. Understanding these mechanisms opens up new possibilities for therapeutic intervention either by targeting PPP-derived factors that promote tumor growth or by modifying plasma composition to enhance anti-tumor immunity. Additionally, analysing PPP may guide personalized therapy decisions by revealing systemic signatures associated with drug resistance or sensitivity.

Despite advancements in the field of metastasis research, still it accounts for majority of morbidities and mortalities. This may be attributed to the existence of many more cross-talks which are yet to be deciphered. One such axis is the interaction between platelets and cancer stem cells to mediate EMT and metastasis. Not much is currently known about the involvement of platelets in CSC mobilisation and invasion, or the possible differential ability of cancer cell subpopulations like CSCs to stimulate platelet aggregation. In this current study, we present a report of elaborate mechanism of platelet mediated stemness and invasion of breast CSCs in luminal-A and triple negative breast cancer subtypes. It highlights the importance of thrombocytosis as a prognostic marker of breast cancer along with the importance of platelet P-selectin as a non-invasive biomarker. Further, we demonstrate that breast CSCs are an important source of P-selectin ligand PSGL1. PSGL1 has an extremely crucial role in bridging EMT with stemness. Cumulatively, the study acquiesces platelets as an important player of tumorigenesis. Their alliance with vicious CSCs to promote disease advancement acknowledges them as a novel restorative agent.

Therefore, platelet directed therapies can prove beneficial to the patients. In contrast to the tumor-supportive role of TEPs, our preliminary findings suggest that PPP exerts a suppressive effect on CSCs. PPP interferes with key hallmarks of stemness, including spheroid and colony formation, chemoresistance and metastatic potential. However, the molecular mechanisms underlying PPP's inhibitory action on CSCs are not yet fully understood and its potential as an anti-stemness therapeutic has not been adequately evaluated. This thesis aims to address critical gaps in our understanding of how TEPs enhance CSC characteristics and more importantly, how PPP can counteract these effects. By investigating the impact of PPP on stemness-related pathways and functional properties of CSCs, this work seeks to establish PPP as a promising candidate for targeting the stem cell-like subpopulation within breast tumors. Furthermore, this research could provide mechanistic insights into how disrupting TEP-CSC crosstalk may reduce metastasis and improve therapeutic outcomes. Given the paucity of effective interventions targeting cancer stemness, the findings from this study have the potential to pave the way for novel anti-metastatic and anti-relapse strategies in breast cancer treatment.

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Objectives

Deciphering the novel role of tumor educated platelets in generating self-renewing, tumorigenic and multi-drug-resistant cancer stem cells in breast cancer and its modulation by aspirin along with the influence of platelet poor plasma on stem-like traits of breast cancer stem cells.

Breast cancer remains the most commonly diagnosed cancer among women worldwide, accounting for approximately 2.3 million new cases and over 685,000 deaths annually, as reported by the World Health Organization. A significant proportion of these deaths are attributed to metastasis, the primary cause of treatment failure and poor prognosis in breast cancer patients. Mounting evidence suggests that a rare population within the breast tumor cells, the breast cancer stem cells (BCSCs), play a critical role in driving metastasis and disease recurrence. Recent studies have uncovered that tumor-educated platelets (TEPs) may serve as potent modulators of cancer progression. However, their role in promoting stem-like traits, multidrug resistance and metastasis in BCSCs remain largely unexplored. Further, the therapeutic potential of aspirin in modulating TEP-induced oncogenic properties, with particular focus on the disruption of TEP~BCSC interactions is also an obscure field of research and therefore these areas require immediate investigations. Also, the impact of platelet-poor plasma on the maintenance and behaviour of BCSCs represents an uncharted frontier in the context of stemness and metastasis. By delineating these mechanisms, this research aspires to offer new insights into oncogenic signalling and contribute to the development of more effective treatment strategies for the management of breast cancer phenotypes.

The proposed study will be undertaken with the following objectives:

Primary Objectives:

2.1 To elucidate the phenotypic and functional differences between normal and tumor educated platelets.

2.2 To investigate how tumor educated platelets promote stemness, EMT and metastasis in breast cancer.

2.3 To decipher the influence of tumor educated platelets on angiogenesis.

2.4 To determine how tumor educated platelets influence stemness, EMT and angiogenesis related signalling cascades.

Secondary Objectives:

2.5 To investigate the impact of platelet poor plasma on stemness and multi-drug-resistant phenotype of breast cancer stem cells.

Materials and Methods

3.1. Materials:

3.1.1. *Consumables:*

3.1.1.1. *Laboratory Reagents:*

Sl. No	Name of Reagents	Product Description	Manufacturers
1.	Acetic acid	CH_3COOH	Merck, Germany
2.	Acrylamide	Acrylamide/ bis-Acrylamide, 37.5:1	Sigma, USA
3.	Acetylsalicylic acid	Aspirin	Sigma Aldrich, USA
4.	Agarose	For nucleic acid analysis	GIBCO,USA
5.	Acetone	C3H6O	Merck, Germany
6.	AEC substrate	For color development in immuno-histochemistry	Vector Laboratories USA
7.	Agar Powder	For soft agar colony formation	Himedia, India
8.	APS	Ammonium persulfate	Sigma, USA
9.	Boric acid	H_3BO_3	Sigma, USA
10.	β-Mercaptoethanol (BME)	$1000X:5.5x10^{-2}$Min D-PBS	Life Technologies, USA
11.	BSA	Albumin bovine fraction V powder	SRL, India
12.	Bradford reagent	Coomassie brilliant blue G-250 to protein solution	Sigma, USA
13.	Chloroform	Anaesthetic, For RNA isolation	SRL, India
14.	Citric acid	C6H807	Merck, India
15.	Collagenase type IV	Used for gentle tissue regeneration	SRL, India

16.	Crystal Violet	Methyl violet 10B or hexamethyl pararosaniline chloride, is a triaryl methane dye used as a histological stain	Sigma Aldrich, USA
17.	CytoFix/CytoPerm solution	Fixation and permeabilization, BD Perm/wash™ Buffer (10X), solution (1X), Golgistop	BD Pharmigen, USA
18.	DEPC	Diethyl pyrocarbonate, $O(COOC2HS)_2$	Sigma, USA
19.	Dextrose	C6H12O6	Merck, India
20.	DPX	Mounting media	SRL, India
21.	Dimethyl sulfoxide (DMSO)	Methyl sulfoxide, C_2H_6OS	Sigma, USA
22.	DAPI-shield	4',6-diamidino-2-phenylindole, mounting media	Sigma, USA
23.	DMEM HG	Dulbecco's Modified Eagle Medium, High	Himedia, India
24.	DMEM: F12K	Cell culture medium	Himedia, India
25.	Ethanol	C_2H_5OH(Absolute)	Merck, Germany
26.	Ethidium bromide	2,7-Diamino-10-ethyl-9phenylphenanthridinium bromide	Sigma, USA
27.	Eosin	Stain for histology	Merck, India
28.	FBS	Fetal bovine serum	Himedia, India
29.	Glycine GR	H_2NCH_2COOH	Merck, India
30.	Glycerol	Used to enhance refractive index during imaging	Merck, USA
31.	HEPES	N-2-hydroxyethyl piperazine N-2 othane sulphonic acid	Sigma, USA
32.	Hydrochloric acid	HCl	Merck, India

33.	Hematoxylin (Delafield's) stain	Stain for histology	Merck, India
34.	KCl	Potassium chloride purified	Merck, India
35.	Lipofectamine	Transfection medium	Sigma, USA
36.	Magnesium dichloride	MgCl2; salt for cell lysis	SRL, India
37.	Matrigel	Commercially available matrix	Corning, USA
38	Mayer's Hematoxylin solution	Stain for histology	Merck, India
39.	Methanol	CH_3OH	Life Technologies, USA
40.	Minimum Essential Medium (MEM)	Cell culture medium	Himedia, USA
41.	Na2HPO4.2H2O	Di-sodium hydrogen phosphate-2-hydrate	Merck, India
42.	Na3C6H5O7	Sodium Citrate	Merck, India
43.	NaCl	Sodium chloride crystal	Merck, India
44.	$NaHCO_3$	Sodium bi-carbonate	SRL, India
45.	Nitrocellulose membrane	Western blot membrane	Merck, Germany
46.	Nuclease free water	H2O	Sigma, USA
47.	Opti MEM	Cell culture medium for siRNA transfection	Gibco, USA
48.	Para-formaldehyde	Polyoxymethylene; $(CH_2O)n$	Merck, India
49.	PBS	Phosphate buffered saline	Himedia, India
50.	Pen-strep	Penicillin, Streptomycin, Neomycin solution	Himedia, India
51.	Phenol	Denatures protein	Merck, India
52.	Poly-l-lysine	Natural polymer used to adhere tissue sections to slide	Sigma Aldrich, USA
54.	Proteinase K	Protein digestion	Merck Millipore, USA
55.	RPMI 1640	Cell culture medium	Himedia, USA

56.	Sheath fluid	Fluid used in flow-cytometer	BD Pharmingen, USA
57.	Sodium dodecyl sulphate (SDS)	Detergent	SRL, India
58.	Sulphuric acid	H2SO4	Merck, India
59.	TEMED	N, N,N',N'-Tetra methylethylenediamine	Life Technologies, USA
60.	Tissue-Trek O.C.T compound	Formulation f water soluble glycols and resins	Sakura Fintek, Tokyo, Japan
61.	TMB substrate	3,3',5,5'-Tetramethylbenzidine or TMB is a chromogenic substrate used in ELISA	BD Biosciences, USA
62.	TRIS buffer	Tris [hydroxymethyl] Aminomethane	Merck, India
63.	TritonX-100	Octyl phenoxy poly ethoxyethanol	Sigma, USA
64.	Trizol Reagent	Mono-phasic solution of phenol and guanidine isothiocyanate	Life Technologies, USA
65.	Trypan blue	Dye content~40% anhydrous M.W. 960.8	Sigma, USA
66.	Tween-20	Polyoxyetene	Sigma, USA
67.	Trypsin EDTA	Trysin conjugated to Ethylene diaminetetra acetic acid disodium salt	Himedia, India
68.	VEGFR2 Kinase Inhibitor I	Selectively inhibits VEGFR2	Abcam, UK
69.	Vecta Mount^{R}	Mounting media	Vector Laboratories
70.	Xylene	Dimethybenzene	Merck, Germany

3.1.1.2. *List of antibodies:*

Sl. No	Antibody/Antibody-cocktail kit	Make	Catalogue
1.	Purified CD41 Antibody	Genetex	GTX113758
2.	Ultra-leaf purified anti-human-CD62P	Biolegend	304947
3.	APC-mouse anti human CD62P	BD Biosciences	561920
4.	Purified PSGL1 Antibody	Santa Cruz	SC-10172
5.	Purified anti ALDH1L1 Antibody	Biolegend	856802
6.	Lineage Cell Depletion Kit, human	Miltenyi Biotec	130-092-211
7.	CD44 Micro Beads, human	Miltenyi Biotec	130-095-194
8.	CD24 Micro Bead Kit, human	Miltenyi Biotec	130-095-951
9.	FITC anti-mouse/human CD44 Antibody	Biolegend	103005
10.	PE anti-mouse/human CD24 Antibody	Biolegend	101807
11.	Purified Nanog Antibody	Santa Cruz	SC-374103
12.	Purified SOX2 Antibody	R & D Systems	MAB2018
13.	Purified OCT4 Antibody	R & D Systems	MAB1759
14.	Purified Anti-mouse E-cadherin	Novus	NBP2-19051
15.	Purified Anti-mouse Vimentin	R & D Systems	MAB2105
16.	Purified Twist Antibody	Genetex	GTX127310
17.	Purified Cofilin Antibody	Cell Signaling Technology	5175
18.	Purified WNT Antibody	Santa Cruz	SC-6266
19.	Purified β-catenin Antibody	Elabscience	ENT0672

20.	Purified Frizzled Antibody	Santa Cruz	SC-7429
21.	Purified VEGF Antibody	Santa Cruz	SC-507
22.	Purified Flt1 (VEGFR1)	Santa Cruz	SC-316
23.	Purified Anti-Flk-1/KDR/VEGFR2 Antibody	Santa Cruz	SC-6251
24.	Purified anti-human IL2 Antibody	Santa Cruz	SC-7896
25.	Purified anti-human IL4 Antibody	Pepro Tech	500-P24
26.	Purified anti-human IL6 Antibody	Biolegend	504501
27.	Purified NA/LE rat anti-human IL10 Antibody	BD Biosciences	554703
28.	Purified anti-human IL12 Antibody	Biolegend	511801
29.	Purified anti-human IL13 Antibody	Biolegend	501901
30.	Purified anti-human TGFβ1 Antibody	BD Biosciences	555052
31.	Purified anti-human TNFα Antibody	Santa Cruz	SC-1351
32.	HRP- Anti-Mouse IgG Antibody	Sigma	A5278
33.	HRP-Anti-Rat IgG Antibody	Abcam	ab6734
34.	HRP-Anti-Rabbit IgG Antibody	Sigma	A0545
35.	HRP-Anti-Goat IgG Antibody	Sigma	A5420
36.	FITC Anti-Rabbit IgG Antibody	Santa Cruz	SC-2012
37.	PE Goat Anti-Mouse IgG Antibody	BD Pharmingen	550589
38.	Donkey Anti-Goat IgG PE	Santa Cruz	SC-3743
39.	PerCP-Cy 5.5 Anti-Mouse Antibody	BD Biosciences	560668
40.	APC/Cy7 Anti-Mouse Antibody	Abcam	AB130785
41.	FITC Anti-Mouse IgG Antibody	Sigma	F5897
42.	TRITC - Anti-Rat Antibody	Abcam	ab6841
43.	Goat Anti-Rabbit FITC Antibody	Santa Cruz	SC 2012
44.	Goat Anti-Rat FITC Antibody	Abcam	AB6840

3.1.1.3. *List of primers:*

Sl. No	Primer Name	Forward primer sequence 5'- 3'	Reverse primer sequence 3'- 5'
1.	Human *β-actin*	AGCGAGCATCCCCCAAAGTT	GGGCACGAAGGCTCATCATT
2.	Human *bcrp1*	TCAGGAGGCCTTGGGATACT	AGTTCCACGGCTGAAACACT
3.	Human *abcb1*	ATTTTCAATGTTTCGCTATT	TTCATGAAGAACCCTGTATC
4.	Human *abcc1*	TCGTGTGGGTGCCTTGTTT	AACAGCAGCACGGTGTAGAA
5.	Human *snail*	CTTCGTCCTTCTCCTCTACT	ATTCCTTGTTGCAGTATTTG
6.	Human *slug*	TACAGTCCAAGCTTTCAGAC	GCTCACATATTCCTTGTCAC
8.	Human *cxcr3*	ATGGAGTTGAGGAAGTACG	CACTCTCGTTTTCTCCATAG
9.	Human *cxcr4*	ACCAACAGTCAGAGGCCAAG	ACACAACCACCCACAAGTCA
10.	Human *cxcr5*	CCATGCTCTACACTTTCGCC	AGAACGTGGTGAGAGAGGTG
11.	Human *mmp7*	GAGTGCCAGATGTTGCAGAA	AAATGCAGGGGGATCTCTTT
12.	Human *mmp9*	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCGTCCTTAT
13.	Human *mmp11*	TAGGTGCCTGCATCTGTCTG	TGGCTTTGGAGGATAGCAGT
14.	Human *wnt*	AGAGAGGGTAGAAGACGTTG	GAGGAACACTGACCTAGTCC
15.	Human *notch1*	GAACTGTGAGGAAAATATCG	GACACACACGCAGTTGTAG
16.	Human *notch4*	AGAAAGACTCCACCTTTCAC	GTCTCACACTCATCCACATC
17.	Human *vegf*	GGCCTCCGAAACCATGAACT	GCTGCGCTGATAGACATCCA
18.	Human *vegfr1*	CTGGGCAGCAGACAAATCCT	CACAACCAAGGTGCTAGCCA
19.	Human *vegfr2*	GTACACCTGTGCAGCATCCA	AATCGTCAGTACATGCCCCG

3.1.1.4. *Primers for siRNA silencing:*

Sl. No	Primer Name	Sense primer sequence 5'- 3'	Anti-sense primer sequence 3'- 5'
1.	Human *WNT3Asi*	AAGCAGGCTCTGGGCAGCTACCCTGTCTC	AAGTACTGCCCAGAGCCTGCCCTGTCTC
2.	Human *VEGFR2si*	AAGGCGCTGCTAGCTGTCGCTCCTGTCTC	AAGCGACAGCTAGCAGCGCCTCCTGTCTC

3.1.1.5. *Buffers:*

Sl. No	Name	Composition
1.	Bradford solution	Coomassie brilliant blue G-250 protein solution
2.	Blocking buffer	5-8% BSA solution
3.	Citrate buffer	0.01 M Sodium solution pH 6.2 citrate and citric acid 0.02 pH 6.2
5.	Electrode buffer	0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3
6.	ELISA blocking buffer	0.15 M PBS, 5% BSA, pH 7.4
7.	ELISA wash buffer	0.15 M PBS, 0.1% Tween 20, pH 7.2
8.	FACS buffer	0.15 M PBS, 2% FBS, 0.09%sodium azide, pH 7.4
9.	HEPES 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid	140mM NaCl, 2.7Mm KCl, 3.8Mm HEPES, 5mM EDTA, pH 7.4
10.	Laemmli buffer	0.06M Tris HCl, containing 2% SDS, 10% glycerol, 0.025% Bromophenol blue and 5% 2-mercaptoethanol (2ME), pH 6.8
11.	Lowry's solution A	4.5% Sodium carbonate, 1% SDS
12.	Lowry's solution B	2% Anhydrous copper sulphate
13.	Nuclear extraction buffer	20mM HEPES, 0.4M NaCl, 1mM EDTA, 25% glycerol, PI cocktail, pH 7.9
14.	PBS	0.15M Phosphate buffered saline, pH 7.4
15.	PBB	0.5% BSA, PBS
16.	Protein lysis buffer	15 mM Tris, 2mM EDTA, 50mM 2-mercaptoethanol (2ME), 20% Glycerol, 0.1% Triton-X, 1mM sodium fluoride, 1mM sodium orthovanadate, PMSF, Aprotonin, Leupeptin, Pepstatin

17.	Resolving gel buffer	1.5M Tris HCl, pH 8.8
18.	Stacking gel buffer	0.5M Tris HCl, pH 6.8
19.	TBE	10X Tris boric acid EDTA buffer
20.	TBS	50 mM Tris HCl, 0.15M NaCl, pH 7.4
21.	TBST	50 mM Tris HCl, 0.15M NaCl, pH 7.4 with 0.1% Tween 20
22.	TE buffer	10 mM Tris, 1 mM EDTA, pH 8
23.	Transfer buffer	0.025 M Tris, 0.194 M Glycine, 0.025% methanol, pH 8.3
24.	Washing buffer for western blot	0.01 M Tris HCl, 0.09% NaCl, 0.1% Tween-20, pH 7.2
25.	Cell dissociation buffer	Distilled water 400 mL + 20X PBS stock solution + 0.5M EDTA 54mL
26.	ACD buffer	39 mM Citric acid, 135 mM dextrose, 75mM Sodium citrate
27.	Platelet wash buffer	10mM Sodium citrate, 150mM Sodium chloride, 1mM EDTA, 1% w/v dextrose

3.1.1.6. *Assay Kits:*

Sl. No	Assay Kits	Product Description	Manufacturers
1.	BD Cytofix/Cytoperm™ Fixation and permeabilization solution	BD Perm/Wash ™ Buffer (10X)	BD Pharmingen, USA Catalog Number 554714
2.	BD IMagnet ™	Plastic test tube rack surrounding a strong permanent rare earth magnet	BD Biosciences, USA Catalog Number 552311
3.	ECL reagent	Solution A and Solution B	Advansta, CA, USA Catalog Number K12045-D20
4.	GoTaq® Green PCR mix 2X	Green master mix containing taq DNA polymerase, dNTPs, MgCl2, reaction buffers with optimum concentration	Promega, USA Catalog Number M7123
5.	Revert Aid ™ first strand cDNA synthesis kit	Revertaid reverse transcriptase (RT), RiboLock RNAse inhibitor, oligo (dT), random hexamer primers	Thermo Scientific, USA Catalog Number K1622
6.	Silencer siRNA construction kit	T7 promoter primer, Exo-klenow, T7 enzyme mix, De-hybridization buffer and sample buffer	Life Technologies, USA Catalog Number AM1620
7.	*In-vitro* angiogenesis assay kit	EC Matrix ™ Gel solution, EC Matrix ™ Diluent buffer	Merck, Germany Catalog Number ECM 625

3.1.2 *Plastic wares:*

3.1.2.1 *Tissue Culture Grade Plastic wares:*

Sl. No	Item Name	Manufacturer	Catalogue No
1.	T-25 vented cell culture flask	Tarson	156367
2.	T-75 vented cell culture flask	Thermo	353136
3.	6 well plate, treated	Tarson	140675
4.	6 well plate, non-treated	Thermo	150239
5.	Ultra-low adherent 6 well plate	Corning	3471
6.	24 well plate, treated	Thermo	143982
7.	24 well flat bottom ultra-low adherent	Corning	3437
8.	12 well plate, treated	FALCON	353043
9.	96 well U bottom plate, non-treated	Thermo	268200
10.	96 well U bottom plate, treated	FALCON	353077
11.	96 well flat bottom plate, non-treated	Thermo	243656
12.	96 well flat bottom plate, treated	NUNC	167008
13.	96 well U bottom plate, ultra-low with black bottom	Corning	4515
14.	35mm petridish	Tarson	460035
15.	35mm tissue culture dish	FALCON	353001
16.	0.22µm PES Millex-GP Filter unit	Merck millipore	SLGP033RS
17.	0.45µm PVDF Millex-HV Filter unit	Corning	SLHV033RS
18.	0.4µm transwell (24 well)	Corning	3413
19.	Cell culture insert (0.4µm)	Himedia	TCP078-2X6NO
20.	Chamber slide (8 well)	Thermo	177402
21.	Cryotube	Thermo	368632
22.	ELISA plate, Immunomodule	NUNC	469949

23.	ELISA strip, Immunomodule	NUNC	469943
24.	Flow cytometric tube (8ml)	FALCON	352054
25.	Pasteur pipette	Tarson	520064
26.	Cell strainer (70 µm)	Genetix	93070

3.1.2.1 *General Culture Grade Plastic wares:*

Sl. No	Item Name	Manufacturer	Catalogue No
1.	0.2 ml Flat cap microcentrifuge tube	Tarson	510051
2.	0.2-10µL Microtip box	Tarson	524052
3.	0.2-10µL Microtips	Tarson	521000
4.	1.5 mL Microcentrifuge tubes	Tarson	500010
5.	1000 mL beaker	Tarson	421060
6.	1000µL Microtips	Tarson	521020
7.	2 mL microcentrifuge tubes	Tarson	500020
8.	200µL Microtip box	Tarson	522020
9.	200µL Microtips	Tarson	524053
10.	5 mL microcentrifuge tubes	Eppendorf	30119401
11.	5 mL Microtips	Tarson	521032
12.	50 mL centrifuge tube	Tarson	546041
13.	500 mL beaker	Tarson	421050
14.	Coplin jar	Tarson	480000
15.	Cryo tube box	Tarson	202060
16.	15 mL centrifuge tube	Tarson	546021

3.1.3 *Instruments:*

Sl. No	Name of the Instruments	Description	Manufacturer
1.	Autoclave	SS with 42 cm diameter	Indo Scientific, India
2.	Balance	XP56 Microbalance	Metller-Toledo, Switzerland
3.	Biosafety cabinet	Vertical Laminar Flow, Stage-II	Klenzoids, India
4.	Centrifuge	Heraeus Biofuge Plate-3K-10 Optima™ L-XP Series	Hereus, Germany REMI, India Sigma, Germany Thermo, USA
5.	Cold room	4ºC constant	Blue Star, India
6.	Cryo-microtome	Leica CM 1850	Leica
7.	Cryo-tank	60L	Thermo Scientific, USA
8.	Electrophoresis apparatus	Vertical, Horizontal	Bio-Rad, India
9.	Flow cytometer	BD LSR Fortessa	Becton Dickinson, USA
10.	Freezer (-20ºC)	Low Temperature Freezer	Vestfrost, India
11.	Freezer (2-8ºC)	Refrigerator	LG; Haier; Electrolux, India
12.	Gel documentation system	Gel Doc XR System	Bio-Rad, USA
13.	Incubator (CO2)	Heracell 240i Tris-Gas Incubators; Cell culture Incubator	Thermo Scientific,USA Esco Technologies, USA
14.	Liquid nitrogen plant	StirLIN-1	Economy Sterling, Netherlands
15.	Micropipettes	20 mL,100 mL, 200 mL, 1000 mL, 5000 mL Volume withdrawal capacity	Gilson Inc. Middleton, WI, USA; Tarson, India; Thermo Scientific, FL, USA;

16.	Microscope	Light-DM1000 Inverted-Phase contrast-DMIL Primo star-Inverted, light microscope	Leica/Leitz (Germany) Zeiss, Germany
		Olympus FLUOVIEW FV3000, Confocal microscope ZEISS, Gemini,	Japan Olympus, Tokyo, Japan
		Scanning electron microscope	Zeiss, Germany
17.	Microtome	Leica BM2125RT	Leica/Leitz (Germany)
18.	Nano-Drop spectrophotometer, NABI	UV/Vis Nano spectrometer	MicroDigital Co.,Ltd. Korea
19.	PCR Thermal Cycler	C1000 Touch Thermal Cycler	Bio-Rad, USA
20.	Plate reader	Infinite 200 PRO multimode reader, Spectramax i3X	Tecan (Switzerland), BioTek, USA, Molecular devices, India
21.	Spectrophotometer (UV-Vis)	Cary 300 (190-900nm)	Agilent Technologies, USA
22.	Stirrer	Magnetic, 2MLH	REMI, India
23.	Syringe	1mL, 2 mL, 5mL, 10mL, 20 mL, 50mL	Dispo Van, India
24.	Vortex	CM 101 Plus	REMI, India
25.	Ultra-low freezer (-80ºC)	FW 227 F, TS586e ULT Freezer	Heto, USA Thermo, USA Esco Technologies, Germany
26.	Western blotting apparatus with power pack	Wet/Tank blotting systems	Bio-Rad, USA
27.	Water bath	37ºC-80ºC water bath	Benchmark, Scientific, USA

3.2. *Human Patient Sample Details:*

Sl. No	AGE	Hormone Receptor Status	TNM Stage
1.	56	TNBC	T4N1MX
2.	50	TNBC	T3N1MX
3.	52	TNBC	T2N2MX
4.	58	TNBC	T2N0MX
5.	56	TNBC	T4N1MX
6.	50	TNBC	T3N1MX
7.	52	TNBC	T2N2MX
8.	58	TNBC	T2N0M0
9.	54	TNBC	T2N1M0
10.	50	TNBC	T2N1M0
11.	58	TNBC	T2N0M0
12.	52	TNBC	T2N0M0
13.	54	TNBC	T2N1M0
14.	53	TNBC	T3NIM0
15.	60	TNBC	T2N1MX
16.	58	TNBC	T4N1M0
17.	51	TNBC	T4N1MX
18.	55	TNBC	T2N1MX
19.	58	TNBC	T2N0M0
20.	48	TNBC	T3N1MX
21.	60	Luminal-A	T4N1MX
22.	62	Luminal-A	T2N1MX
23.	45	Luminal-A	T2N1MX
24.	54	Luminal-A	T3N1M0
25.	55	Luminal-A	T4N1MX
26.	41	Luminal-A	T4N2M0
27.	56	Luminal-A	T3N2M1

28.	48	Luminal-A	T2N1MX
29.	67	Luminal-A	T2N1MX
30.	66	Luminal-A	T3N0MX
31.	59	Luminal-A	T4N1MX
32.	58	Luminal-A	T4N1M2
33.	41	Luminal-A	T2N0MX
34.	52	Luminal-A	T4N1M1
35.	48	Luminal-A	T2N1MX
36.	50	Luminal-A	T2N0M0
37.	47	Luminal-A	T2N1MX
38.	51	Luminal-A	T2N0M0
39.	51	Luminal-A	T2N1MX
40.	41	Luminal-A	T4N1M2

3.3. Methods:

3.3.1 Human solid tumors procurement: Pre-treatment and post-operative breast tumor samples (luminal-A n=20; TNBC n=20) with proven histo-pathological normalcy were collected from Chittaranjan National Cancer Institute (CNCI), Kolkata, India following approval from patients and Institutional Ethical Committee (Approval no: CNCI-IEC-SB-2020-30). TNM staging status was noted during collection.

3.3.2 Human blood collection: Blood (5ml) was procured from pre-treatment and post-operative breast carcinoma patients (n=40) by venepuncture method from Chittaranjan National Cancer Institute (CNCI), Kolkata, India following approval from patients and Institutional Ethical Committee (Approval no: CNCI-IEC-SB-2020-30). 5ml blood was also collected from healthy donors (n=20) who were not under any medications and did not consume any platelet lowering drugs for atleast 6 months were used as controls. Patient details are provided in section 3.2

3.3.3 Isolation of tumor educated platelets (TEPs): TEPs were isolated from whole blood by double centrifugation method. Briefly 5ml blood was collected in ACD anticoagulant and initially centrifuged at 260g x 20 minutes. This separates the straw-coloured platelet rich plasma (PRP) from the underlying RBC zone. The PRP was divided to two parts. One part was untreated and to the other part 100µm/ml aspirin was added for 30 minutes to maintain the resting condition of platelets (resting platelets-RP). The PRP was further centrifuged at 800g x 20 minutes without brakes to separate platelet pellet from platelet poor plasma (PPP). The pellet was then washed with platelet wash buffer thrice and dissolved in Tyrode buffer and was ready for use [1].

3.3.4 SEM Microscopy: Ultrastructure of platelets was visualized by SEM microscopy. Approximately 20µL of PRP was taken on coverslip and a uniform smear was drawn (n=6). These coverslips were then placed on moist filter paper and incubated at 37ºC for 5 minutes. This allowed the smear to dry in a humid environment. Post incubation, the samples were washed with 1X PBS to remove excess plasma. Samples were fixed in 2.5% glutaraldehyde for 30 minutes followed by triplicate washing with 1 X PBS for 5 minutes each. Further fixation was done in 1% osmium tetroxide for 1 hour.

The samples were thoroughly washed and dehydrated in ascending grades of ethanol starting with 30%, 50%, 70%, 90% and

finally 100% with atleast 15 minutes incubation at each step. Samples were then transferred to carbon tapes on aluminium stubs, gold coated [2] and visualized using Zeiss Gemini Scanning Electron Microscope.

3.3.5 Cell lines and culture: MCF-7, MDA-MB-231, 4T1 cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in complete media supplemented with 10% (v/v) heat inactivated FBS, 2mM L- glutamine, 100 U/ml penicillin and 100µg/ml streptomycin in incubator at 37°C and 5% CO_2. Cell lines were monitored regularly for any changes in morphology. Mycoplasma contamination if any was removed by EZkill™ Mycoplasma Elimination kit procured from Himedia (Mumbai, India). Cells were maintained for 10-12 passages and all experiments were performed within 6 months of purchase.

3.3.6 Co-culture: Single cell suspensions of luminal-A and TNBC subtypes and MCF-7, MDA-MB-231, 4T1 and EC were cultured with TEPs and RP in 1:100 (BCSC: platelet) ratio for 24 hours. After the incubation period, the culture media with platelet suspension was removed and the cells were collected by trypsinization and centrifuged for further analysis.

3.3.7 Magnetic cell sorting: From the co-culture setup BCSCs were sorted by using magnetically labelled $CD44^+/CD24^-$ cocktail antibodies and cell purification was carried out according to manufacturer's protocol (MicroBead kit, Miltenyi Biotech, Germany). Purity of cells was checked by flow cytometry.

3.3.8 CSC enrichment culture and tumorsphere assay: CSC enrichment media was prepared by supplementing serum free DMEM: F12K (1:1) media with 1% B27™ supplement (50X). To it heparin (40ng/mL), human-rEGF (20ng/mL), human-rbFGF (20ng/mL) were added freshly. CSCs ($1x10^4$) were cultured in this media and plated on ultra-low adherent plate (Corning, New York, USA). Cells were incubated in 5% CO_2 humified atmosphere at 37°C for 7 days and fresh media was supplemented to the culture after every three days. Tumorspheres were micrographed and their count from 5 random fields was documented. Their area was calculated using ImageJ software. Tumorspheres so formed were dissociated with trypsin and were centrifuged to produce single cell suspensions which were further analysed.

3.3.9 Mice and tumors: Wild- type female BALB/c and Swiss albino mice (age: 4-6 weeks; body weight: 18-22 g average) were obtained from Institutional

Animal Care and Maintenance Department. All experimental animals were maintained in pathogen free environment and fed with autoclaved food (Epic Laboratory, West Bengal Government, Kalyani, India) and water *ad libitum.* All experiments were performed after approval from Institutional Animal Care and Ethics Committee (Approval No: IAEC-1774/SBn-4/2021/9). 4T1 cells were maintained *in-vitro* and EC cells (Ehrlich Carcinoma) were maintained in Swiss albino mice as intraperitoneal passage.

3.3.10 Spontaneous metastasis model: Post co-culture, sorted CSCs ($2x10^5$) of EC and 4T1 cells were inoculated into the mammary fat pads of female Swiss albino and BALB/c mice respectively for development of solid tumors. Tumor growth was monitored twice a week and measured using Verniercallipers. Tumor area was measured (length x width) and presented in mm^2. Health of the animals was monitored daily. When the tumor size reached 20mm, the mice were euthanized by overdose of ketamine HCL (160 mg/kg) and xylazine (20mg/kg) according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals.

3.3.11 Experimental metastasis model: Post co-culture, sorted BCSCs ($2x10^5$) of 4T1 and EC cells were injected through the tail vein of BALB/c and Swiss albino mice respectively for development of lung metastasis. The animals were monitored for 4 weeks after which they were euthanized as described above and the organs were harvested.

3.3.12 Processing of tumors: Procured tumors were digested with 1% collagenase for generating single cell suspensions. The cells were then washed with 1X PBS and were then used for various *in-vitro* assays.

3.3.13 Cryo-sectioning: Freshly procured tumor samples were fixed in 4% para-formaldehyde for 2 hours at room temperature followed by incubation at 4°C overnight in 30% sucrose. They were then snap chilled in liquid nitrogen at stored in -80°C for later use.

For cryo-sectioning, the frozen tissue samples were embedded in OCT (optimal cutting temperature compound, Leica biosystems, Wetzlar, Germany) and cut into 5µm sections using cryostat (Leica CM1950, Wetzlar, Germany). The sections were collected on poly-L-lysine coated slides and stored at -80°C for further use.

3.3.14 Histology and HE staining: The cryo cut tissue sections were stained with hematoxylin-eosin (HE) following standard staining protocol. Briefly, the sections were rehydrated in 1X PBS. This was followed by staining with hematoxylin

for 2-5 minutes and then rinsing in running tap water for another 5 minutes to allow for bluing. Subsequently, the sections were stained with eosin for another 1-2 minutes and then dehydrated in ascending grades of alcohol (70%, 90%, 100%; 5 minutes each). The slides were then dipped in xylene and mounted with DPX. Stained sections were examined using Carl Zeiss Plan Achromat bright field microscope along with Axiocam color camera.

3.3.15 Soft agar colony formation assay: Briefly $5x10^3$ cells were cultured on upper 0.35% soft agar layer along with 1mL culture media. This layer was placed upon bottom agar bed of 0.7% agar and cell culture medium. The culture setup was maintained for 21 days with fresh media supplementation on every third day. The colonies were finally micrographed and the number of colonies was quantified.

3.3.16 Contact independent transwell assay: For transwell assay, magnetically sorted BCSCs were co-cultured in presence of 0.4µm transwell membrane (Hi-Media, Mumbai, India) in 1:100 (BCSC: platelet) for 24 hours. Following the incubation period, the transwell was removed and the cells were undertaken for further analysis.

3.3.17 Wound healing assay: Post co-culture, sorted BCSCs of MCF-7 and MDA-MB-231 were grown until completely confluent. A scratch or wound was drawn using a cell scratcher and wound healing was observed by taking micrographs at different time points for 24 hours. Percentage wound closure was calculated as final area/initial area X 100% [3].

3.3.18 Matrigel invasion assay: Post co-culture, sorted BCSCs were serum starved for 3 hours after which they were layered on matrigel coated transwell inserts (8µm) (Corning-354480, New York, USA). These inserts with the cell suspension were placed in 24 well plate containing FBS as chemo-attractant. This entire setup was maintained for 24 hours following which the invaded cells were fixed with paraformaldehyde and permeablized by absolute methanol followed by staining with 0.2% crystal violet. Migrated cells from five random fields were photographed and quantified.

3.3.19 Vascular mimicry (VM) assay: For VM assay, 96 well flat bottom plates were coated with 60µL of growth factor reduced basement membrane (Matrigel; R&D System, MN, USA) and incubated at $37^{\circ}C$ for solidifying. Roughly $5x10^3$ BCSCs in 150 µL of complete media were rested on the plates and incubated at $37^{\circ}C$ for 24 hours. Following the incubation, the plates were micrographed and number of

interconnected tubes per field was counted. Length and width of the tubes were quantified in ImageJ software.

3.3.20 RT PCR: Total RNA content of single cell suspensions was extracted by Trizol (Ambion, Thermo Fisher Scientific, MA, USA). cDNA was synthesized according to manufacturer's protocol from it using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA). Reverse transcriptase PCR was performed using 2X Go Taq Green Mix (Promega, WI, USA). Electrophoresis was done using 1.5% agarose gels and stained with ethidium bromide. PCR bands were visualized on Chemi Doc XRS+ (BioRad Laboratories, CA, USA), identified by image Lab softwareV5.1 and quantified using ImageJ software. List of gene specific primers used in PCR are given in section 3.1.1.3.

3.3.21 Flow cytometry: Briefly, cells were stained with fluorescently tagged antigen specific antibodies and incubated in dark at 4°C for 30 minutes. For intracellular molecules, cells were treated simultaneously with 0.2% saponin (permeabilization buffer) followed by addition of either the primary antibody (1:100 ratio) or fluorescently tagged antigen specific antibody. In case of primary antibody, post incubation at 4°C for 30 minutes, fluorescently tagged secondary antibody (1:1000 ratio) was added. The cells were then incubated at room temperature for 30 minutes in the dark. Following incubation, cells were finally washed with FACS buffer to remove any unbound antibodies. Cells were then fixed with 1% paraformaldehyde and data was acquired using BD LSR Fortessa X-20 Cell Analyzer (Becton Dickinson, New Jersey, USA). For data collection Cell Quest Pro 5.1 and for analysis FlowJo™ Version 11 (Becton Dickinson, New Jersey, USA) was used. Cell morphology was determined by FSC-A and SSC-A.

3.3.22 Extraction of cytosolic and nuclear proteins: Ice cold nuclear extraction buffer was added to the cell pellets and incubated at 4°C for 1 hour. The cells were centrifuged at 6000 RPM for 5 minutes and the supernatant was collected as cytosolic fraction. The pellet was dissolved in nuclear extraction buffer and vortexed for 30 minutes at 4°C. The samples were centrifuged at 12,000 RPM for 10 minutes. The supernatant thus obtained was the nuclear fraction.

3.3.23 Extraction of total proteins and Western Blot: Cells were lysed by incubating in RIPA buffer for 30 minutes at 4°C. This was followed by centrifugation for 30minutes at 12,000 RPM at 4°C. Protein concentration of the

lysates so obtained was determined by Bradford assay. 30-50µg of the protein lysates was separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane using BioRad Gel Transfer system and bands were developed using ECL Kit (Advansta, CA, USA). Band intensity was quantified using Image Lab 6.2 software (Bio-Rad, California, USA).

3.3.24 Immunofluorescence microscopy and analysis: Targeted samples were harvested on poly-L-lysine coated glass slides and initially blocked with 5% BSA at RT. Cells were perforated with 0.15% Triton X-100 prior to blocking for staining intracellular molecules. Antigen specific primary antibodies were added to the section and incubated overnight at 4°C. Fluorescently tagged secondary antibodies were added next and incubated for 3 hours at RT. Finally, the sections were thoroughly washed and mounted with Fluoroshield DAPI (Abcam, Cambridge, UK). Images were acquired using Olympus-BX53 microscope (Olympus Life sciences, Tokyo, Japan). Fluorescence intensity was evaluated using Image-J software and corrected total cell fluorescence (CTCF) was calculated using the formula

CTCF= Integrated density- (Area of selected cell x Mean fluorescence intensity of background readings) [4].

3.3.25 Confocal microscopy: Platelet-CSC interaction was visualized by confocal microscopy. Briefly, mammospheres were initially cultured in chamber slide. Binding of TEPs and RPs to mammospheres was allowed for another 24 hours. The cells were fixed in 2% PFA for 30 minutes, followed by perforation with 0.5% Triton X for 10 minutes. Nonspecific interaction was blocked by 5% BSA treatment. Cells were stained with primary antibody (1:100 ratio) and incubated in dark at 4°C overnight. The samples were washed thrice with 1XPBS to remove unbound molecules and then fluorescently tagged secondary antibody (1:1000 ratio) was added and incubated at room temperature for 30 minutes in the dark. Post incubation, the sample was washed with 1XPBS atleast thrice, mounted with DAPI and visualized with Olympus Fluoview FV3000. Images were analysed and quantified using ImageJ software.

3.3.26 Immunohistochemistry: Tissue sections were initially harvested on poly-L-lysine coated glass slides and kept in 1XPBS until the OCT was removed. Sections were treated with 3% H_2O_2 (Merck 17544) in methanol for 30 minutes, to block endogenous peroxidase. Non-specific sites were blocked by incubating the sections with 5% BSA at

RT for 30 minutes followed by incubation with primary antibody (1:100 ratio) overnight. After thorough washing with PBS-Tween 20, HRP tagged secondary antibody (1:1000 ratio) was added to the samples. AEC Substrate (Vector Laboratory SK4200) was used to develop chromogenic colour according to manufacturer's protocol. Counterstaining was performed using Hematoxylin (Merck-HX 68597049) for 40 seconds and then mounted with VectaMount (Vector Laboratories H5501). Image was acquired using Carl Zeiss Plan Achromat bright field microscope along with Axiocam color camera.

3.3.27 Isolation of platelet poor plasma: For isolating platelet poor plasma (PPP), initially, 5ml of blood was collected in ACD anticoagulant from healthy donors who have not consumed anti-platelet drugs for the last six months. Blood was centrifuged initially at 260g for 20 minutes to separate the platelet rich plasma. This plasma was again centrifuged for 20 minutes at 800g to separate the platelet pellet and platelet poor plasma. The PPP was separated carefully to prevent any contamination from the underlying platelet pellets. The collected PPP was aliquoted in sterile vials of 1ml each and stored at -80ºC for later use.

3.3.28 Co-culture of CSCs with PPP and enrichment in CSC enrichment media: Magnetically sorted $CD44^{+}/CD24^{-}$CSCs ($1x10^{5}$) were cultured in stem cell enrichment media (serum free DMEM: F12K (1:1) media with 1% B27^{TM} supplement (50X), heparin (40ng/mL), human-rEGF (20ng/mL), human-rbFGF (20ng/mL)) were added freshlyand plated on ultra-low adherent plate (Corning, New York, USA) and maintained for 5 days in presence/absence of 50µL of PPP. The plates were micrographed and the results of 5 random fields was documented and analysed via ImageJ software.

3.3.29 Ki67 Proliferation assay: Experimental samples were immediately fixed in 4% PFA and incubated for 15 mins at RT to preserve the phosphorylation status. Fixed cells were then permeabilized by adding absolute methanol drop wise to the sample while vortexing it at 10-15 s at 2500 rpm at RT. These permeabilized samples were stored at -20ºC for a minimum 1hr prior to staining. The cells were then stained with Ki67 primary antibody (1:100 ratio) and incubated at 4°C for 30 minutes. Post incubation the cells were thoroughly washed to remove unbound molecules and fluorescently tagged secondary antibody (1:500 ratio) was added and incubated at room temperature for 30 minutes in dark.

The cells were then thoroughly washed with 1XPBS and data was acquired using FACS Calibur and LSR Fortessa X-20 Cell Analyzer (Becton Dickinson, New Jersey, USA) as per requirement along with suitable negative isotype controls. For data collection, Cell Quest Pro 5.1 and for data analysis FlowJo^{TM} Version 11(Becton Dickinson, NewJersey, USA) were used. Cellular morphology was inferred from FSC-A vs. SSC-Agates, while singlet population was determined from FSC-A vs. FSC-H gates

3.3.30 Cell Cycle Analysis: Cells from experimental groups were harvested, fixed in chilled 70% methanol while vortexing, followed by incubation at −20◦C overnight. Following day, the cells were washed twice with PBS containing 0.1% sodium azide, and followed by a treatment of 50 µL (100 ng/mL) RNase A. Finally, the cells were stained with 200 µL (50µg/mL) propidium iodide (PI), incubated for 10 minutes and then analysed by flow-cytometry without any further delay.

3.3.31 ELISA: 96 well plates were immobilized with co-culture supernatants collected from different experimental groups. Primary antibody (1:500 ratio) was added to these plates and incubated overnight at 4ºC. HRP-conjugated secondary antibody (1:1000 ratio) was added next and incubated for 3 hours at 37ºC. Finally, TMB substrate (BD OptEIA, BD Biosciences) was added and the resultant colorimetric output was measured by Spectra-max i3X (Molecular Devices, San Jose, USA) at 450 nm and accounted via SoftMax Pro 7.1 software.

3.3.32 siRNA mediated silencing *in-vitro*: siRNA for human *WNT3A* and human VEGFR2 were constructed *in-vitro* using Silencer R siRNA construction kit (Life Technologies, USA), according to the manufacturer's protocol. Primers utilized are mentioned in section 3.1.1.4.

Briefly, $CD44^{+}/CD24^{-}$breast CSCs were magnetically sorted from MCF-7 and MDA-MB-231 cells cultured in the monolayer. These CSCs were then serum starved for 2 hours in presence of lipofectamine-2000 reagent (Invitrogen, USA). This was followed by addition of both the target specific and scramble control siRNA (Sigma-Aldrich) to the *in-vitro* setup at a final concentration of 50nM. Following the incubation period, the CSCs were thoroughly washed with 1 X PBS and co-cultured with TEPs and RPs was performed as described in section 3.3.6. Post co-culture, the cells were layered on 3D stem cell enrichment set-up and allowed to form primary tumorspheres for 7 days. The tumorspheres were micrographed from 5 random fields and

finally collected and analysed for various parameters.

3.3.33 Protein-protein interaction visualization: String (Search Tool for the Retrieval of Interacting Genes/Proteins) database version 8.0 RRID: SCR_005223 was utilized to decipher the interactions between various proteins of interest. Based upon former reports of direct and indirect interactions, an interactome map illustrating this interrelationship between the proteins was generated. Each protein is assigned a colour and their interaction with the other proteins is represented by multicolour lines. The network properties include; nodes which represents the number of proteins in the interactome, edges depicting the number of interactions, followed by node degree which refers to the average number of interactions and finally clustering coefficient indicating the tendency of the network to form clusters [5].

3.3.34 Statistical Significance: Statistical significance was drawn from either Student *t*-test (for 2 groups) or one-way/two-way analysis of variance. Mean±SD of the results have been represented. For *in-vivo* (n=6) and for *in-vitro* (n=3-6) independent experiments were performed. All statistical analysis was performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, USA). Experimental results with $p \leq 0.05$ have been considered as significant.

3.4. References:

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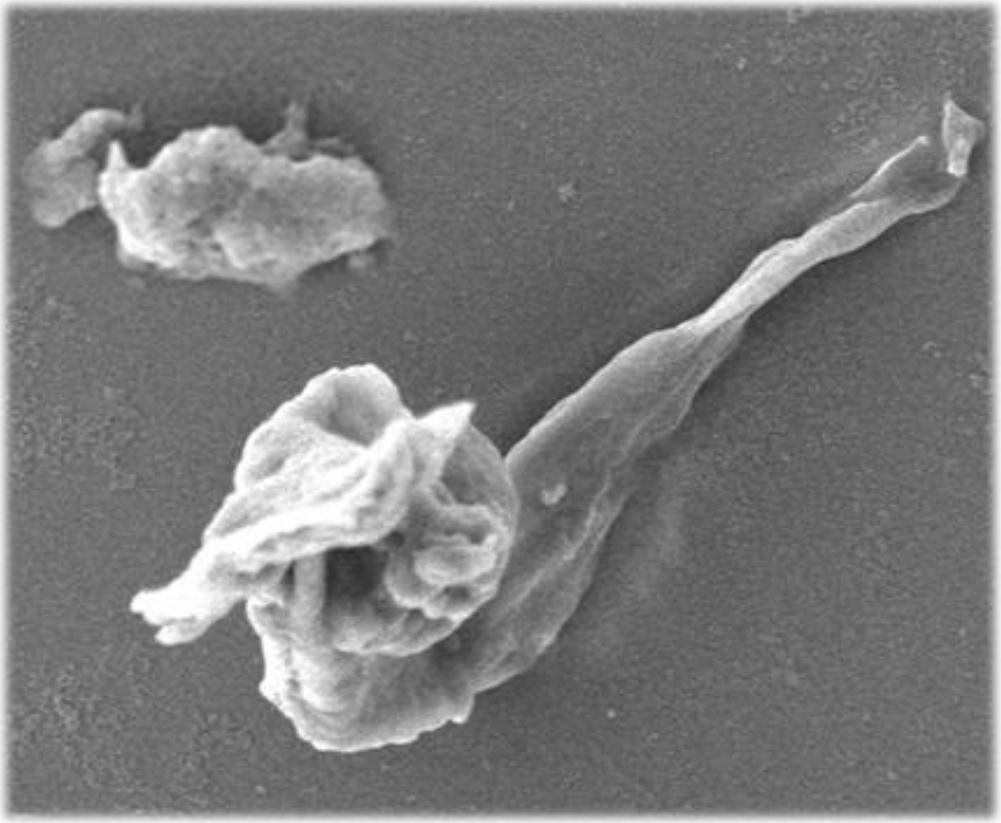
Chapter 1

Structural and functional differences between normal and Tumor educated platelets: Intervention by Aspirin



Scanning electron microscopy image of healthy platelet and tumor educated platelet from breast cancer patients at 100X magnification

Structural and functional differences between normal and Tumor educated platelets: Intervention by Aspirin

Abstract:

Apart from their role in blood clotting and maintaining haemostasis in the body, platelets play an intriguing additional role in cancer that favours the survival and progression of the tumor cells. While the pro-tumorigenic role of platelets has been recognized, comprehensive morphological, phenotypic and functional characterization of tumor educated platelets (TEPs) in luminal-A and TNBC breast cancer (BC) patient's remains limited. Moreover, the interaction between TEPs and cancer stem cells (CSCs) for stemness, metastasis, therapy resistance and recurrence has not yet been reported in BC. Additionally, there is insufficient understanding of how pharmacological interventions, such as aspirin, modulate the behaviour of TEPs and impact their role in metastasis in these two subtypes with special emphasis on stemness and metastasis. To address these gaps, the present study was designed to characterize the morphological and phenotypic differences between TEPs from luminal-A and TNBC patients and platelets from healthy individuals. Finally, the therapeutic potential of aspirin in modulating TEP activation was elucidated. SEM imaging revealed large TEP aggregates in both luminal-A and TNBC subtypes with elongated filopodia and lamellipodia indicating towards an activated state. Further, flow cytometry analysis confirmed elevated expression of activation marker P-selectin in patients compared to healthy donors. Moreover, immunofluorescence imaging demonstrated close association between TEPs and $ALDH1^+$ CSCs in mammospheres, suggesting a previously unrecognized interaction. Finally, aspirin treatment significantly reduced TEP activation by targeting P-selectin and disrupted TEP-tumor cell associations. These results confirmed that TEPs in BC exhibit distinct structural and functional alterations that support metastasis and potentially interact with CSCs to drive tumor progression. Targeting TEPs with aspirin disrupts these interactions and mitigates their pro-tumorigenic effects. These findings underscore the potential of TEP-targeted therapeutic strategies in BC treatment.

Introduction:

Platelets were first discovered in 1882 by, Giulio Bizzozero. However, the first accurate description of platelets was proposed by Schultz who described them to be as uncoloured spherules or granules and proposed the term granular masses for

these structures [1]. The primary function of platelets has long been thought to be to wound healing and ensure uninterrupted blood flow in the body [2]. They develop from the megakaryocytes which mature to form pseudo membrane blebs that eventually extend and breakout to form platelets [3]. With an average life span of 7-10 days, they maintain haemostasis by aggregating with other platelets and attaching to the vascular endothelium and thereby initiating the coagulation cascade that form fibrin mesh, which eventually prevents blood loss [3]. The presence of several receptors on their outer membrane is critical for its functioning. These receptors facilitate their attachment during aggregate formation as well as to endothelial surfaces [3].

The normal platelet count of healthy individuals is 1.5-2.5 lakhs per microlitre of blood [4]. When this number goes above the normal range the condition is termed as 'thrombocytosis' or 'thrombocythemia' and when the average count falls below the expected level the condition is termed as thrombocytopenia [4].

According to the National Heart, Lung and Blood Institute, USA, the major difference between thrombocythemia and thrombocytosis is that in the former the increase in platelet count is not attributed to another health condition whereas in the latter, this upsurge is diagnosed when there is another underlying disease or condition. Thrombocytosis has been reported to be a diagnostic factor for undetected cancer [5]. Solid tumors induce the production of IL-6, which in turn stimulates the level of thrombopoietin or TPO [5]. TPO in turn promotes platelet production from the megakaryocytes [5]. Gasic et al. first described the association between platelet number and potential for metastasis. This swarm of platelets help tumors escape the surveillance process by the immune cells in circulation.

Once in the circulation, cancer cells induce direct activation of platelets by facilitating the interaction between integrins on the surface of tumor cells and corresponding receptor on the surface of platelets and this promotes the formation of tumor cell induced platelet activation and aggregation [6]. One such interaction is the association between platelets P-selectin with PSGL1 on the surface of tumor cells. This interaction allows the formation of bio-shield by activated platelets around the tumor cells, allowing them to escape the process of immunosurveillance.

In the late 1960's Dr. Harvey Weiss reported the anti-thrombotic effect of acetylsalicylic acid or aspirin which inhibits platelet activity by acetylation of

cyclooxygenase at amino acid serine 529. This prevents the substrate arachidonic acid from accessing the catalytic site and thereby preventing the formation of thrombus [10]. Along with-it aspirin also prevents the production and secretion of various pro-angiogenic factors like VEGF to prevent angiogenesis and also have an inhibitory effect on the production of P-selectin [11]. Overall low dose aspirin has been proven to reduce the risk of several cancers [12].

All studies till date have been restricted to the interaction of whole tumor cells with platelets. Our investigation reports the status of platelets in peripheral blood and TME with respect to the less aggressive luminal-A and most aggressive TNBC subtypes, which is an area of paucity of research along with their overall interactome to promote stemness, metastasis and angiogenesis in breast cancer scenario.

Objectives:

- To study the morphological and functional differences between platelets of healthy individuals and breast cancer patients
- To decipher the impact of aspirin on platelet activity
- To elucidate the role of TEPs in tumor progression

In comparison to healthy controls, platelets in BC patients exhibited distinct morphological peculiarities by forming aggregates of extensive size. Also, an elaborate system of lamellipodia was observed in these platelets, indicating towards their activation. To confirm this, status of activated platelet marker P-selectin was analysed via flow-cytometry on these platelets. It was revealed that expression of P-selectin was elevated in BC patients in comparison to healthy controls, thus validating their existence as activated 'tumor educated platelets or TEPs' in BC. Additionally, by blocking TEPs P-selectin with aspirin, this activation could be reverted, thereby divulging the anti-cancer properties of aspirin in BC. Functionally, these TEPs form an alliance with the vicious CSCs by interacting with PSGL1 on the surface of CSCs thereby augmenting the outspread of tumor cells.

Materials and Method:

Isolation of platelets

Platelets were isolated from peripheral blood by double centrifugation method. Briefly, 5ml of blood was collected in ACD anticoagulant from breast cancer patients as well as from healthy donors who have not consumed anti-platelet drugs for the last six months. Blood was

centrifuged initially at 260g for 20 minutes to separate the platelet rich plasma. This plasma was again centrifuged for 20 minutes at 800g to separate the platelet pellet and platelet poor plasma. The platelet poor plasma was discarded and the pellet was thoroughly washed thrice with platelet wash buffer and finally dissolved in Tyrode's buffer.

Morphological analysis by SEM

To elucidate the changes in morphology of platelets from healthy individuals and breast cancer patient's SEM microscopy was performed as described in section 3.3.4 of materials and methods. Briefly, Platelet rich plasma around 20µL was taken on a cover slip and a uniform smear was drawn. This smear was fixed and dehydrated followed by critical point drying, coating with carbon and visualization using Zeiss Gemini scanning electron microscope.

Flow cytometric analysis

CD41, P-Selectin, PSGL1 and ALDH1 expression of resting and activated platelets were analysed by flow cytometry. Briefly, the samples were initially stained with primary antibodies. Following incubation period of 30 minutes at 4ºC the samples were washed to remove unbound molecules. Fluorescently tagged secondary antibody was then added and the samples were incubated for 30 minutes at room temperature, followed by washing and fixation in 1% paraformaldehyde. Data acquisition was done using BD LSR Fortessa and analysed using flow jo.

Confocal microscopy

Cryo-fixed tumor samples were cut into 5µm thick sections and stained with CD41 and P-selectin primary antibodies post blocking with 5% BSA. The sections were washed and stained with fluorescently tagged secondary antibody, mounted with DAPI and visualized using Olympus Fluoview FV3000.

Immunofluorescence microscopy

For mammosphere staining, post co-culture, spheres were stained with ALDH1 and TEPs with P-selectin. Following the incubation period, around 20µL of the samples were taken on glass slide and the droplet was allowed to air dry. These were then mounted with DAPI and visualized with Olympus BX 53 microscope. Images were analysed and quantified using ImageJ software.

Immunohistochemistry

Tissue sections were stained for CD41 and P-selectin as described in section 3.3.26 of materials and method. Briefly, sections were initially treated with 3% H_2O_2 (Merck 17544) in methanol for 30 minutes, to inhibit endogenous peroxidase

followed by non-specific inhibition by 5% BSA at RT for 30 minutes. Post blocking the samples were incubated with primary antibodies CD41 and P-selectin overnight. After thorough washing with PBS-Tween 20, HRP tagged secondary antibody was added to the samples and AEC substrate was used to develop chromogenic colour according to manufacturer's protocol. Counterstaining was performed using Hematoxylin (Merck-HX68597049) for 40 seconds and then mounted with Vectamount (Vector Laboratories H5501). Image was acquired using Carl Zeiss Plan Achromat bright field microscope along with Axiocam color camera.

Blocking platelets with aspirin

To block the activity of tumor educated platelets (TEPs), antiplatelet agent aspirin was added at a concentration of 100μm/mL to the platelet rich plasma isolated as described above. Following incubation for 30 minutes at 37ºC, the platelet rich plasma was again centrifuged. The pellet so obtained was washed with platelet wash buffer thrice and finally dissolved in Tyrode's buffers.

Protein-protein interaction visualization

String (Search Tool for the Retrieval of Interacting Genes/Proteins) database version 8.0 was utilized to decipher the interactions between various proteins of interest.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software 8.4.2. Significance was drawn from unpaired Student T-test or One-way ANOVA / Two-way ANOVA followed by Tukey's multiple comparison. To ensure normal distribution pattern, normality and log normality tests were performed. All data passed the Shapiro-Wilk test of normal distribution. Entire statistical analysis was performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, USA). Experimental results with $p \leq 0.05$ have been considered as significant.

Results:

Thrombocytosis is prevalent in breast carcinoma and these platelets are morphologically different from normal

Retrospective study of patient records admitted at Chittaranjan National Cancer Institute, Kolkata, India, revealed that

breast carcinoma patients had platelet count >3lakhs/microL of blood (n=20 for healthy control, luminal-A and TNBC patients). According to National Heart, Lung and Brain institute, USA, the average platelet counts of healthy females between the age groups 34-49 and 50-64 is ~2.5lakhs/microL [13] [14]. Further, upon

comparison between luminal-A and TNBC subtypes, it was observed that this augmentation in platelet count was more in TNBC (average platelet count ~ 4.7lakhs/microL) than luminal-A (average platelet count ~ 3.1 lakhs/microL). Interestingly, in benign breast tumor patients (n=2), the mean platelet count was in the normal range (average platelet count of benign tumor patients ~ 2.2 lakhs/ microL, average platelet counts of healthy females ~ 2.17 lakhs/ microL). This further strengthened our observation of thrombocytosis due to underlying cancer condition (Fig 1.1A). Additionally, thrombocytosis was also observed in other malignancies like rectal, stomach, buccal mucosa, penis and prostate condition (Fig 1.1A). All samples followed log normal distribution (Fig 1.1A). Moreover, it was also observed that with time there was a significant increment in platelet count in BC patients (Fig 1.1B). Also, a positive co-relation between platelet count and time was noted in both luminal-A and TNBC patients (Fig 1.1B). Further, when compared between the stages this increment in platelet count was more in stage IV BC followed by stage III and stage II in both luminal-A and TNBC subtypes (Fig 1.1C).

Next, morphological analysis revealed that, in comparison to healthy resting platelets which have a discoid appearance with 1.5μm^2 area, platelets of cancer patients were of altered morphology and formed huge aggregates. The average area of the aggregates in luminal-A patients was ~9.5μm^2, whereas in TNBC it was ~70μm^2 (n=6).

Additionally, they underwent cytoskeletal rearrangement producing numerous filopodia and lamellipodia. Presence of these structures facilitates the adhesion of platelets to each other during aggregate formation as well as to the tumor cells, thereby protecting them from immunosurveillance process (Fig 1.1D).

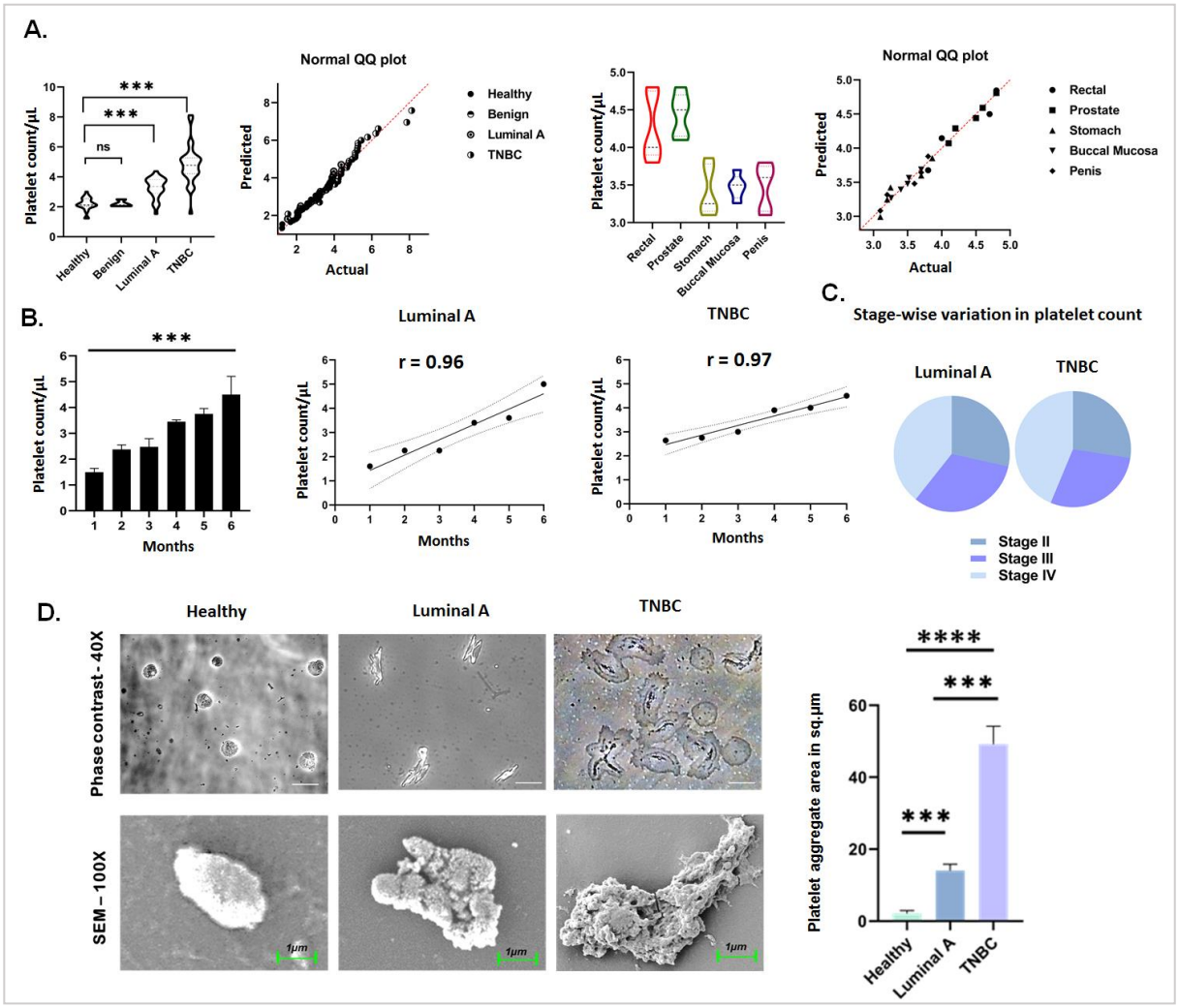


Figure 1.1: Thrombocytosis is prevalent in breast carcinoma and these platelets are morphologically different from normal - A. Representative box-violin plot of platelet counts in healthy (n=20), benign (n=2), luminal-A (n=20) and TNBC (n=20) patients. Scatter plot denoting log normal distribution of samples under analysis is portrayed. One-way ANOVA followed by Tukey's multiple comparison tests was performed to draw statistical significance. Multi-colour box-violin plot depicting platelet count in rectal, prostate, stomach, buccal mucosa and penis carcinoma. Scatter plot show-casing log normal distribution of the sample. **B.** Bar graph demonstrating increase in platelet count (count/µL of blood) with time (in months) is presented. Individual scatter plot delineating positive correlation between platelet count (count/µL of blood) and time (in months). **C.** Stage-wise variation in platelet count in luminal-A and TNBC patients is presented in pie-chart, luminal-A (n=20) and TNBC (n=20). **D.** Representative phase contrast (40X) and scanning electron microscope images (100X) respectively of healthy platelets and platelets from luminal-A and TNBC patients. Scale bar 1µm of each image. In bar-graph, (mean±SD) of platelet area in μm^2 of all the three groups is provided. Statistical significance drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=6) *****p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Platelets of breast carcinoma patients are in an activated state in the peripheral blood and TME- Modulatory effect of aspirin

Given the prevalence of thrombocytosis and cytoskeletal rearrangement in cancer, we next sought to investigate the functional alteration of these platelets. Following their isolation from peripheral blood by double centrifugation method from healthy donors and breast cancer patients, flow cytometric analysis of P-selectin (platelet activation marker) and CD41 (healthy platelet marker) was performed. In comparison to healthy normal (n=11), expression of P-selectin was elevated in both luminal-A (n=11) and TNBC patients (n=11) than CD41. Further, amongst these subtypes, this upsurge was more prominent in TNBC than luminal-A. On the other hand, normal healthy platelets were predominantly $CD41^{+}$ (Fig 1.2A). In addition to peripheral blood, functional status of platelets within the tumor microenvironment (TME) was investigated by screening of breast tumor sections. Immunohistochemical analysis of CD41 and P-selectin-stained micrographs showcased infiltration of $CD41^{low}$P-$selectin^{high}$ platelets within TME of both luminal-A and TNBC patients, with higher infiltration percentage in TNBC than luminal-A (Fig 1.2B). This was further confirmed by confocal microscopy imaging that also demonstrated a similar trend of infiltration of $CD41^{low}$P-$selectin^{high}$ TEPs into the TME of both luminal-A and TNBC subtypes (Fig 1.2C)

In an attempt to revert this activation, platelets were treated with acetylsalicylic acid or aspirin as described. Post blocking the platelet pellet was analysed for P-selectin expression by flow cytometry. Aspirin treatment reduced P-selectin expression in both the subtypes, but could not bring down the level to normal (Fig 1.2D). Taken together, from this report it can be concluded that both in the peripheral blood and TME platelets of breast cancer patients remain activated and treatment with aspirin can partially revert this state.

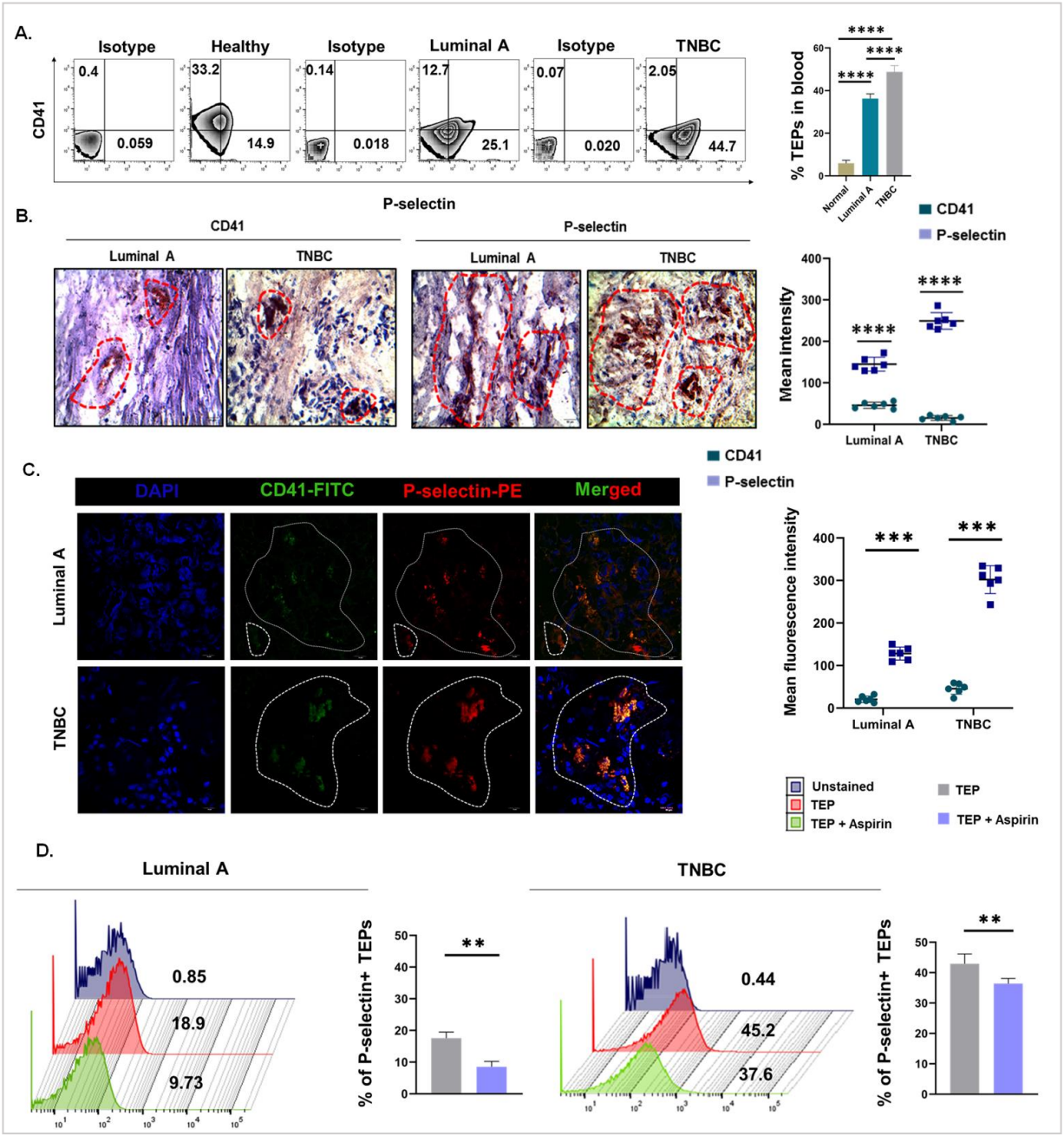


Figure 1.2: Platelets of breast carcinoma patients are in an activated state in the peripheral blood and TME - Modulatory effect of aspirin - A. Flow cytometric zebra plots showcasing frequencies of CD41, P-selectin, in healthy donors (n=11) and luminal-A (n=11), TNBC patients (n=11). Bar diagrams representing % of TEPs across all the three groups keeping (mean±SD) and statistical significance established from one-way ANOVA followed by Tukey's multiple comparison test. **B, C**. Representative immunohistochemical and confocal microscopy images at 40X and 100X magnification respectively of breast tumor sections from luminal-A and TNBC patients stained with CD41 and P-selectin. Dotted lines representing stained zones are provided. In graph, (mean±SD) of mean intensity is provided with statistical significance inferred from unpaired non-parametric t-test, followed by two-tailed p value. **D**. Flow cytometric histogram plots depicting reduction in expression of P- selectin in TEPs upon treatment with aspirin in both luminal-A (n=11) and TNBC (n=11). In bar-graph, (mean±SD) is presented with statistical significance inferred from unpaired non-parametric t-test, followed by two-tailed p value was performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Platelets P-selectin interacts with PSGL1 on the surface of BCSCs to promote disease progression

Numerous studies have reported the interactions between tumor cells and platelets leading to the formation of TCIPA (tumor cell induced platelet aggregation) and further their prominent impact on metastasis. We wanted to investigate whether TEPs interact with the cells that are the initiators of metastasis, that is the cancer stem cells (CSCs) and if this synergism has a role to play in promoting the overall aggressiveness. As it has already been proven that TEPs express P-selectin, the next target was to elaborate if CSCs express its corresponding ligand PSGL1. However, prior to this, it was first investigated if at all an interaction occurs between TEPs and CSCs. For this, BCSCs of MCF-7 and MDA-MB-231 were grown into mammospheres in 3D stem cell enrichment setup and co-cultured with TEPs. Following this BCSCs were stained with ALDH1 and TEPs with P-selectin and observed by immunofluorescence microscope. It was revealed to us that TEPs remained in close proximity to BCSCs in both MCF-7 and MDA-MB-231 depicting their physical interaction (Fig 1.3A). The expression of PSGL1 on CSCs was investigated next. Pseudocolour flow cytometric plots depicted that both MCF-7 and MDA-MB-231 tumor cells and their corresponding BCSCs express PSGL1. However, this expression was more pronounced in BCSCs than the whole tumor. Additionally, it was also observed that the expression of PSGL1 was comparatively more on BCSCs of TNBC than luminal-A (Fig 1.3B). As PSGL1 was more prominent on BCSCs of TNBC, we investigated if it has any role to play in promoting aggressiveness by mediating metastasis and stemness. String analysis disclosed direct interaction of PSGL1 with vimentin and CD44 which further interacts with the other genes and transcription factors that are pivotal for both the events (Fig 1.3C). Finally, to prove the intra-tumoral interaction between BCSCs and TEPs via P-selectin-PSGL1 axis, single cell suspension of breast tumor tissue samples of luminal-A and TNBC subtypes were stained for $ALDH1^{+}PSGL1^{+}$population and analysed via flow cytometry. In TNBC, greater population of $ALDH1^{+}$ $PSGL1^{+}$ BCSCs was noted than luminal-A (Fig 1.3D). This proved that TEPs within the TME interacts vividly with the CSCs, mediated by P-selectin-PSGL1 which in turn promoted overall aggressiveness and advancement of the metastatic disease.

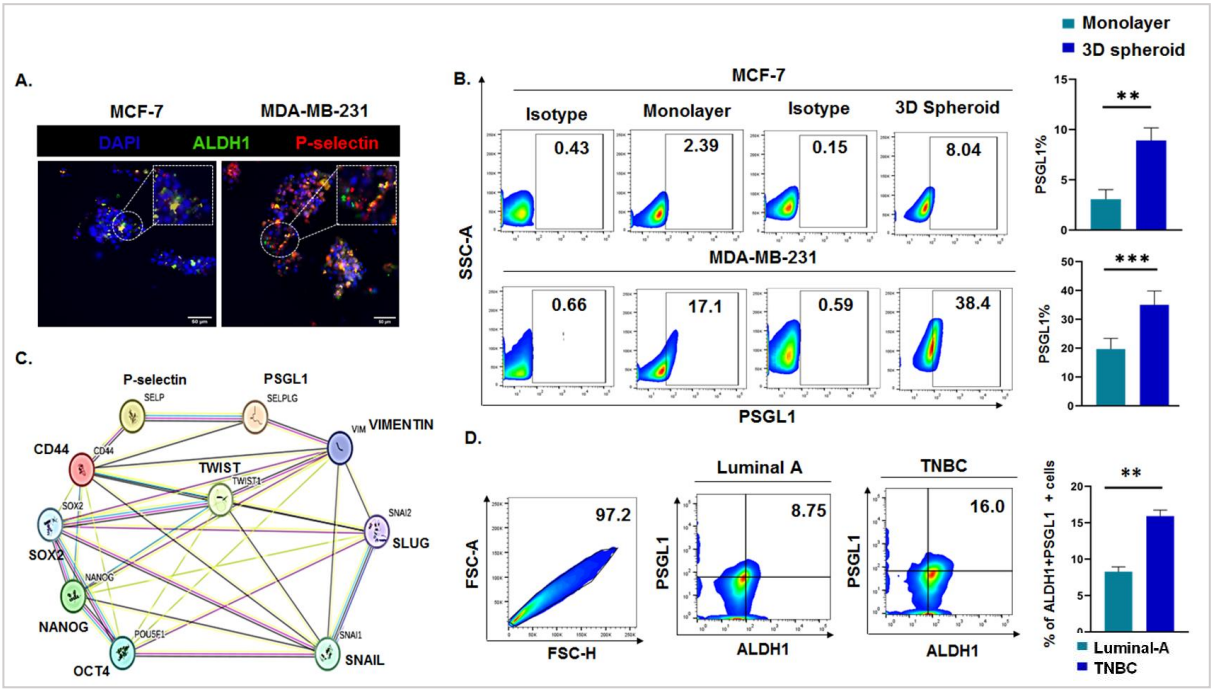


Figure 1.3: Platelets P-selectin interacts with PSGL1 on the surface of BCSCs to promote disease progression – A. Representative immunofluorescence micrographs at 40X magnification of mammospheres of MCF-7 and MDA-MB-231 stained with ALDH1-FITC and P-selectin-PE. Enlarged image of their interaction is provided in inset. **B.** Pseudocolour flow cytometric plots showcasing frequency of PSGL1in monolayer and 3D spheroids of MCF-7 and MDA-MB-231. In bar-graph, (mean±SD) of PSGL1% is provided and statistical significance is inferred from unpaired non-parametric t-test, followed by two-tailed p value. **C.** Identification of the genes responsible for linking PSGL1 with stemness and metastasis using STRING database is given. **D.** Flow cytometric dot plots representing frequency of $PSGL1^{+}ALDH1^{+}$ fraction BCSCs of both luminal-A and TNBC (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Discussion:

The pivotal role of platelets has been studied in several malignancies including ovarian, myeloma, colon and lewis lung carcinoma [15]. It has been reported that activated platelets protect circulating tumor cells from shear stresses and immune surveillance, promote the formation of metastatic niches by recruiting stromal cells, accelerate tumor angiogenesis and vascular remodelling [16]. On the other hand, tumor cells may also "educate" platelets to an active state. Platelets respond to cancer in a systematic and local way during the 'bidirectional' tumor-platelet interactions [16]. They continuously absorb and enrich free proteins, nucleic acids, vesicles and particles, which causes changes in their RNA and proteomics expression profile. As a result, these platelets are referred to as 'tumor educated platelets' (TEPs) [16].

In this current study, we describe how platelets contribute to the progression of BC in two subtypes: luminal-A (the less aggressive type) and TNBC (the most aggressive type). Patients of both the

subtypes presented higher than normal platelet count suggesting the possibility of thrombocytosis or thrombocythemia. Analysis of their medical records revealed that the patients under consideration did not suffer from any other health conditions at the time of diagnosis that could boost the production of platelets. This confirmed that they did not suffer from thrombocythemia, but it is the prevalence of thrombocytosis in them.

Also, a notable increment in platelet counts with the progression of time across different stages of BC, specifically within luminal-A and triple-negative breast cancer (TNBC) subtypes was noted. Notably, this increase in platelet count was more pronounced in advanced disease stages, with stage IV patients exhibiting the highest levels, followed by stage III and stage II, respectively.

This trend suggests that as BC progresses; there is a corresponding elevation in platelet count, which may reflect underlying biological processes such as tumor-induced thrombopoiesis or thrombocytosis. The greater elevation observed in stage IV patients may be attributed to a more aggressive tumor phenotype, greater tumor burden, or metastatic activity, all of which have been associated with heightened platelet activation and production.

Moreover, the positive correlation observed between platelet count and time progression further reinforces the potential role of platelets in the tumor microenvironment and disease evolution. Platelets have been implicated in tumor growth, angiogenesis and metastasis through the secretion of growth factors and cytokines. Therefore, a temporal increase in platelet count may not only be a consequence of cancer progression but might also actively contribute to it.

Moreover, platelets of cancer patients formed huge aggregates and had an elaborate system of filopodia and lamellipodia. The distinctive feature of activated platelets is the formation of aggregates. This demonstrated that platelets of luminal-A and TNBC subtypes remain active in the peripheral blood. An important molecule for the formation of aggregates is P-selectin. Platelet's α-granules typically include P-selectin. They degranulate after activation, revealing P-selectin on their surface, making it an important biomarker of activated platelets. Furthermore, TNBC had elevated percentage of P-selectin^{+} platelets than luminal- A. Therefore, a higher percentage of P-selectin^{+} activated platelets may correspond to an increased level of malignant aggression. Along with the peripheral blood, P-selectin^{+} platelets were

also noted within the breast TME of luminal-A and TNBC patients, confirming their integral role in malignancy.

Given the prevalence of TEPs in BC, we next sought to ascertain how TEPs function within the TME to promote overall aggressiveness of the disease. We discovered that BCSCs express high levels of PSGL1 (P-selectin Glycoprotein Ligand 1), which is the ligand of P-selectin. Surprisingly, PSGL1 expression was comparatively higher on BCSCs than the whole tumor cells in both MCF-7 and MDA-MB-231. As PSGL1 was more pronounced on the surface of BCSCs, it suggested that PSGL1 may have a role in augmenting the aggressiveness of the disease. String database analysis confirmed that PSGL1 directly interacts on one hand with stem cell marker CD44, which in turn interacts with the pivotal CSC transcription factors OCT4, SOX2, NANOG and on the other hand with EMT marker VIMENTIN. VIMENTIN in turn forms an interactome with TWIST, SNAIL and SLUG. Thus, PSGL1 turns out to be an important linker between stemness and EMT. TEPs and BCSCs physically interact with each other in both the subtypes of BC. Thus, BCSCs PSGL1 binds with TEPs P-selectin and this kinship facilitates tumor progression. Further, previous studies have also demonstrated that PSGL1 has prominent role in metastasis and stemness [17]. This further strengthened our observation of high PSGL1 expression in TNBC than luminal-A. This study advocates for the importance of TEPs as potential biomarker in BC diagnosis. Their alliance with vicious BCSCs to promote disease advancement acquiesce them as a novel restorative agent. Accordingly, novel anti-platelet agent, aspirin was used to block the activation of TEPs. Aspirin works by irreversibly inhibiting the enzyme cyclo-oxygenase (COX-1) which is required to make the precursors of thromboxane within platelets. This reduces thromboxane synthesis. Thromboxane is required to facilitate platelet aggregation and to stimulate further platelet activation.

Therefore, the overall observations support the dominating role of platelets in promoting disease aggression and further advancement.

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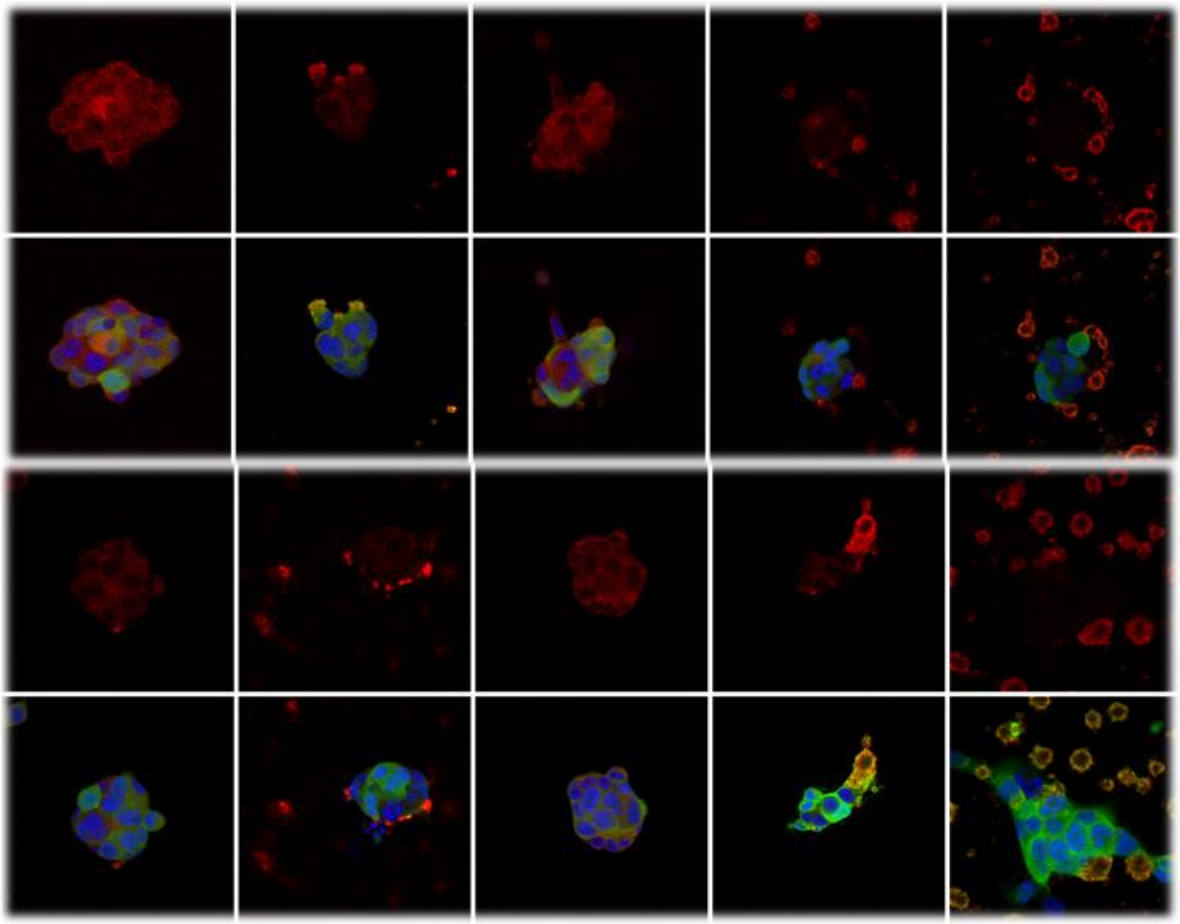
Chapter 2

Effect of Tumor educated platelets on stemness of breast cancer stem cells



Confocal microscopy image of PSGL1^{+}(FITC) mammospheres interacting with P-selectin^{+} (PE) tumor educated platelets from breast cancer patients at 100X magnification

Effect of Tumor educated platelets on stemness of breast cancer stem cells

Abstract:

The role of tumor educated platelets (TEPs) in promoting metastasis is widely established. Along with TEPs numerous other cell types within the TME are also accountable for progression of the disease from their primary to secondary site. A rare population of quiescent cells within the tumor, known as cancer stem cells (CSCs) play pivotal role in initiation as well as encourage aggressiveness of the disease. Given the shared similarities in properties between TEPs and CSCs, we hypothesized that if TEPs form any alliance with the CSCs to augment the metastatic outspread of tumor cells. Through numerous *in-vitro* and *in-vivo* assays, we uncovered that TEPs interact with breast cancer stem cells (BCSCs) to promote stemness of these cells. The physical interaction between TEPs and BCSCs was confirmed via confocal imaging. Further, results of tumor mammosphere assay revealed that in stem cell enrichment set-up TEPs elevated the number of spheroids in MCF-7, MDA-MB-231 cell lines as well as in luminal-A and triple negative breast cancer (TNBC) samples. Moreover, these TEP influenced BCSCs were highly clonogenic and produced increased number of colonies in soft agar bed, indicating towards influence of TEPs on tumorigenic potential of BCSCs. Interestingly, analysis of MDR-phenotype related genes showcased uptrend in the expression of *bcrp1, abcc1 and abcb1* in BCSCs co-cultured with TEPs, suggesting their involvement in therapy resistance. Upon further investigation of the transcription factors regulating stem cell fate it was revealed that impact of TEPs on BCSCs resulted in surge of NANOG-OCT4-SOX2. Blocking the activity of TEPs with aspirin reduced this uptrend in stemness, tumorigenicity and MDR phenotype of BCSCs along with the concomitant reduction in the expression of NANOG-OCT4-SOX2.These observations thus strengthen our hypothesis and validate the dominant influence of TEPs on the tumorigenic properties of aggressive CSCs, thereby directing the overall progression of the disease.

Introduction:

As, TEPs directly promote expansion of BCSCs, characterization of these TEP influenced BCSCs is crucial in determining its overall impact on tumor

progression. It has been established that a crucial element in the growth of malignancy is cancer stem cells [1] [2]. They are thought to be a unique population with strict hierarchical organisation and strong evidence suggests that these cells have a flexible cellular state regulated by dynamic CSC-niche interactions [1] [2] [3].The ability of a cell to reproduce its lineage, proliferate into differentiated cells and interact with its surroundings to balance quiescence, proliferation and regeneration is what is referred to as stemness [3]. Adult stem cells exhibit these characteristics during tissue regeneration; however, CSCs act more like their malignant counterparts. CSCs display stemness in various circumstances, including the sustaining of cancer progression and the interaction with their environment in search for key survival factors. As a result, CSCs can recurrently persist after therapy [4].The primary contributing factor of cancer chemotherapy's failure is multidrug resistance. Through the hydrolysis of ATP, ATP-binding cassette transporters, also known as ABC transporters, move a variety of substrates across biological membranes. Chemotherapeutic drugs are rapidly effluxed by high concentrations of active ABC transporters, which contributes to multidrug resistance [4] [5]. Due to the fact that CSCs can continue to exist and multiply even after the majority of cancer cells have been eradicated, the density of CSCs within the tumor is enhanced after chemotherapy. CSCs express many proteins in common with early embryonic stem cells, especially OCT4, NANOG, and SOX2 [4] [5] [6]. These transcription factors play a key role in maintaining CSC pluripotency and its self-renewal property. Multiple research studies have confirmed the pro-tumorigenic role of TEPs in cancer. TEPs have been found to shield circulating tumor cells (CTCs) with a protective "bio-armour," aiding their escape from immune surveillance. Despite these advancements, metastasis continues to be the primary cause of cancer-related mortalities. This highlights the urgent need to identify additional non-conventional biological components and signalling pathway crosstalk's that contribute to tumor cell dissemination.

In this context, we investigated the interplay between TEPs and CSCs, as both of these players have been reported to be the key drivers of metastasis. In order to decipher the impact of TEPs on the key attributes of BCSCs like stemness and tumorigenicity *in-vitro,* 3D mammosphere formation and soft agar colony formation assays were performed.

A significant upregulation in the number and size of spheroids as well as agar

colonies was noted in presence of TEPs. Additionally, TEPs assure tumor relapse through development of multidrug-drug-resistance intrinsically. A notable increment in the expression of ABC drug efflux pumps such as *bcrp1*, *abcb1* and *abcc1* was noted in TEP influenced CSCs. These cells will thus remain elusive to commonly administered treatments and continue to thrive. However, targeting TEPs with aspirin (resting platelet RP), demonstrated positive response by downregulating the expression of MDR genes and also exhibited a significant decline in the number of mammospheres and soft agar colonies.

Objectives:

- To evaluate and characterize TEPs influence on stemness, tumorigenicity and MDR phenotype of BCSCs and thus, delineate their role in tumor aggressiveness.
- To validate the involvement of P-selectin-PSGL1 axis in mediating the interaction between TEPs and BCSCs.
- To elucidate the impact of TEPs on stemness regulating transcription factors like NANOG, OCT4 and SOX2

Materials and Method:

Cell lines and culture

MCF-7 (luminal-A) and MDA-MB-231(TNBC) cells were cultured in MEM and RPMI complete media respectively supplemented with 10% (v/v) heat inactivated FBS, 2mM L- glutamine, 100 U/ml penicillin and 100µg/ml streptomycin in incubator at 37°C and 5% CO_2. Cells were maintained for 10-12 passages and all experiments were performed within 6 months of purchase.

Processing of tumors

Procured breast tumors were processed as described in section 3.3.12 of Materials and methods.

Co-culture

Single cell suspensions of MCF-7 and MDA-MB-231 cell lines and luminal-A and TNBC patient samples were cultured with TEPs and RP in 1:100 (BCSC: platelet) ratio for 24 hours. After the incubation period, the culture media with platelet suspension was removed and the cells were collected by trypsinization and centrifuged for further analysis.

Magnetic cell sorting: From the co-culture setup BCSCs were sorted by using magnetically labelled $CD44^+/CD24^-$ antibodies and cell purification was carried out according to manufacturer's protocol (MicroBead kit, Miltenyi Biotech, Germany).

CSC enrichment culture and tumorsphere assay

CSCs ($1x10^4$) were cultured in stem cell enrichment media and plated on ultra-low adherent plates (Corning, New York, USA) and maintained for 7 days. Tumorspheres were micrographed and their count from 5 random fields was documented. Their area was calculated using ImageJ software. Tumorspheres so formed were dissociated with trypsin and were centrifuged to produce single cell suspensions which were further analysed.

Soft agar colony formation assay

Briefly, $5x10^3$ cells were cultured on upper 0.35% soft agar layer along with 1mL culture media. This layer was placed upon botton agar bed of 0.7% agar and cell culture medium (MEM and RPMI complete media respectively for MCF-7 MDA-MB-231). The culture setup was maintained for 21 days. The colonies were finally micrographed and the number of colonies was quantified.

RT PCR

Co-culture samples were processed for RNA extraction and cDNA preparation according to manufacturer's protocol. Briefly, total RNA content of single cell suspensions was extracted by Trizol (Ambion, Thermo Fisher Scientific, MA, USA). cDNA was synthesized from it using Revert Aid First Strand cDNA Synthesis Kit. Reverse transcriptase PCR was performed using 2X Go Taq Green Mix. Electrophoresis was done using 1.5% agarose gels and stained with ethidium bromide.

Flow cytometry

Cells were stained with fluorescently tagged antigen specific antibodies against CD44, CD24, NANOG, OCT4, SOX2 and incubated in dark at $4^{\circ}C$ for 30 minutes. For intracellular molecules, cells were treated simultaneously with 0.2% saponin (permeabilization buffer). Cells were finally washed, fixed with 1% paraformaldehyde and data was acquired using BD LSRFortessa X-20 Cell Analyzer (Becton Dickinson, New Jersey, USA).

Confocal microscopy

Binding of P-selectin$^+$ TEPs to PSGL1$^+$BCSCs was visualized by confocal microscopy as described in section 3.3.25 of Materials and methods and images were analysed using ImageJ software.

Statistical analysis

All data passed the Shapiro-Wilk test of normal distribution.Statistical significance was drawn from either One-way/ Two-way analysis of variance followed by Tukey's multiple comparison test. Entire statistical

analysis was performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, USA). Experimental results with $p \leq 0.05$ have been considered as significant.

Results:

TEPs augment stemness in BC by upregulating $CD44^{+}/24^{-}$ population of BCSCs

Given the possible synergism of TEPs and BCSCs in tumor advancement, their coaction was further analysed. We investigated whether this mutual association between TEPs and BCSCs have any role in augmenting the specific hallmarks of CSCs specifically for stemness, tumorigenicity, clonogenicity and MDR phenotype. For this, post co-culture, $CD44^{+}CD24^{-}$ BCSCs were magnetically sorted from BC cell lines MCF-7, MDA-MB-231 and single cell suspensions of solid breast tumor samples of both luminal-A and TNBC subtypes (Fig 2.1A). These cells were propagated in 3D-CSC-enrichment setup for 7 days for primary tumorsphere formation. A significant upsurge of tumorsphere count along with an increment in surface area was noted in presence of TEPs with respect to non-treated control CSCs across the cell lines and tumor samples. Further, blocking TEPs with aspirin, followed by co-culturing them with BCSCs, significantly lowered this surge, exhibiting lesser tumorsphere formation with reduced surface area (Fig 2.1B).

To investigate the changes in BCSC percentage post co-culture in 3D setup, cells were analysed via flow cytometry. It was revealed that there was a prominent increment in $CD44^{+}CD24^{-}$ BCSC-percentage in presence of TEPs with respect to non-treated control and this uptrend was down-regulated in aspirin treated TEPs or RPs in MCF-7, MDA-MB-231, luminal-A and TNBC samples (Fig 2.1C).

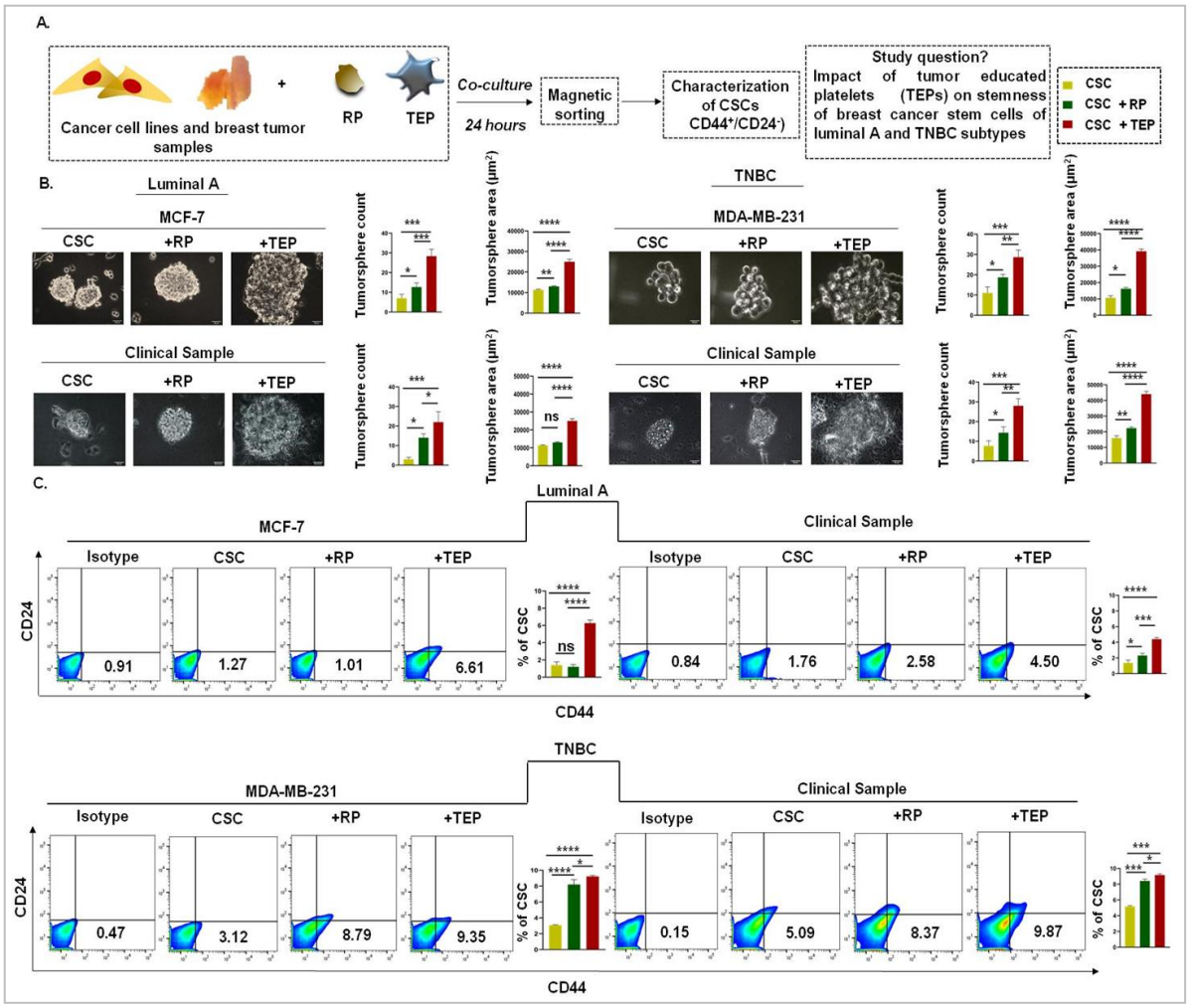


Figure 2.1: TEPs augments stemness in BC by upregulating $CD44^{+}/24^{-}$ population of BCSCs: A. Schematic representation of magnetic sorting for isolation and characterization of $CD44^{+}CD24^{-}$ BCSCs from the co-culture setup of cancer cell lines and single cell suspensions of breast tumor samples of luminal-A and TNBC subtypes with TEP and RP. *Inset*: yellow, green and red column denoting CSC, CSC+RP, CSC+TEP. **B.** Representative images of BCSCs magnetically sorted, post co-culture and layered in 3D enrichment setup for primary tumorsphere formation at 40X magnification for CSC, CSC+RP, CSC+TEP groups of MCF-7, MDA-MB-231, luminal-A and TNBC patient samples. In bar-graph, (mean±SD) for tumorsphere count and area is depicted. Statistical significance is inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=6). **C.** Pseudo-colour flow cytometric plots portraying changes in BCSC frequency in MCF-7, MDA-MB-231, luminal-A and TNBC across all the groups, post co-culture. Bar diagrams illustrating changes in frequencies of CSC, CSC+RP, CSC+TEP across the cell lines and patient samples keeping (mean±SD). Statistical significance is drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=6).*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

TEPs physically interact with BCSCs to mediate stemness via P-selectin-PSGL1 axis

With the established role of TEPs in promoting mammosphere formation and upregulating $CD44^{+}/24^{-}$ BCSC population in luminal-A and TNBC subtypes of BC, we next sought to verify if TEPs physically interact with BCSCs, mediated by P-selectin-PSGL1 axis, to promote stemness. For this, $CD44^{+}CD24^{-}$ BCSCs of MCF-7 and MDA-MB-231 were enriched in stem cell enrichment media to form mammospheres and co-cultured with TEPs and RPs, keeping non-treated (NT) mammospheres as control. Confocal microscopy imaging revealed that TEPs physically interact with BCSCs in both luminal-A and TNBC and this interaction is facilitated by P-selectin and PSGL1 (Fig 2.2A). Furthermore, it was noted that the administration of RP had a considerable impact on this binding, which in turn decreased the mean fluorescence intensity (Fig 2.2B).

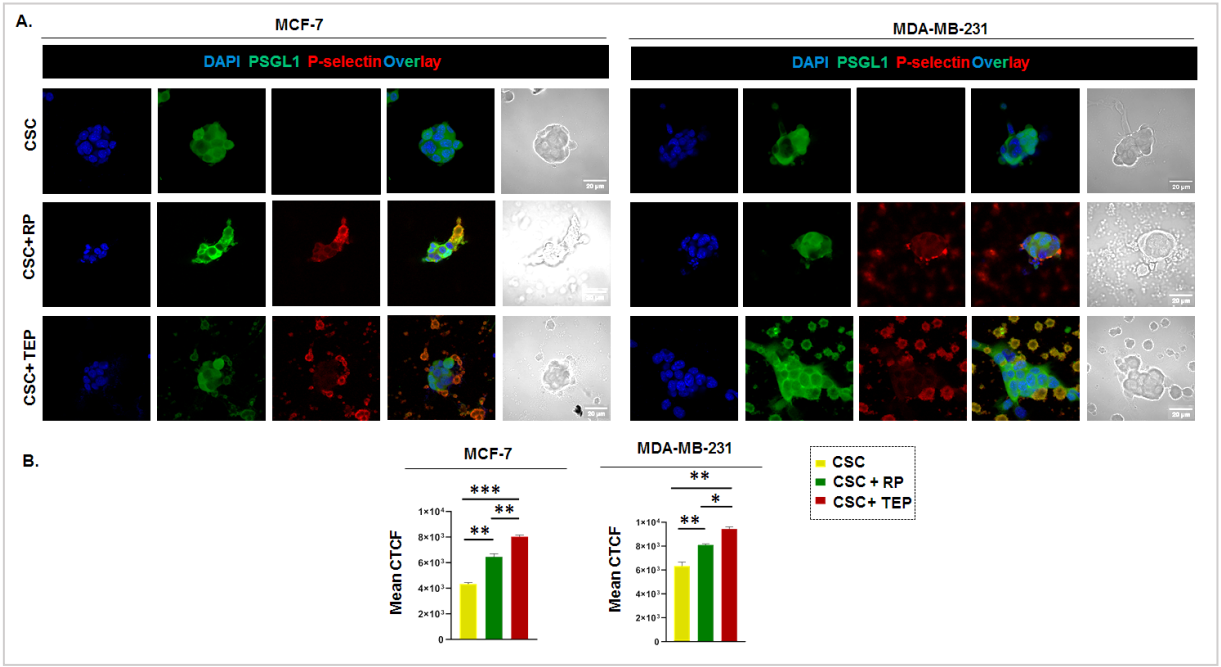


Figure 2.2: TEPs physically interact with BCSCs to mediate stemness via P-selectin-PSGL1 axis: A. Representative confocal microscopy images at 100X magnification of mammospheres stained with PSGL1 (FITC) and TEP and RP stained with P-selectin (PE) of MCF-7 and MDA-MB-231 is given. Bright field images of each respective panel at 100X magnification are presented. **B.** In bar-graph, (mean±SD) for corrected total cell fluorescence (CTCF) is depicted. Statistical significance is inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=3). *Inset*: yellow, green and red column denoting CSC, CSC+RP, CSC+TEP. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

TEP induces tumorigenicity and MDR phenotype along with orchestration of NANOG-OCT4-SOX2:

One of the key attributes of CSCs is tumorigenicity, which is their ability to produce clonogenic colonies from single cell. To delineate the possible role of TEPs in clonogenicity of CSCs, soft agar colony formation assay was performed *in-vitro*. Post co-culture, magnetically sorted, BCSCs were placed on double layer soft agar bed for 21 days for colony formation. TEP influenced BCSCs showed increased colony formation compared to non-treated BCSCs or RP influenced BCSCs across the cell lines (Fig 2.3A). The status of transcription factors like NANOG, OCT4, SOX2 which orchestrate the various attributes of BCSCs were evaluated next. A significant escalation in the expression of these molecules in presence of TEPs was noted from the flow cytometry analysis of the co-cultured samples (Fig 2.3B).

Resistance towards therapeutic agents make cancer treatment very much challenging. CSCs are wholly responsible for this therapy resistance. Analysis of MDR phenotype regulating genes via RT-PCR revealed increment in the expression of *bcrp1*, *abcc1* and *abcb1* in TEP influenced BCSCs of luminal-A and TNBC thus affirming the potential role of TEPs in therapy resistance of cancer (Fig 2.3C). These data suggest that tumor infiltrating TEPs do not remain dormant but interact with the highly malicious BCSCs and thereby generate extremely tumorigenic, clonogenic and therapy resistant variants.

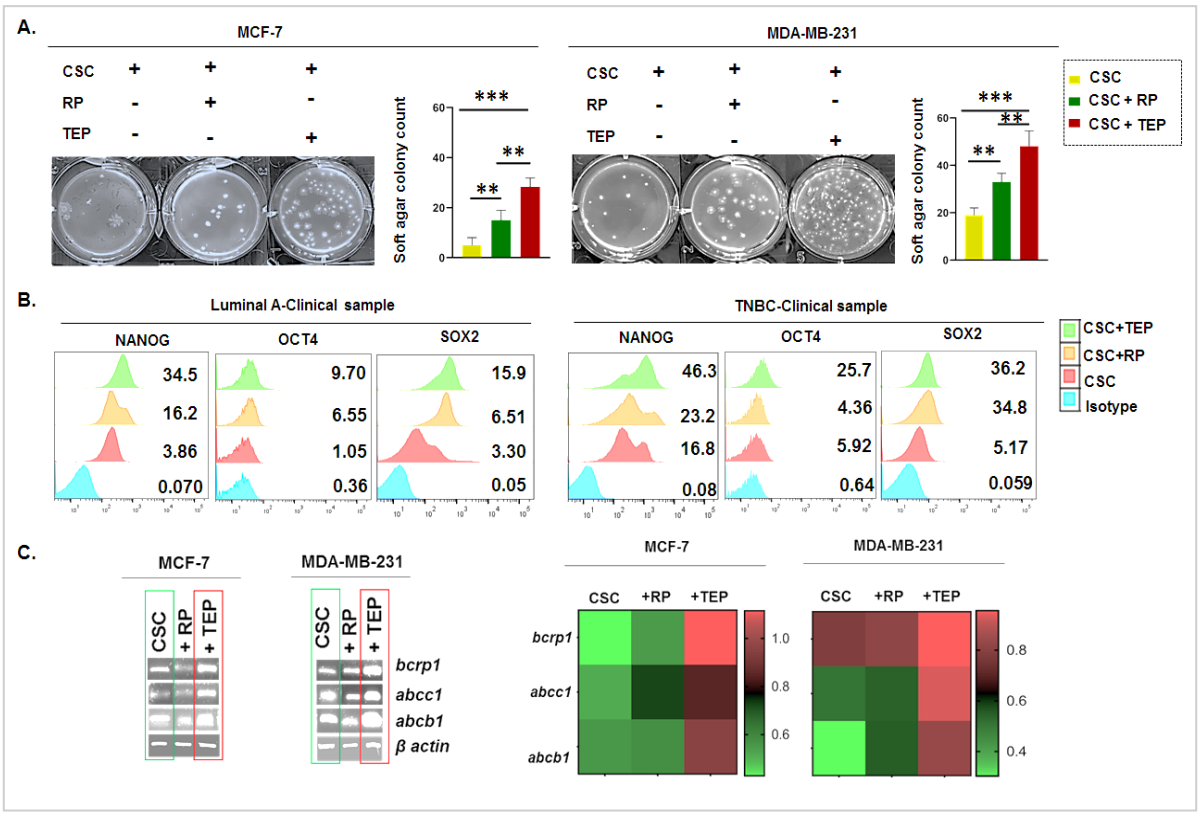


Figure 2.3: TEP induces tumorigenicity and MDR phenotype along with orchestration of NANOG-OCT4-SOX2: A. Representative soft agar colony images of NT-CSC, CSC+RP, CSC+TEP across the cell lines is presented. Number of colonies per field in each cohort is depicted in bar-graph (mean±SD) with statistical significance drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=3). *Inset*: yellow, green and red columns denoting NT-CSC, CSC+RP, CSC+TEP. **B.** Representative flow-cytometric offset histogram plots showcasing changes in the frequencies of transcription factors regulating stem cell fate NANOG, OCT4, SOX2 upon treatment with TEP and RP keeping NT-CSC as control (n=6), in both luminal-A and TNBC subtypes. *Inset*: blue, red, orange and green column denoting Isotype, CSC, CSC+RP, CSC+TEP respectively. **C.** mRNA expression of *bcrp1*, *abcc1*, *abcb1* is represented keeping *β-actin* as control in CSC, CSC+RP, CSC+TEP cohorts of MCF-7 and MDA-MB-231. Illustrative, heatmap depicting relative gene expression of MDR genes *bcrp1*, *abcc1*, *abcb1* is presented. Shades of green denote lower expression while red colour signifies higher concentration respectively. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Discussion:

Our preliminary investigation revealed that TEPs mediate disease aggressiveness by interacting with BCSCs physically via P-selectin-PSGL1 axis. To ascertain TEPs precise role on tumor progression, TEP influenced BCSCs were thoroughly characterized. Upon, in-depth investigation of this TEP and BCSC interplay using *in-vitro* cell lines and clinical breast tumor samples, it was found that TEPs elevated stemness of BCSCs, as observed from the increased number of mammospheres, with

enhanced surface area per field. Formerly it has been described that mamamosphere count is an in direct method of assessing stemness *in-vitro* [7] [8]. Thus, corroborating with their study, it can be concluded that TEPs promote stemness of BCSCs.

Along with stemness, CSCs are pivotal players of tumorigenesis [9] [10]. Several reports have mentioned that mutual interaction between CSCs and their niche play a critical role in the regulation of CSCs self-renewal and tumorigenesis as well [11]. Once the regulatory balance is destroyed, uncontrolled CSCs ultimately lead to tumor formation [11]. TEPs tend to hijack this regulatory mechanism, thereby leading to enormous production of CSCs that eventually lead to tumorigenesis, as evidenced from extensive generation of colonies in *in-vitro* soft agar colony formation assay.

Further, analysis of transcription factors that regulate various attributes of CSCs disclosed that under the influence of TEPs there was significant upregulation of NANOG, OCT4 and SOX2. Previous studies have observed that OCT4 and NANOG are overexpressed among numerous malignant solid tumor types that are immortal, undifferentiated and invasive [12]. Knockdown of these two factors may inhibit tumor development and growth [12]. On the other-hand, SOX2 is the master regulator of pluripotency and maintenance of tumor stem cell properties [13]. It is thought to play a significant role in maintaining characteristics of CSCs that produce heterogeneity within tumor and confer resistance to chemotherapy and radiation [13] [14].

Also, these TEP-BCSCs overexpressed genes related to MDR phenotype such as *abcc1, abcb1 and bcrp1*, making it quite difficult to target them by conventional therapies. Elevation of NANOG and OCT4 might play multi-faceted role by maintaining stemness and at the same time promote invasion and metastasis along with MDR phenotype [15]. Additionally, interaction of NANOG with metastasis promoting transcription factor TWIST, may enhance stemness and resistance to drugs by augmenting the expression of *bcrp1* [16].

However, it is interesting to note that in RP, where the activity of TEPs was blocked by aspirin, a significant downregulation was noted in stemness as well as tumorigenicity. Also, the uptrend in the expression of NANOG, OCT4 and SOX2 in TEP influenced CSCs was appreciably reduced in RP~CSC cohort.

Previous reports have demonstrated the anti-platelet role of aspirin in numerous

other diseases along with cancer. Consistent with these findings, we observed that aspirin directly suppressed P-selectin expression on the surface of TEPs, which in turn hindered the P-selectin-PSGL1 axis and barred TEPs from interacting with BCSCs, thereby regulating the progression of the disease.

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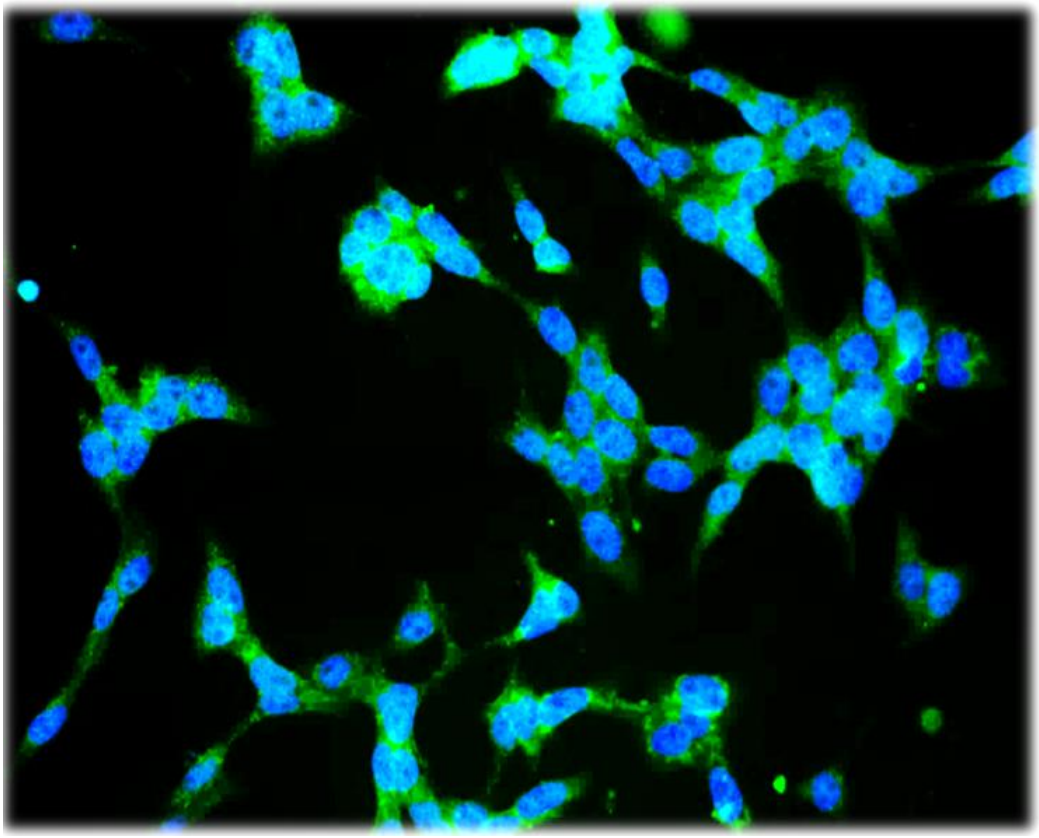
Chapter 3

Tumor educated platelets direct invasive and metastatic nature of BCSCs in contact-dependent manner



Immunofluorescence microscopy image showcasing Cofilin 1^{+}(FITC) tumor educated platelet influenced breast cancer stem cells of MCF-7 at 100X magnification

Tumor educated platelets direct invasive and metastatic nature of BCSCs in contact-dependent manner

Abstract:

Metastasis is responsible for majority of cancer related deaths. However, this multi-step cascade is a very inefficient process whereby only 0.01% (approx) of tumor cells can form metastatic foci. Cancer stem cells (CSCs) are thought to be the 'initiators of tumor, also they are regarded to be the basis of metastatic foci generation. In this regard, the impact of tumor educated platelets (TEPs) on invasion and metastasis of breast cancer stem cells (BCSCs) were delineated. Through numerous *in-vitro* assays it was revealed that under the influence of TEPs, BCSCs of MCF-7 and MDA-MB-231, adopted a typical mesenchymal like architecture producing numerous invadopodia depicting their invasive nature. Also, TEP~BCSCs were more migratory and capable of healing wounds faster. Additionally, analysis of proteins responsible for EMT (epithelial to mesenchymal transition) and metastasis showcased downregulation of epithelial marker (E-CADHERIN) and concomitant upregulation of mesenchymal markers (VIMENTIN, TWIST, SNAIL) further strengthening their metastatic nature. Taken together our results support TEPs capability to generate highly invasive and metastatic BCSC sub-variants. Moreover, this TEP mediated effect was observed only when they were in close proximity with BCSCs, thus diminishing the role of any soluble factors in encouraging TEP influenced BCSCs invasion and metastasis.

Introduction:

The process of epithelial-mesenchymal transition (EMT) is crucial for both tissue regeneration and appropriate embryonic development [1] [2]. Nevertheless, during cancer progression and metastasis, abnormal reactivation of EMT is linked to malignant characteristics of tumor cells, including higher resistance to chemotherapy and immunotherapy, increased tumor stemness, and enhanced motility and invasiveness [2]. During the course of cancer development, epithelial cells acquire some unique mesenchymal characteristics that can be distinguished in the original tumor and allow them to penetrate neighbouring tissues before proceeding distantly [2] [3] [4] [5]. The phenotypic state of tumor cells going through this process can be assessed using a combination of mesenchymal and epithelial biomarkers [6]. Within the tumor, individual cells that move to

various states along the E to M spectrum can produce a large deal of phenotypic heterogeneity [1] [6]. This phenotypic plasticity and heterogeneity can provide cancer cells more resilience and adaptability [6]. However, EMT is an extremely laborious process for the tumor cells and only a small fraction of highly competent cells with stem cell like properties only succeed in establishing colonies in another organ [7]. There is an intriguing relationship between EMT and stem cells, as reports have shown that cells undergoing EMT can have traits similar to those of CSCs [7].

Considering, the established role of TEPs in promoting the expansion and proliferation of CSCs, we next elucidated the EMT status of these cells under influence of TEPs, for determining its overall impact on disease progression. One of the pivotal attributes of CSCs is their capacity to initiate secondary migration to distal organ. They dissolve the extracellular matrices by means of matrix metalloproteinases like MMP7, MMP9, MMP 11 etc. and with the protective shield provided by TEPs, they navigate through the blood vessels to their target site.

Metastatic CSCs develop a typical invasive architecture, aided by cytoskeletal rearrangement resulting in the formation of invadopodia that facilitates focal degradation [8]. Circulating CSCs have elevated expression of chemokine receptors like CXCR3, CXCR4, CXCR5, CCR7 etc. and depending upon the chemokine signals, they travel to distal organs such as lymph nodes, spleen, lungs to generate secondary tumors [9]. Within TEP influenced CSCs, all of these traits such as morphology, migratory and invasive potentialities, EMT state and its regulating transcription factors were ascertained both *in-vivo* as well as *in-vitro*.

Objectives:

- To delineate TEPs role on EMT and metastasis of BCSCs
- To elucidate if TEP~BCSC interaction occurs via contact-dependent or contact-independent mechanism

Materials and Method:

Co-culture

Single cell suspensions of luminal-A and TNBC tumor samples and MCF-7, MDA-MB-231, were cultured with TEPs and RP in 1:100 (BCSC: platelet) ratio for 24 hours. After the incubation period, the culture media with platelet suspension was removed and the cells were collected by trypsinization and centrifuged for further analysis.

Magnetic cell sorting

From the co-culture setup BCSCs were sorted by using magnetically labelled $CD44^+/CD24^-$ antibodies and cell purification was carried out according to manufacturer's protocol (Micro Bead kit, Miltenyi Biotech, Germany).

Matrigel invasion assay

Post co-culture, serum starved, magnetically sorted $CD44^+/CD24^-$BCSCs were layered on 8µm matrigel coated transwell inserts. These inserts with the cell suspension were placed in 24 well plate containing FBS as chemoattractant. This entire setup was maintained for 24 hours following which the invaded cells were fixed and stained with 0.2% crystal violet. Migrated cells from five random fields were photographed and quantified.

Contact independent transwell assay

For transwell assay, magnetically sorted $CD44^+/CD24^-$ BCSCs were co-cultured in presence of 0.4µm transwell membrane (Hi-Media, Mumbai, India) in 1:100 ratio (BCSC: platelet) for 24 hours. Following the incubation period, the transwell was removed and the cells were undertaken for further analysis.

Wound healing assay: Post co-culture, sorted $CD44^+/CD24^-$ BCSCs of MCF-7 and MDA-MB-231 were grown until completely confluent. A scratch or wound was drawn using a cell scratcher and wound healing was observed by taking micrographs at different time points for 24 hours. Percentage wound closure was calculated as final area/initial area X 100%

RT PCR

Total RNA content of single cell suspensions was extracted by Trizol (Ambion, Thermo Fisher Scientific, MA, USA). cDNA was synthesized according to manufacturer's protocol from it using Revert Aid First Strand cDNA Synthesis Kit. Reverse transcriptase PCR was performed using 2X Go Taq Green Mix. Electrophoresis was performed using 1.5% agarose gels and stained with ethidium bromide.

Western Blot

Total protein concentration of the lysates so obtained was determined by Bradford assay. 30-50µg of the protein lysates was separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane using BioRad Gel Transfer system and bands were developed using ECL Kit (Advansta, CA, USA). Band intensity was quantified using Image Lab 6.2 software (Bio-Rad, California, USA).

Immunofluorescence

Targeted samples were harvested on poly-L-lysine coated glass slides and initially blocked with 5% BSA at RT. Antigen specific primary antibody (Cofilin1, OCT4, P-selectin) were added to the section and incubated overnight at 4°C. Fluorescently tagged secondary antibody was added next and incubated for 3 hours at RT. Finally, the sections were thoroughly washed with 1XPBS and mounted with Fluoroshield DAPI (Sigma,USA) and images were acquired using Olympus-BX53 fluorescence microscope. Fluorescence intensity was evaluated using Image-J software.

Protein-protein interaction visualization

String (Search Tool for the Retrieval of Interacting Genes/Proteins) database version8.0 RRID: SCR_005223 was utilized to decipher the interactions between various proteins of interest.

Statistical analysis

To ensure normal distribution pattern, normality and log normality tests were performed. All data passed the Shapiro-Wilk test of normal distribution. Statistical significance was drawn from One-way ANOVA / Two-way ANOVA followed by Tukey's multiple comparison test. Entire statistical analysis was performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, USA). Experimental results with $p \leq 0.05$ have been considered as significant.

Results:

TEP influenced BCSCs adopt an invasive architecture

TEPs are able to generate highly malicious BCSC sub-variants and their alliance with these cells indicated towards possible disease progression. Additionally, CSCs being the basis of tumor invasion and metastasis, insights into the significance of BCSCs in BC metastasis under the influence of TEPs was elucidated. For this, following co-culture, magnetically sorted BCSCs were collected and evaluated for many key characteristics associated with the process (Fig 3.1A).

Morphological analysis through phase-contrast microscopy revealed that these sorted BCSCs (from both MCF-7 and MDA-MB-231) in the monolayer adopted a very linear architecture with increased cellular length and reduced cellular width under influence of TEPs. Whereas, non-treated BCSCs had a framework similar to that of normal MCF-7 and MDA-MB-231. Conversely, in the presence of RP, these cells had almost similar cellular length like non-treated controls, but their width was reduced significantly in their comparison (Fig 3.1B). Furthermore, TEP influenced BCSCs had an invasive character owing to the presence of numerous invadopodia on their surface. These structures facilitate focal degradation for invasion and metastasis (Fig 3.1B). Immunofluorescence staining of Cofilin 1, an actin binding cytoskeletal protein revealed increased nuclear expression in TEP~BCSCs than the control groups of MCF-7, suggesting its role in supporting the development of invadopodium (Fig 3.1C).

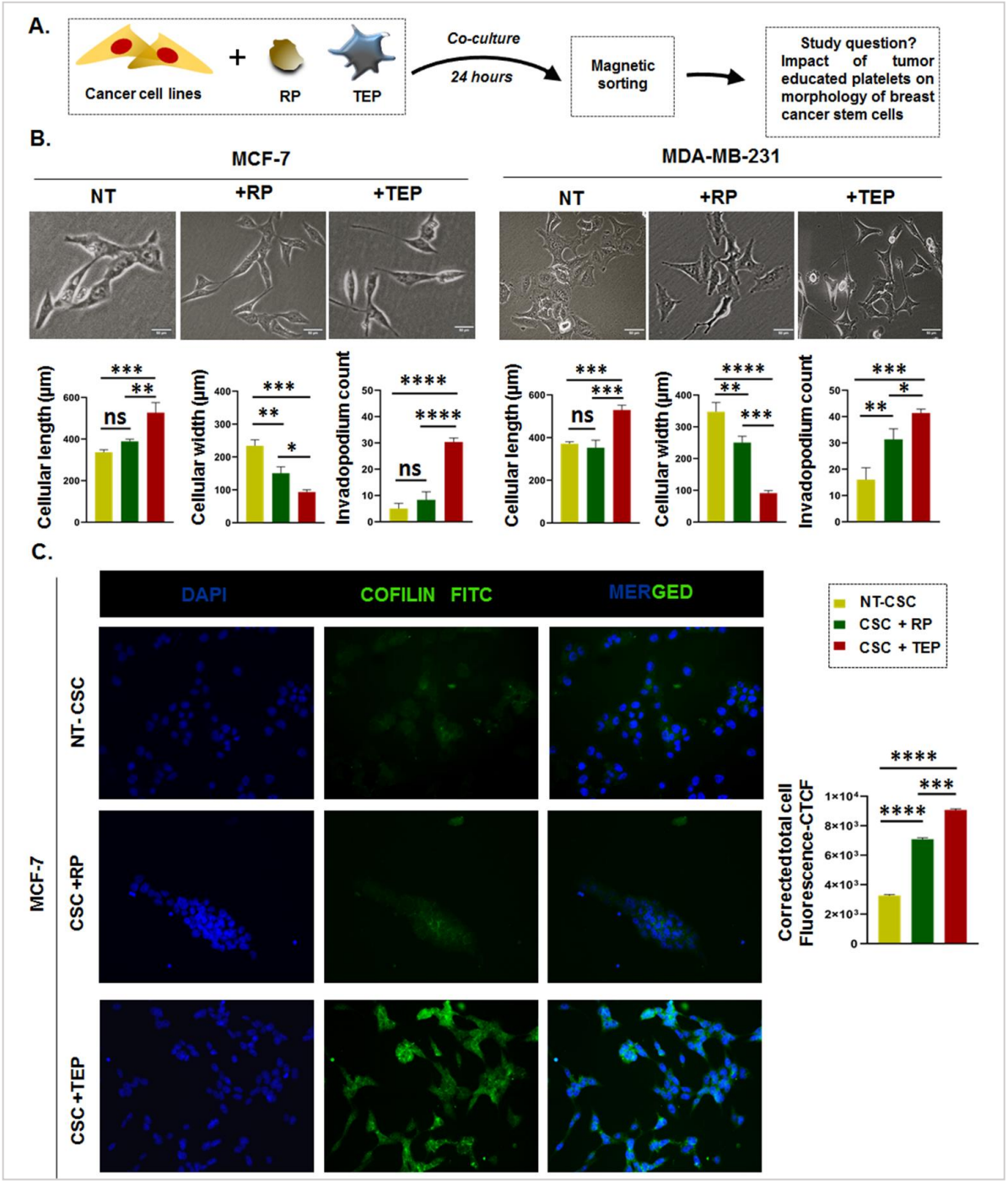


Figure 3.1: TEP influenced BCSCs adopt an invasive architecture: A. Schematic representation of isolating $CD44^{+}CD24^{-}$ BCSCs by magnetic sorting from the co-culture setup of cancer cells with TEP and RP followed by illustration of the study questions. **B.** Representative micrographs at 40X magnification of post co-culture, sorted BCSCs of all groups of MCF-7 and MDA-MB-231 showcasing changes in morphological features. Changes in cellular length and width and invadopodia count in NT-CSC, CSC+RP, CSC+TEP of MCF-7 and MDA-MB-231 is presented in bar graph (mean±SD) with statistical significance drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=4). **C.** Representative immunofluorescence images at 100X magnification of NT-CSC, CSC+RP, CSC+TEP of MCF-7 stained with Cofilin 1 FITC. Quantified corrected total cell

fluorescence intensity (CTCF) is displayed in bar-graph, (mean±SD) with statistical significance inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=3). *Inset*: yellow, green and red column denoting NT-CSC, CSC+RP, CSC+TEP. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

TEP augments migratory potential of BCSCs

Given the invasive architecture of BCSCs under the influence of TEPs, we next investigated the capacity of these cells to penetrate through the extracellular matrix and initiate invasion and metastasis by performing in-*vitro* matrigel invasion assay and transwell migration assay (Fig 3.2A). Microscopic imaging from invasion assay revealed that TEP-impacted BCSCs had an enhanced invasive potency with higher number of invading cells per field compared to non-treated CSCs of MCF-7 and MDA-MB-231.

Interestingly this uptrend was significantly reduced in RP~BCSC cohort compared to TEP~BCSC group (Fig 3.2B).

Additionally, the migratory capacity of these cells was elucidated by transwell migration experiment. TEP-influenced BCSCs were extremely mobile than RP-treated BCSCs or non-treated controls. They exhibited a greater number of migratory cells per field in both MCF-7 as well as MDA-MB-231 (Fig 3.2C). In line with these findings, the observations of wound healing assay provided more evidence for this.

It was uncovered that TEPs elevated the migratory potentialities of these invasive BCSCs, as evidenced by greater extent of wound closure in these cells than controls (Fig 3.2D).

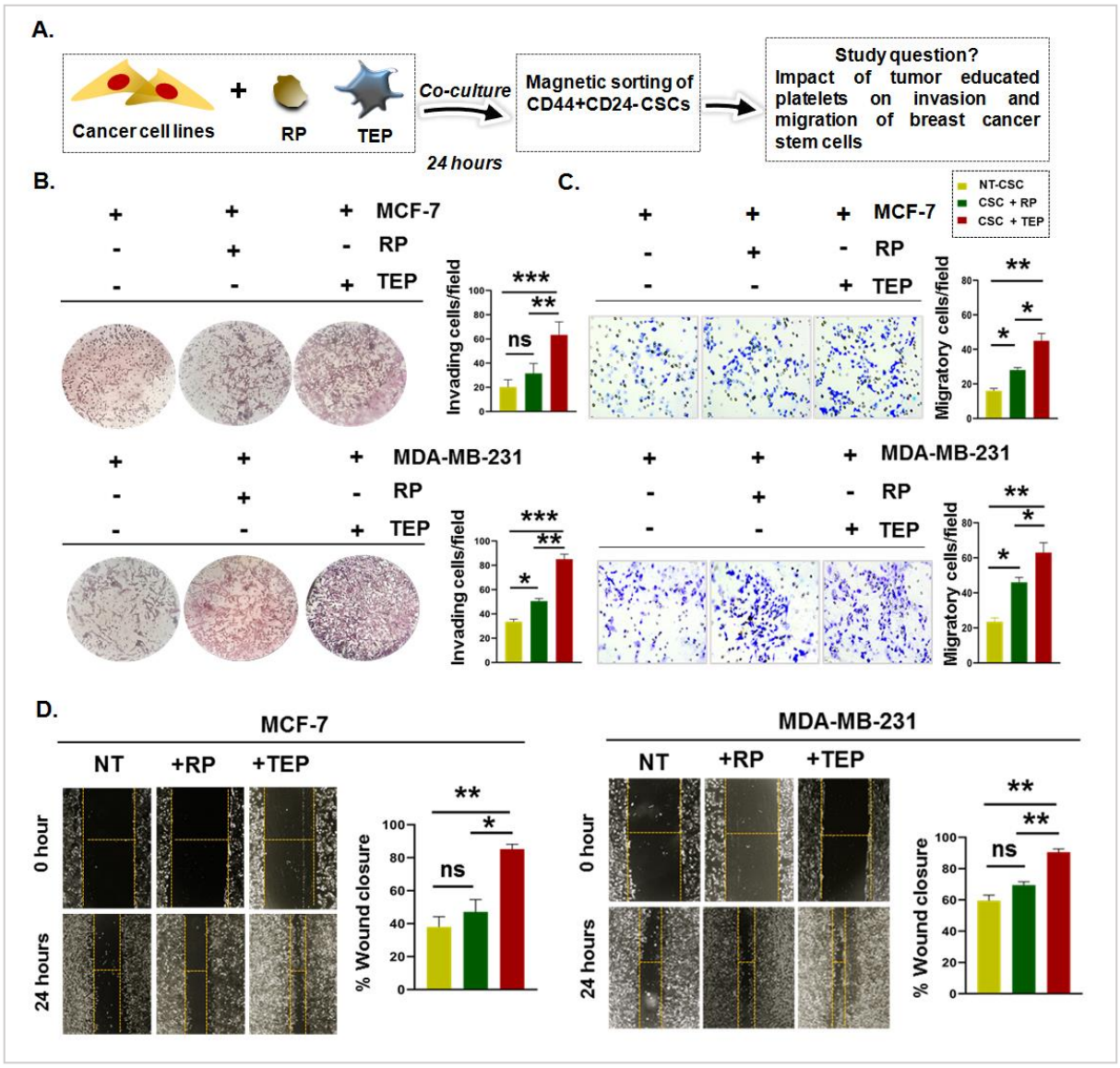


Figure 3.2: TEP augments migratory potential of BCSCs: A. Schematic representation of isolating $CD44^{+}CD24^{-}$ BCSCs by magnetic sorting from the co-culture setup of cancer cells with TEP and RP followed by illustration of the study questions. **B.** Representative micrographs of cells from matrigel invasion assay at 10X magnification of BCSCs from each group of MCF-7 and MDA-MB-231. In bar graph, invading cell count per field (mean±SD) is displayed and statistical significance inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=3). **C.** Representative illustrations of migratory cells from transwell migration assay of NT-CSC, CSC+RP, CSC+TEP of MCF-7 and MDA-MB-231. In bar graph, count of migratory cells per field (mean±SD) is given with statistical significance performed using one-way ANOVA followed by Tukey's multiple comparison test (n=3). **D.** Microscopic images at 10X magnification of wound healing assay at 0 hour and 24 hours across all the groups of both the cell lines. Bar diagram (mean±SD) representing percentage wound closure in all the study groups of both the cohorts. One-way ANOVA followed by Tukey's multiple comparison test was performed to draw statistical significance (n=3). *Inset*: yellow, green and red column denoting NT-CSC, CSC+RP, CSC+TEP respectively**.** *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

TEP influenced BCSCs are skewed towards mesenchymal lineage

Now that the critical role of TEPs in facilitating invasion and metastasis of BCSCs has been demonstrated, we likewise attempted to clarify the roles played by transcription factors, proteins, chemokines and *mmps* in the process. In light of this, post co-culture magnetically sorted BCSCs were examined in order to decipher modifications in the expression of relevant molecules (Fig 3.3A).

Western blot analysis of EMT- related transcription factors revealed that extent of upsurge in the expression of VIMENTIN, TWIST and reduction in expression of E-CADHERIN was more in TEP treated BCSCs than controls across the cell lines confirming their mesenchymal lineage (Fig 3.3B). This was further corroborated by the results of RT-PCR analysis of *snail* and *slug* which demonstrated elevation in the expression of *snail* in BCSCs upon TEP treatment, validating their mesenchymal predisposition. Nevertheless, *slug* remained at a steady level throughout the groups (Fig 3.3C). Next, a panel of chemotaxis-associated genes was evaluated for expression status using RT-PCR in order to determine the likely migration sites of these invasive, metastatic TEP-influenced BCSCs. The TEP-treated group exhibited increased expression of *cxcr4* and *mmp9* amongst these molecules (Fig 3.3D). Additionally, to ascertain the link between stemness and metastasis, along with the association between *cxcr4* and *mmp9*, string data base was utilized which confirmed the interactions between the various molecules that have been reported to be involved in interconnecting stemness with metastasis (Fig 3.3E, F).

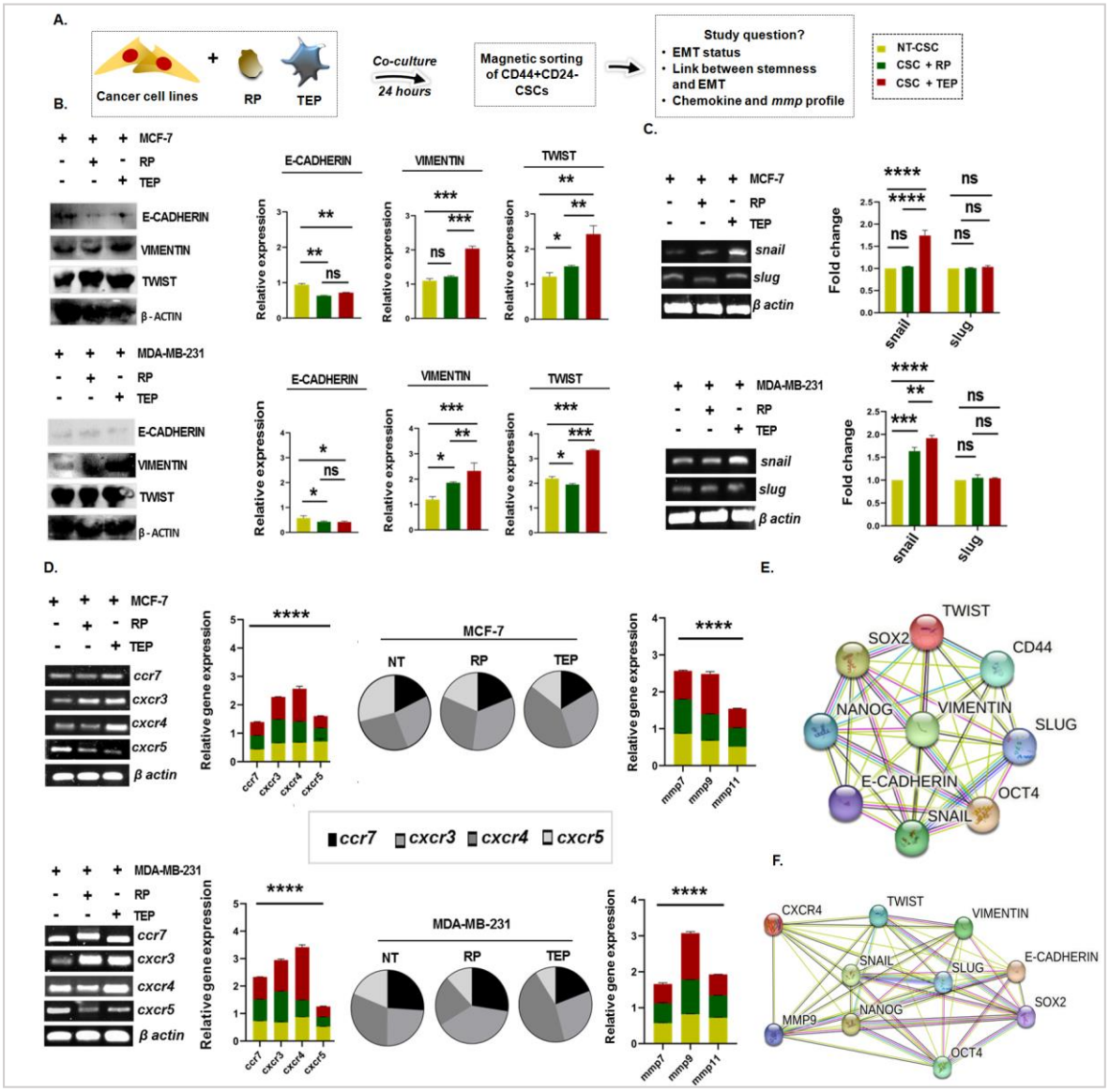


Figure 3.3: TEP influenced BCSCs are skewed towards mesenchymal lineage: A. Illustration of isolating $CD44^{+}CD24^{-}$ BCSCs by magnetic sorting from the co-culture setup of cancer cells with TEP and RP followed by depiction of the study questions. *Inset*: yellow, green and red column denoting NT-CSC, CSC+RP, CSC+TEP. **B.** Representative western blots of EMT and metastasis related proteins E-CADHERIN, VIMENTIN, TWIST keeping β-ACTIN as control is presented in all the three groups of MCF-7 and MDA-MB-231. Bar diagram (mean±SD) depicting relative protein expression is delineated. Two-way ANOVA followed by Tukey's multiple comparison test was the source of statistical significance (n=3). **C.** mRNA expression of genes *snail* and *slug* by RT-PCR, with β-ACTIN as control is presented in all the three groups of MCF-7 and MDA-MB-231. In bar graph **(**mean±SD) quantified values of relative fold change is represented with statistical significance inferred from two-way Anova followed by Tukey's multiple comparison test (n=3). **D.** mRNA expression of related chemokine genes in NT-CSC, CSC+RP, CSC+TEP of both the cell lines by RT-PCR is depicted along with summary bar graphs of relative gene expression. Statistical significance inferred from two-way ANOVA followed by Tukey's multiple comparison (n=3). Cumulative chemokine profile of BCSCs of all the groups of both MCF-7 and MDA-MB-231 is displayed as parts-of-whole-pie-chart. mRNA expression of matrix metalloproteinases (*mmp*7, 9, 11) across all the groups is represented in summary bar-graphs. Two-way ANOVA followed by Tukey's multiple comparison was performed to draw statistical significance (n=3). **E.** Identification of the genes

responsible for linking stemness and metastasis using STRING database is provided. **F.** Representative interactome of string analysis depicting link between CXCR4 and MMP9 and the other EMT and stemness regulating transcription factors and proteins. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

TEP promotes stemness and metastasis predominantly by contact-dependent manner

In light of TEPs important contribution in BCSC aided disease progression, we next investigated whether such influence was mediated by soluble factors (contact independent) or receptor-ligand interaction dependent (cell-cell contact dependent) mechanisms. For this, $CD44^{+}CD24^{-}$ BCSCs were magnetically sorted from single cell suspensions of solid breast tumor samples of both luminal-A and TNBC subtypes post co-culture with TEP and RP, keeping non-treated cells as control. For contact dependent interaction, BCSCs and TEPs were cultured together in the same well, whereas in the other setup they were physically separated using 0.4µm transwell inserts. This ensured that the only means of communication between BCSCs and TEPs was through soluble cellular secretions (Fig 3.4A). In both luminal-A and TNBC, TEP-induced BCSC were skewed towards the mesenchymal state predominantly in the contact dependent setup as indicated by the strong expression of VIMENTIN and low expression of E-CADHERIN (Fig 3.4B).

In-vivo, luminal-A and TNBC breast carcinoma tissue sections were examined for any indication of physical contact-dependent interaction between TEPs and BCSCs in order to further corroborate the *in-vitro* results. For this, BCSCs were tagged with stem cell marker OCT4 and TEPs with activated platelet marker P-selectin (Fig 3.4C). The relationship between BCSCs and TEPs within the TME was confirmed using immunofluorescence microscopy, which showed that these cells remain in very close proximity, thereby, further validating their contact-dependent interaction. Their co-localization was affirmed by Mander's co-localization coefficient (Luminal-A; M1-0.907 and M2-0.580) (TNBC; M1-0.958 and M2-0.657).

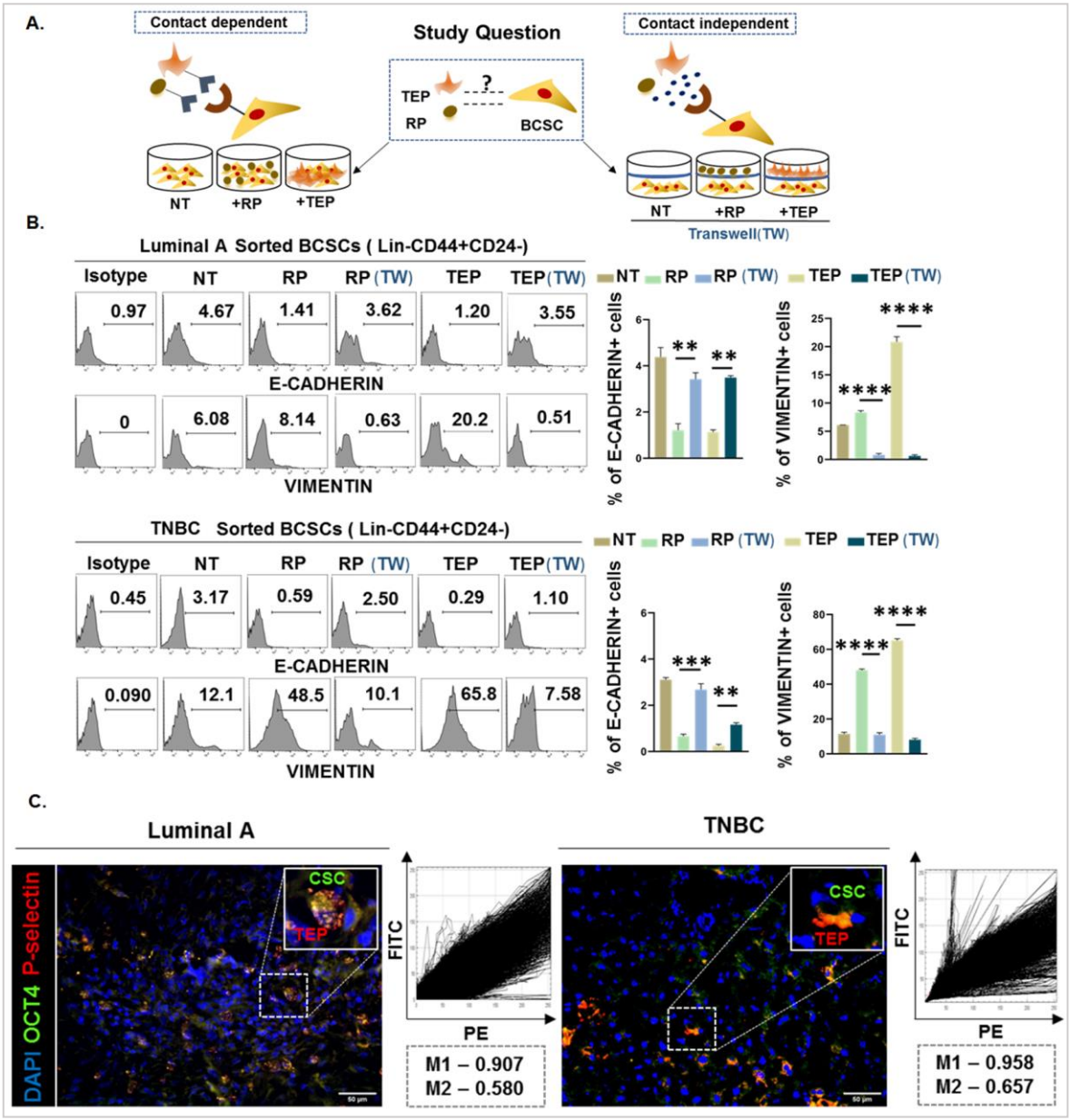


Figure 3.4: TEP promotes stemness and metastasis predominantly by contact-dependent manner: A. Schematic illustration of probable contact-dependent and contact-independent interactions between BCSCs +/- TEP/RP in luminal-A and TNBC subtypes. **B.** Representative flow-cytometric histograms of E-CADHERIN and VIMENTIN expression of BCSCs isolated from co-culture setups of contact-dependent and contact-independent arrangements of all the groups across luminal-A and TNBC sub-types. Bar graphs (mean±SD) depicting changes in expression of E-CADHERIN and VIMENTIN in contact-dependent and independent setups of all the groups in both the cohorts is presented. Statistical significance is drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated. **C.** Representative immunofluorescence images of breast tumor sections of luminal-A and TNBC subtypes at 40X magnification depicting proximity of BCSCs (stained with OCT-FITC) and TEPs (stained with P-selectin PE). In *inset* enlarged image of their close adjacency is presented. Cytofluoromicrographs and Mander's co-localization coefficient (M1- overlap of PE over FITC and M2- overlap of FITC over PE) of both the sub-types is presented.

Discussion:

Epithelial-to-mesenchymal transition (EMT) is associated with metastasis formation as well as with generation and maintenance of cancer stem cells. In this way, EMT contributes to tumor invasion, heterogeneity and chemoresistance [10]. Our primary investigation with luminal-A and TNBC BC subtypes disclosed a direct upregulation of metastatic markers on the invasive CSCs. To ascertain TEPs precise role on tumor invasion and metastasis, TEP influenced CSCs were characterized.

It was observed that TEP ~ CSCs of both the subtypes mostly exists in mesenchymal state with an architecture, typical of metastatic cells. Previous reports have revealed that EMT bestows stemness, plasticity and metastatic potentiality to CSCs [10]. Corroborating with their study, we observed that TEP~BCSC cohort exhibited significantly elevated rate of invasion and migration in *in-vitro* studies. Moreover, development of invadopodia along with overexpression of Cofilin 1, further confirms their invasive phenotype. Former reports have revealed that migratory cells initiate focal degradation of extracellular matrix with the aid of invadopodia [11]. Additionally, a positive correlation between reduced overall survival rate and increased Cofilin expression has been demonstrated in various malignancies like breast, prostate and NSCLC [11]. Cumulatively, results of matrigel invasion assay, transwell assay and wound healing assay disclosed that TEP~CSCs are assuredly highly invasive and metastatic in nature.

Analysis of transcription factors and proteins involved in EMT and metastasis revealed that TEP influenced CSCs have elevated expression of VIMENTIN, TWIST and SNAIL. TWIST overexpression in TEP~CSCs helps in maintaining stemness via crosstalk with OCT4 [12]. It also facilitates invasion and metastasis by stimulating production of invadopodia [12]. Also, by interacting with SOX2, it allows the CSCs to develop MDR phenotype [13]. Further, TWIST downregulates the expression of E-CADHERIN that aids in detachment of metastatic cells [14]. We next screened a panel of chemotaxis molecules, to elucidate the probable secondary site of these invasive CSCs. TEP induced BCSCs demonstrated increased expression of CXCR4 and MMP9.Elevated CXCR4 has been linked to BC metastasis to the liver, brain, lungs and lymph nodes according to literature reports [15] [16] [17] [18]. In contrast to the very low intensity found in normal breast tissues, majority of breast tumors have higher levels of CXCR4.

According to Salvucci et al. (2006), over 40% of breast tumors express higher CXCR4 levels, but a basal to high expression is present in all breast tumor tissues. Additionally, with the increase in tumor grade the expression of CXCR4 also increases. While normal breast tissue exhibits only 20% CXCR4, it increases to 40% in *in-situ* ductal carcinoma and to 67% in invasive ductal carcinoma [19]. Furthermore, because of its great capacity for ECM breakdown, MMP9 plays a critical role in metastasis and its high expression has been linked to poor prognosis for breast cancer and has been acclaimed by several clinical reports [15] [16] [17] [18]. It has also been reported that MMP9 specifically correlates with high grade breast tumors including basal like and Her2 positive BCs [20].

Therefore, our study demonstrates that TEPs via EMT generates highly invasive and metastatic sub-variants of BCSCs which are capable of migrating to secondary sites. Thus, targeting these TEPs may prove beneficial for the patients and provide better disease management.

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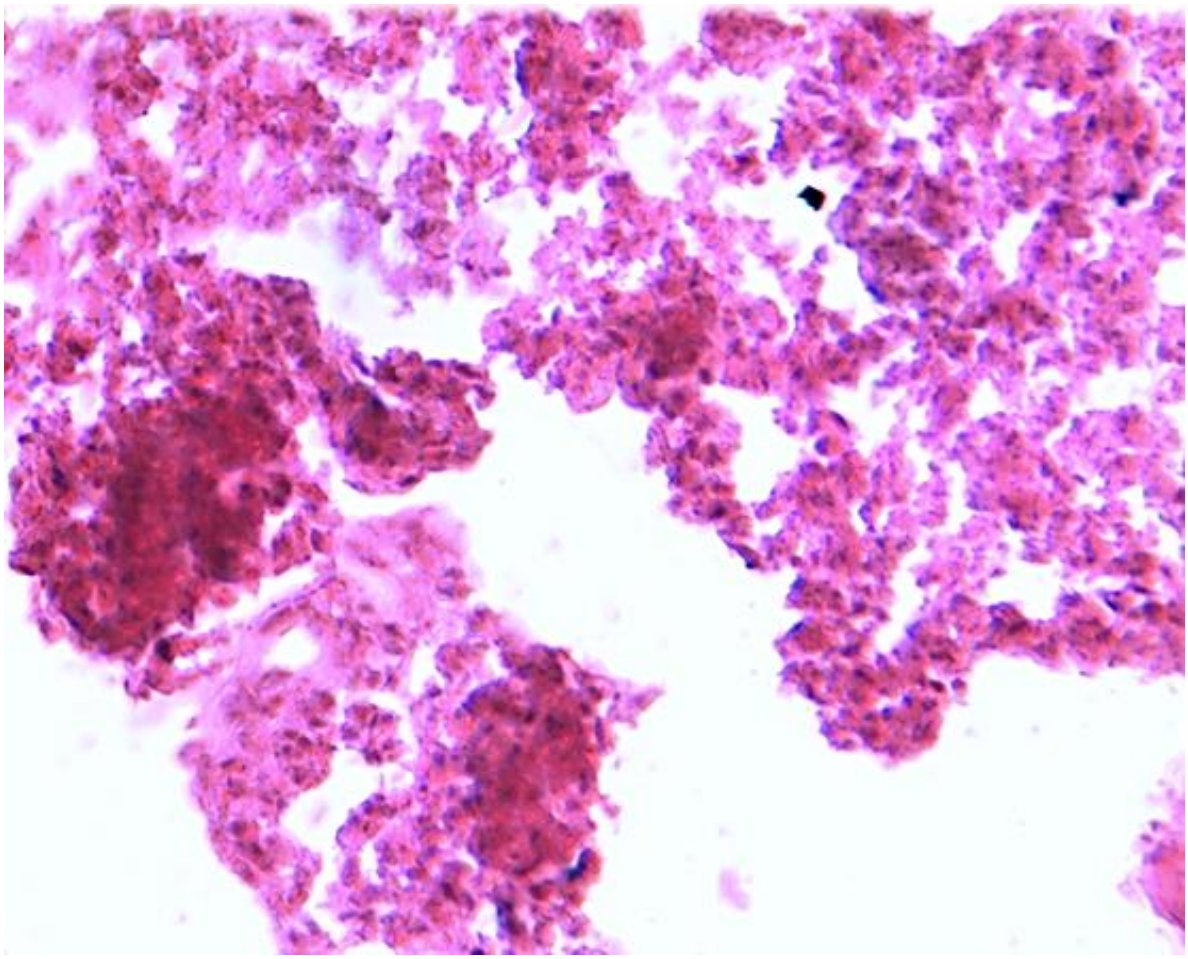
Chapter 4

Tumor educated platelets influenced BCSCs are highly tumorigenic, promotes angiogenesis and induces lung metastasis in-vivo: Intervention by Aspirin



HE stained section of lungs showcasing metastatic foci of 4T1 tumor bearing female BALB/c mice inoculated with tumor educated platelet influenced breast cancer stem cells at 40X magnification

Tumor educated platelets influenced BCSCs are highly tumorigenic, promotes angiogenesis and induces lung metastasis in-vivo: Intervention by Aspirin

Abstract:

Given the critical role of TEPs in fostering stemness and metastasis *in-vitro*, we further, elucidated their influence on overall tumorigenesis *in-vivo*. In line with the *in-vitro* observations, it was noted that TEPs were effective in promoting mammosphere formation in both EC and 4T1 subtypes of BC. Additionally, compared to the corresponding CSCs, TEP-treated EC-CSCs and 4T1-CSCs were more virulent in initiating primary tumors in female Swiss and BALB/c mice, respectively. Furthermore, it was discovered that TEPs tend to trigger metastasis, regardless of the initial tumour growth. Macroscopic imaging of murine lungs, liver, spleen and lymph nodes revealed that metastatic lesion was predominantly perceived in the lungs in the form of nodules in both the murine systems. Moreover, TEPs promoted angiogenesis, which is essential for supporting metastatic outgrowth of tumor cells. Overall, the findings indicate that TEPs and breast CSCs interact intricately to promote primary tumor growth as well as favours metastasis in experimental model, while also playing a significant role in regulating angiogenesis.

Introduction:

A precondition for effective haematogenous metastatic dissemination is the interaction between tumor cells and platelets [1]. To create a permissive milieu, tumor cells instantly activate platelets when they enter the bloodstream. Platelets recruit myeloid cells, mediate an arrest of the tumor cell platelet embolus at the arterial wall and shield them from shear forces and NK cell assault [1]. It then bestows the tumor cells with a mesenchymal-like appearance and open the capillary endothelium to promote extravasation in distant organs. Lastly, growth factors released by platelets promote the development of tumor cells to micro-metastatic foci [1]. However, the lack of blood supply substantially limits the growth potential of avascular tumors. By altering the local balance of

proangiogenic and antiangiogenic factors, tumors 'switch on' the process of angiogenesis in order to grow in size and prepare for metastasis [2].The angiogenic switch is often made possible by the overexpression of proangiogenic factors, such as vascular endothelial growth factor (VEGF), in tumors in comparison to antiangiogenic factors [2]. Numerous *in vitro* and *in vivo* investigations using a variety of angiogenic assays have long acknowledged the significance of platelets in angiogenesis. Platelets contain a number of molecules that have proangiogenic qualities, such as VEGF, platelet-derived growth factor (PDGF), basis fibroblast growth factors (bFGF) and epidermal growth factor (EGF), as well as molecules that have anti-angiogenic effects, such as endostatin, angiostatin, PF4 or thrombospondin [2] [3] [4] [5]. Patients with various malignancies frequently have high serum VEGF levels, which is associated with advanced disease and poor prognosis [6].

The success of cancer metastasis nevertheless, depends on the seeding and successful colonization by specialized cells, the CSCs at distant organs [7]. In-fact, the inherent resistant nature of CSCs, favours their spread from the initial site [7]. Despite such advancements in the field of cancer metastasis research, majority of cancer related fatalities is still due to the detachment and progression of tumor cells from their primary origin.

In order to provide new insights in this field, we elucidated the interplay between the two pioneering players of metastasis, platelets and CSCs through *in-vivo* experiments. Studies on female Swiss albino and BALB/c murine models uncovered the significant role of TEPs in promoting tumorigenesis as well as angiogenesis. Interaction of TEPs and CSCs favoured the growth and as well outspread of CSCs and also ensured the steady supply of oxygen and nutrients to the rapidly proliferating cells. Further, upon targeting TEPs with aspirin, its impact on CSCs could be substantially mitigated resulting in retardation of angiogenesis and metastasis.

Objectives:

- ❖ To elaborate TEPs precise role in tumorigenesis
- ❖ To decipher the influence of TEPs on metastasis irrespective of primary tumor growth
- ❖ To elucidate TEPs role in angiogenesis

Materials and Methods:

Mice and tumors

Wild- type female BALB/c and Swiss albino mice (age: 4-6 weeks; body weight: 18-22 g average) were obtained from Institutional Animal Care and Maintenance Department. Experimental animals were maintained in pathogen free environment and fed with autoclaved food (Epic Laboratory, West Bengal Government, Kalyani, India) and water *ad libitum*. 4T1 cells were maintained *in-vitro* and EC cells (Ehrlich Carcinoma) were maintained in Swiss albino mice as intraperitoneal passage. All experiments were performed after approval from Institutional Animal Care and Ethics Committee (Approval No: IAEC-1774/SBn-4/2021/9).

Co-culture

Co-culture was performed as mentioned in section 3.3.6 of materials and methods. Briefly, EC and 4T1 cells were cultured with TEPs and RP in 1:100 (BCSC: platelet) ratio for 24 hours. TEPs were isolated from peripheral blood of EC and 4T1 tumor bearing mice by double centrifugation method. RPs or resting platelets on the other hand are basically TEPs treated with 100µM/ml aspirin to prevent further activation and restore the functionality of platelets as in resting or non-activated condition. After the incubation period, the culture media with platelet suspension was removed and the cells were collected by trypsinization and centrifuged for further analysis.

Magnetic cell sorting

From the co-culture setup BCSCs were sorted by using magnetically labelled $CD44^+/CD24^-$ antibodies and cell purification was carried out according to manufacturer's protocol (Micro Bead kit, Miltenyi Biotech, Germany) as described in materials and method in section 3.3.7.

***In-vivo* tumorigenicity assay**

EC and 4T1 cells were cultured with TEPs and RP in 1:100 (BCSC: platelet) ratio for 24 hours. Post co-culture, $CD44^+/CD24^-$ magnetically sorted CSCs ($2x10^5$) of EC and 4T1 cells were inoculated into the mammary fat pads of female Swiss albino and BALB/c mice respectively for development of solid tumors according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals. Tumor growth was monitored twice a week and measured using Vernier callipers.

Experimental metastasis model

Post co-culture, $CD44^+/CD24^-$ magnetically sorted BCSCs ($2x10^5$) of 4T1 and EC cells were injected through the tail vein of BALB/c and Swiss albino mice

respectively for development of lung metastasis.

Processing of tumors

Solid tumors were digested with 1% collagenase for preparing single cell suspensions. The cells were then washed with 1X PBS and used for various *in-vitro* assays.

Cryo-sectioning

Freshly collected tumor tissues were fixed in 4% paraformaldehyde for 2 hours at room temperature followed by incubation at 4°C overnight in 30% sucrose for cryopreservation. They were then snap chilled in liquid nitrogen and stored in -80 °C for later use.

For cryo-sectioning, the frozen tissue samples were embedded in OCT (optimal cutting temperature compound, Leica biosystems, Wetzlar, Germany) and cut into 5µm sections using cryostat (Leica CM1950, Wetzlar, Germany). The sections were collected on poly-L-lysine (Sigma Aldrich, USA) coated slides and stored at -80°C for further use.

Histology and HE staining

The cryo cut tissue sections were stained with hematoxylin-eosin (HE) following standard staining protocol as described in section 3.3.14.

Vascular mimicry (VM) assay

For VM assay, 96 well flat bottom plates were coated with 60µL of growth factor reduced basement membrane (Matrigel; R&D System, MN, USA) and incubated at 37°C for solidifying. Roughly $5x10^3$ BCSCs in 150 µL of complete media were rested on the plates and incubated at 37°C for 24 hours. Following the incubation, the plates were micrographed and from five random fields, the number of interconnected tubes per field was counted. Length and width of the tubes were quantified in ImageJ software.

Statistical Significance

Statistical significance was drawn from either one-way/two-way analysis of variance. Mean±SD of the results have been represented.

For *in-vivo* (n=6) and for *in-vitro* (n=3-6) independent experiments were performed. All statistical analysis was performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, USA). Experimental results with $p \leq 0.05$ have been considered as significant.

Results:

TEP promotes primary tumor development in both female Swiss and BALB/c murine models

In context with the fact that TEPs play crucial role in enhancing stemness, clonogenicity of CSCs and their alliance with these cells elevate invasiveness, migration and metastasis of CSCs of BC subtypes *in-vitro*, we further ought to corroborate these findings *in-vivo* (Fig 4.1A). BCSCs isolated from the co-culture (BCSC: TEP/RP; 1:100 ratio for 24 hours) setup and enriched in 3D stem cell enrichment media, showed increased tumorsphere formation in both the less aggressive EC cells as well as in more aggressive 4T1 subtype under influence of TEPs (Fig 4.1B). In an effort to explore the influence of TEP-BCSCs on tumorigenesis *in-vivo*, BCSCs were isolated from the co-culture setup of EC and 4T1 with TEP and RP and $2x10^5$ cells were injected into the mammary fat pads of female Swiss and BALB/c mice respectively to initiate primary tumor growth.

Compared to BCSCs treated with RP or non-treated controls, tumor growth expanded dramatically in mice injected with TEP-BCSCs (Fig 4.1C).

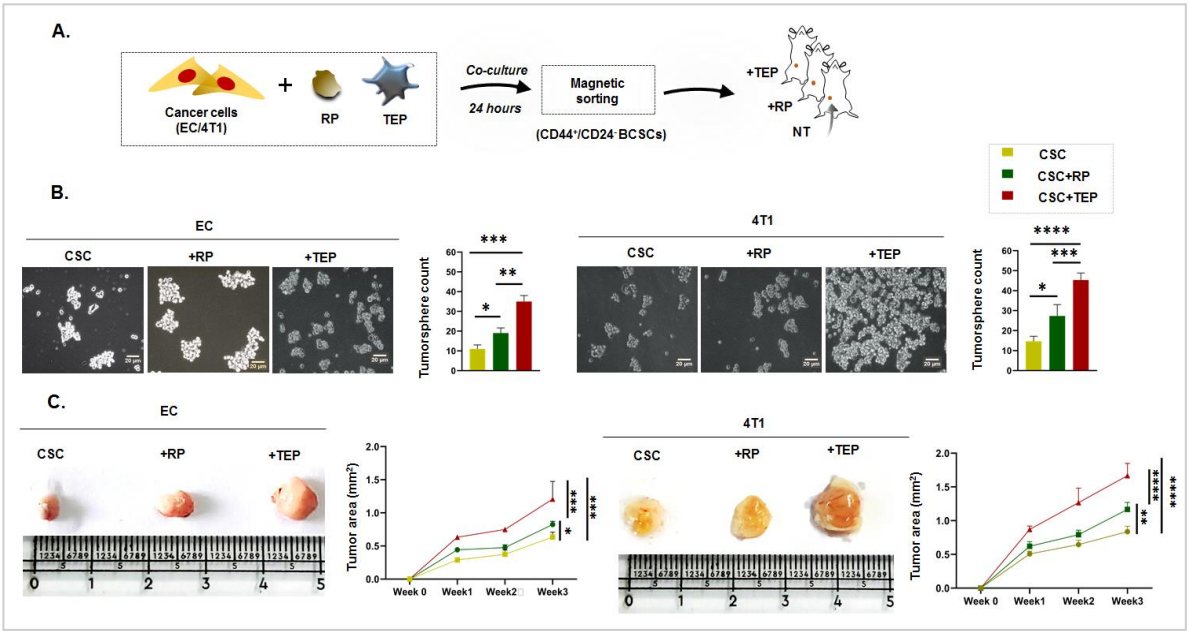


Figure 4.1: TEP promotes primary tumor development in both female Swiss and BALB/c murine models: A. Diagrammatic representation of magnetic sorting for isolation of $CD44^{+}CD24^{-}$ BCSCs from the co-culture setup of cancer cells (EC, 4T1) with TEP and RP and inoculation of CSC, CSC+RP, CSC+TEP into the mammary fat pads of female mice. *Inset*: yellow, green and red column denoting CSC, CSC+RP, CSC+TEP. **B.** Representative images at 10X magnification of primary tumorspheres in CSC, CSC+RP, CSC+TEP cohorts of EC and 4T1 cell lines. In bar-graph, mean±SD

for tumorsphere count is displayed. Statistical significance is inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=6). **C.** *In-vivo*, BCSCs isolated from the co-culture of EC +/- TEP/RP and 4T1+/- TEP/RP was subcutaneously injected into the mammary fat pads of female Swiss mice and BALB/c mice respectively for development of primary tumor. Representative photographs of harvested tumors with scale are presented (n=6). Tumor growth curve depicting tumor area in mm^2 (mean±SD) at each time point is exhibited. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

TEP promotes angiogenesis in both female Swiss and BALB/c murine models and human system: Intervention by aspirin

The development and progression of tumor also requires uninterrupted supply of nutrients and oxygen to the rapidly proliferating cells. This is achieved either by neovascularisation or by angiogenesis and also by vascular mimicry. TEPs influence on these events was analysed by *in-vitro* and *in-vivo* investigations (Fig 4.2A). From the primary tumor experiment, it was also observed that TEP-BCSC group had thicker vasculature with numerous lateral branching draining towards the tumor in comparison to RP (aspirin treated TEP)-BCSC in both EC and 4T1 setup.

Conversely, the thickness, and number of lateral branches from the primary blood vessel were all dramatically less in the non-treated control group (Fig 4.2B) compared to both TEP and RP influenced CSCs. This validated the involvement of TEPs in ensuring the steady supply of oxygen and nutrients to the tumor cells, thereby promoting angiogenesis which was significantly impeded in RP influenced cohort. These data additionally confirms that aspirin effectively neutralizes the ability of TEPs to drive neo-vessel proliferation and complexity *in-vivo* by interacting with BCSCs. To further elaborate TEPs role in angiogenesis, vascular mimicry was performed *in-vitro*. For this, post co-culture sorted BCSCs were rested on matrigel and allowed to form tubes for 24 hours. TEP treated BCSCs in both MCF-7 and MDA-MB-231 showed increment in tube number with an upsurge in tube length than control CSCs or RP-CSCs which produced fewer tubes with reduced dimensions across the cell lines (Fig 4.2C). BCSCs treated with RPs showed a highly significant impairment of channel formation. Compared to TEP treated BCSCs, in BCSC-RP cohort notable reduction in the total number of interconnected channels were observed. Also, most of the cells remained as isolated clusters rather than forming continuous tubular networks. Thus, aspirin

not only diminishes angiogenic vessel sprouting *in-vivo* but also disrupts BCSC-mediated channel formation *in-vitro*, underscoring its dual inhibitory effects on tumor vascularization.

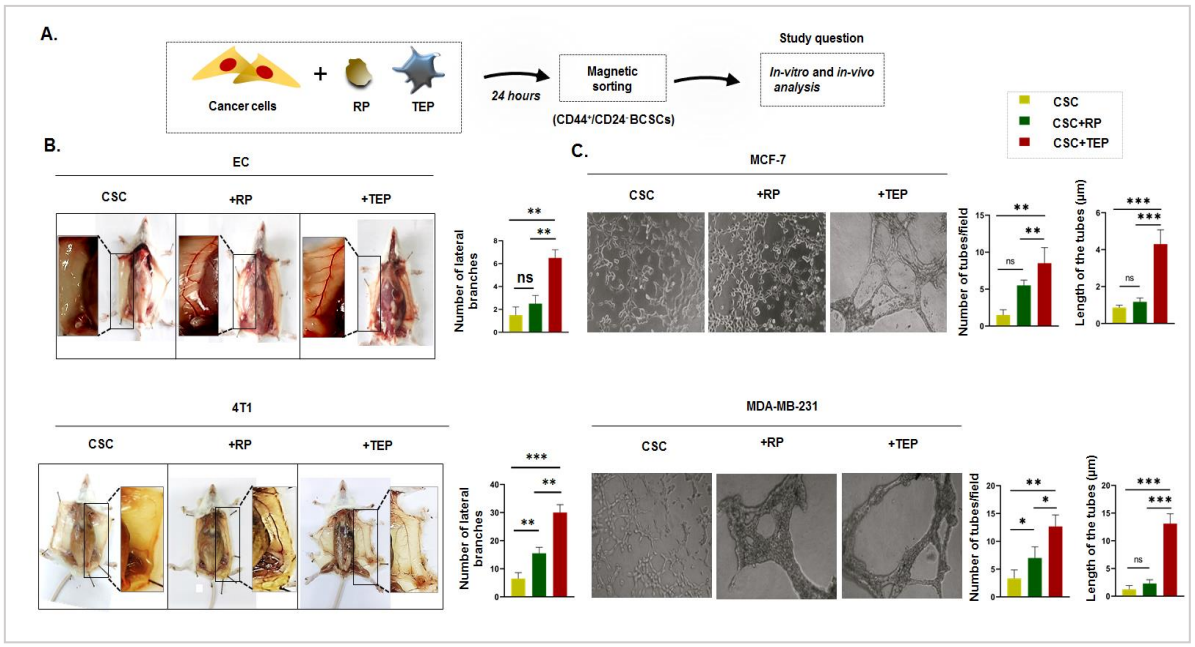


Figure 4.2: TEP promotes angiogenesis in both female Swiss and BALB/c murine models and human system: A. Diagrammatic representation of study question. **B.** Illustrative photographs of tumor draining blood vessel with lateral branches depicting angiogenesis across all groups of ECs and 4T1 is presented (n=6). In bar-diagram, mean±SD of lateral branch count is given. Statistical significance drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=6). **C.** Representative micrographs at 10X magnification of interconnected tube formation on matrigel across the groups of both MCF-7 and MDA-MB-231 is provided. In bar-graph, (mean±SD) of number of tubes/field and tube length in µm is given. Statistical significance is drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=3). *Inset*: yellow, green and red column denoting CSC, CSC+RP, CSC+TEP. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

TEP promotes metastasis irrespective of primary tumor growth

We next sought to determine if TEP-BCSCs may induce metastasis without regards to the primary tumor growth. In light of this, experimental metastasis was performed. To accomplish this experiment, $2x10^5$ BCSCs were intravenously injected into the tail veins of female Swiss and BALB/c mice, which were isolated from the co-culture setup of EC-TEP/RP and 4T1-TEP/RP respectively (Fig 4.3A). A greater number of tumor nodules, with increased surface area of the metastatic

foci were observed from macroscopic imaging as well as HE staining of the affected lungs, in both the murine models of TEP influenced BCSC cohort in comparison to the controls (Fig 4.3B, C). No significant changes were observed in the morphology and weight of liver, spleen and lymph nodes of these mice (Fig 4.3D)

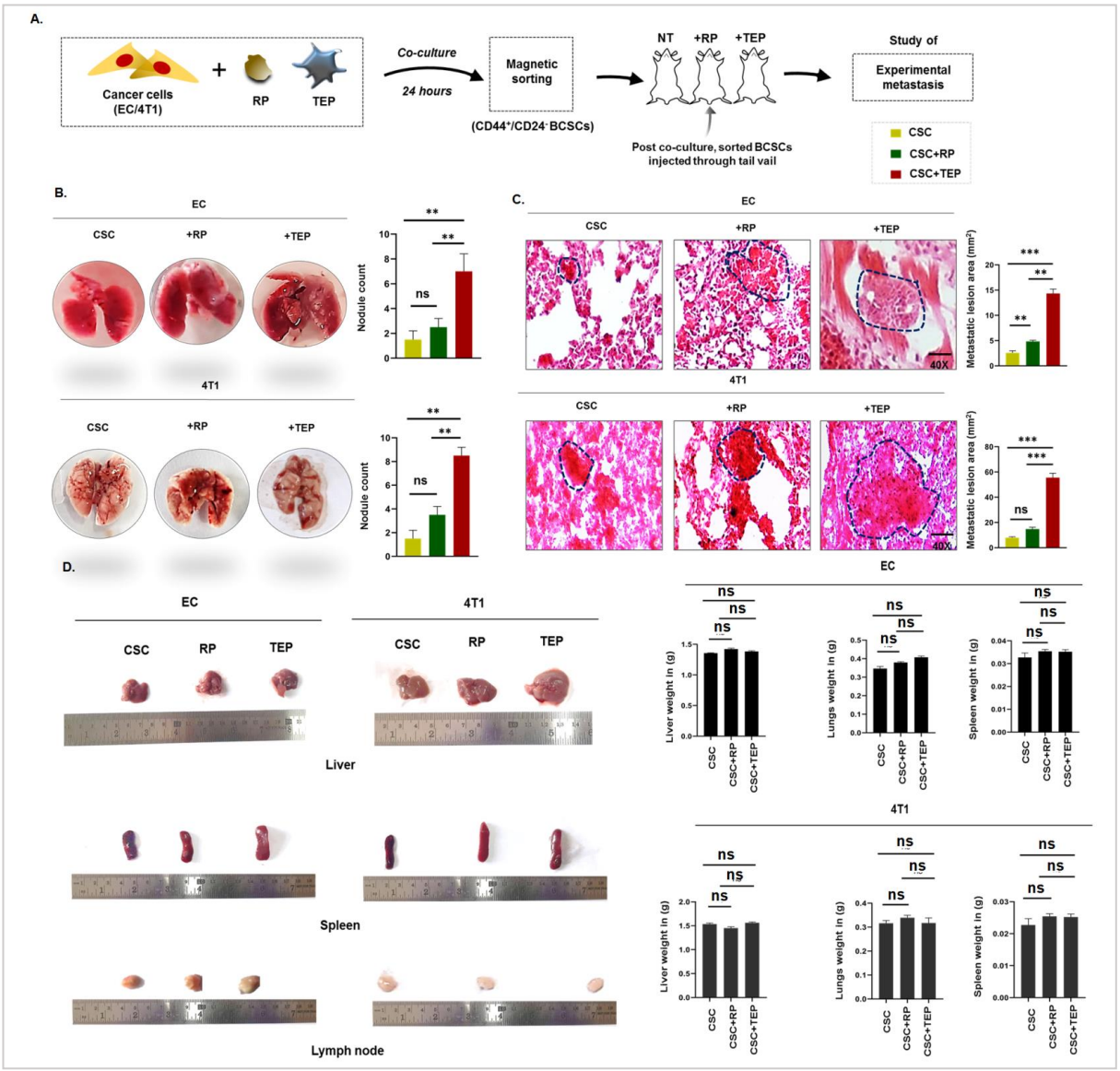


Figure 4.3: TEP promotes metastasis irrespective of primary tumor growth: A. Schematic representation of experimental metastasis. **B.** Photographs of lung nodules across the groups of EC and 4T1 is shown. Bar graphs (mean±SD) representing nodule count is given. Statistical significance (mean±SD) inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=6). **C.** Micrographs of Hematoxylin-Eosin-stained lung tissue sections at 40X magnification depicting metastatic deposition in CSC, CSC+RP, CSC+TEP of EC and 4T1 is presented. Dotted lines depicting boundaries of tumor foci is demonstrated. In bar-graph, mean±SD for metastatic area in

mm^2 is presented and statistical significance drawn from one-way ANOVA followed by Tukey's multiple comparison test (n=6). **D.** Illustrative photographs of unaffected murine liver, spleen and lymph node of experimental metastasis model of CSC, CSC+RP, CSC+TEP of both EC and 4T1. Weight in grams of liver and spleen and lymph node of EC (upper panel) and 4T1 (lower panel) murine models is given. In bar-graph, (mean±SD) of organ weight is provided with statistical significance inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=6). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Discussion:

Here, we primarily investigated the impact of TEPs on metastasis and angiogenesis in an *in vivo* setup, in conjunction with the *in vitro* data. TEPs effectively heightened tumorigenicity and induced the development of breast tumors with an extensive surface area in both female Swiss and BALB/c murine models. This is consistent with our *in-vitro* results where interaction of TEPs with CSCs produced a greater number of soft agar colonies from single cell in both luminal-A and TNBC BC subtypes. There are different mechanisms by which platelets can promote proliferation of tumor cells. Recent studies on ovarian cancer suggested that TGF-β, or transforming growth factor β released by platelets, accelerated the growth of ovarian cancer cells [8].

Additionally, platelet microparticles promoted lung carcinoma cells expansion by stimulating mitogen-activated protein kinases [9]. Several other studies on BCSCs have reported that, in order to facilitate the epithelial-mesenchymal transition, BCSCs are stimulated by the tumor microenvironment, which includes platelets.

The BCSC travels through the circulation or lymphatic system after achieving cell transformation by rupturing the extracellular matrix and intercellular connections [10].

Platelets prevent shear pressure and protect BCSCs from natural killer cells. The secondary metastatic niche is also prepared by the tumor-educated platelets [10] [11]. Not only does TEPs positively influence the process of tumorigenesis, but also stimulate metastasis without the involvement of primary tumor. The BCSCs under influence of TEPs migrated through the blood vessels to the lungs to induce distant metastasis. This was supported by our observation of elevated *cxcr4* and *mmp9* in CSC-TEP cohorts [12]. Previous clinical studies have reported that high *cxcr4* is associated with the metastasis of breast tumors cells to either lungs, liver, brain and lymph nodes which in turn aligns with our results [12] [13]. Aspirin treated TEPs or RPs directly limited the *in-vitro* growth of tumor spheroids formed by BCSCs.

When BCSCs were co-cultured with RPs, a significant reduction in spheroid size was noted. Also, RPs when interacting with BCSCs, not only curb primary tumor growth *in-vivo* but also significantly suppress metastatic dissemination. In addition to EMT and metastasis, TEPs play an indispensable role in angiogenesis [14]. The production of blood vessels within the tumor is stimulated by the diverse proangiogenic molecules found within platelets [15] [16]. As a result, the tumor cells are able to proliferate beyond the avascular bulk and expand significantly. This study uniquely emphasizes the role of TEPs in orchestrating neovascularization and demonstrates that targeting TEPs with aspirin can prevent vessel sprouting at its source. Furthermore, while BCSC vasculogenic mimicry has been linked to aggressive tumor behaviour and metastasis, this is the first study to our knowledge showing that RPs restrains this phenomenon *in-vitro*. The dual blockade of classical angiogenesis and vasculogenic mimicry underscores aspirin's promise as a low-cost anti-vascular cancer therapy.

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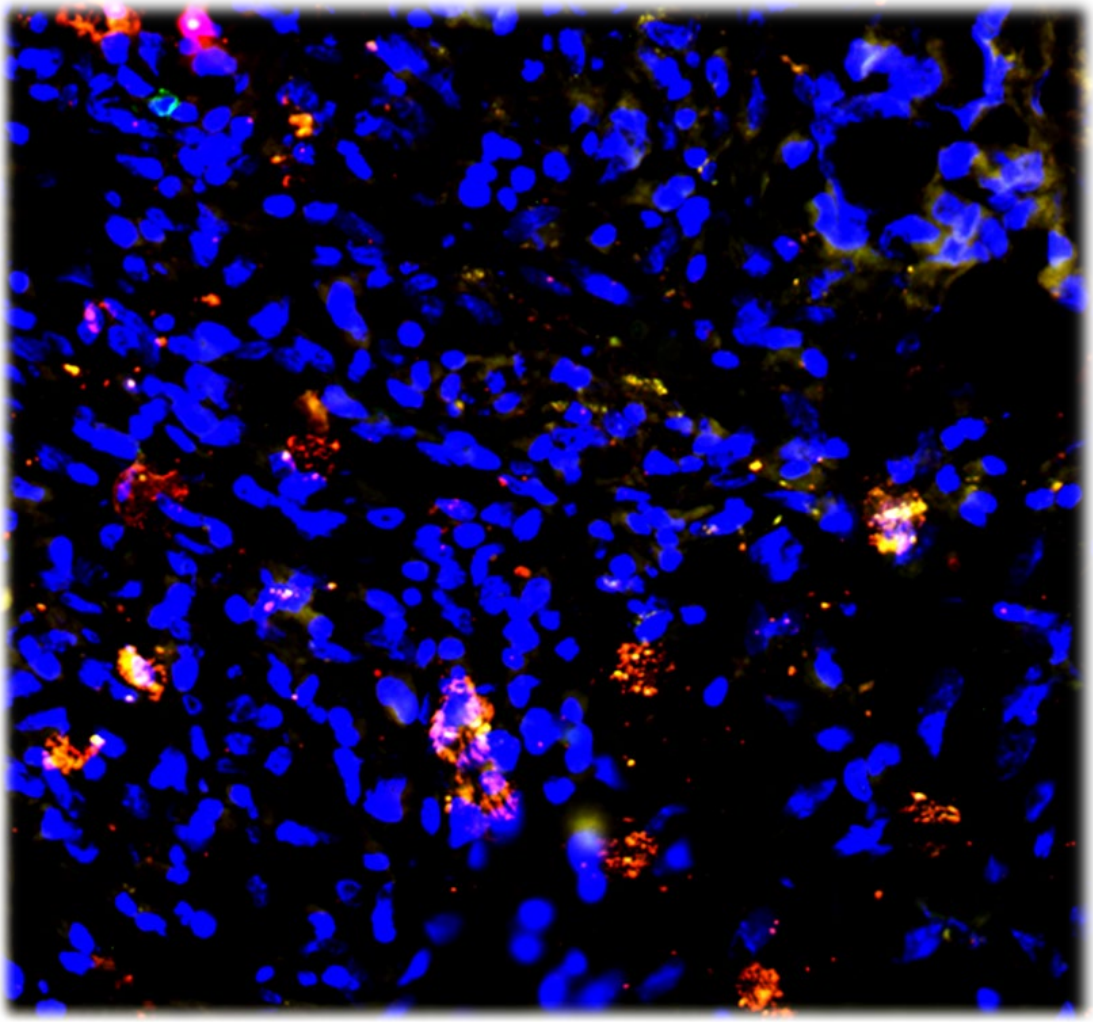
Chapter 5

Tumor educated platelets influenced stemness and metastasis of BCSCs is mediated by WNT-β-Catenin-VEGF-VEGFR2 cascade: Intervention by Aspirin



Confocal microscopy image of human triple negative breast tumor section depicting physical interaction between $OCT4^{+}$ (FITC) cancer stem cells and P-$selectin^{+}$(PE) tumor educate platelets at 40X magnification

Tumor educated platelets influenced stemness and metastasis of BCSCs is mediated by WNT-β-Catenin-VEGF-VEGFR2 cascade: Intervention by Aspirin.

Abstract:

Given all of the experimental data supporting TEPs involvement in tumor progression via their interactions with BCSCs, the next target was to identify the molecules in the signalling cascade that are responsible for downstream transmission of this information. BCSCs were assessed mainly for the receptor-ligand-based signalling pathways that regulate BCSC fate as there is substantial evidence indicating that the interaction between TEPs and BCSCs is mostly dependent on physical contact. RT-PCR analysis of *wnt, notch1* and *notch4* demonstrated up-regulation in the expression of only *wnt*, on BCSCs but not in the levels of *notch1* or *notch 4*. Based on several reports of VEGF mediated regulation of stemness and metastasis, the status of *vegf* as well as its receptors *vegfr1* and *vegfr2* was explored on BCSC surface. Selective upregulation of *vegfr2* along with *vegf* was noted in BCSCs under the influence of TEPs at both the gene and protein levels. Similar trend in the expression of WNT and its receptor FRIZZLED was also noted at the protein level, thus confirming the involvement of this axis in mediating the downstream signalling. Further, cytoplasmic to nuclear translocation of β-Catenin, reinforced our observation. To additionally verify their involvement, WNT and VEGFR2 were silenced by siRNA mediated targeting. Both individual and concomitant knockdown of both these proteins, hampered the tumorsphere forming ability of these CSCs, thereby demonstrating reduction in stemness. Also, flow cytometric analysis revealed that these BCSCs were less metastatic, upon dual blocking. Interestingly, the greatest reduction in stemness and metastasis was noted in the cohort where P-selectin was pharmacologically blocked by aspirin along with the dual inhibition of WNT and VEGFR2. This proved the direct link between P-selectin-PSGL1 with WNT-VEGFR2 axis. Thus, it can be concluded that the interaction between TEPs P-selectin with BCSCs PSGL1, results in 'switching on' of the WNT pathway, which in crosstalk with VEGF-VEGFR2 cascade promotes stemness and metastasis in both the BC subtypes.

Introduction:

A distinctive feature of many cancer types that supports the parallels between embryonic development and carcinogenesis is the deregulation of developmental signalling pathways. A conserved signalling axis involved in a

variety of physiological processes, including proliferation, differentiation, apoptosis, migration, invasion and tissue homeostasis, is the WNT/β-Catenin signalling system [1]. There is mounting evidence that certain solid tumors like colorectal, ovarian, breast and haematological malignancies develop and progress as a result of deregulation of the WNT/β-Catenin cascade [1].

The binding of WNT ligand to its receptor, results in the inhibition of the β-Catenin degradation complex (GSK3β/AXIN /APC). Un-phosphorylated β-Catenin then migrates from the cytosol to the nucleus, resulting in its nuclear accumulation. Within the nucleus β-Catenin interacts with LEF/TCF and triggers the expression of WNT target genes [2] [3] [4]. A number of clinical studies on basal like breast cancer in particular has revealed that high nuclear β-Catenin expression is associated with poor prognosis of the disease [5].

In human mammary epithelial cells, WNT1 expression promotes stem cell self-renewal, apoptotic resistance and senesce failure. Recent research employing the MMTV-WNT-1 mouse model has revealed an expanded pool of mammary stem cells (SC) from a population of committed luminal progenitors, suggesting that both progenitor cells and mammary stem cells may be the cellular targets of WNT-1-induced carcinogenesis [6]. This suggests that WNT-1 activation causes aberrant progenitor cells to appear.

Another crucial cytokine is VEGF that directs the growth of new blood vessels from pre-existing vascular networks and vascular development throughout embryogenesis [7]. VEGF promotes endothelial cell invasion and vascular development and is released by stromal and cancer cells. Due to restricted supply of nutrients and oxygen, tumor expansion is constrained in the absence of new blood vessel development [8] [9]. Numerous tumors express VEGF and over-expression of this protein is linked to poor prognosis and metastasis-related death [10]. The role of VEGF is not limited to angiogenesis and vasculogenesis. Autocrine VEGF has been demonstrated to promote tumor metastasis and works in tandem with EGFR to promote tumor development [11]. In fact, circulating VEGF levels are higher in patients with metastatic breast cancer than non-metastatic patients [11] [12].

In contrast to previous reports demonstrating oscillatory patterns in the expression of WNT and NOTCH to determine the fate of CSCs, in this current study we demonstrate a non-conventional crosstalk between WNT-β-Catenin and VEGF-VEGFR2 cascade, in the

downstream of P-selectin-PSGL1 receptor ligand interaction, to promote TEP mediated stemness and metastasis in luminal-A and TNBC breast cancer subtypes.

Objectives:

- To elucidate the downstream signalling cascade of TEP-BCSC interaction
- si-RNA-mediated silencing of the signalling cascades components to confirm the involvement of the implicated axis

Materials and Methods:

Cell lines and culture

MCF-7 (luminal-A) and MDA-MB-231(TNBC) cells were cultured in MEM and RPMI complete media respectively supplemented with 10% (v/v) heat inactivated FBS, 2mM L- glutamine, 100 U/ml penicillin and 100µg/ml streptomycin in incubator at 37°C and 5% CO_2. Cells were maintained for 10-12 passages and all experiments were performed within 6 months of purchase.

Co-culture

MCF-7 and MDA-MB-231, were cultured with TEPs and RP in 1:100 (BCSC: platelet) ratio for 24 hours. After the incubation period, the culture media with platelet suspension was removed and the cells were collected by trypsinization and centrifuged for further analysis.

Magnetic cell sorting

From the co-culture setup BCSCs were sorted by using magnetically labelled $CD44^+/CD24^-$ antibodies and cell purification was carried out according to manufacturer's protocol (Micro Bead kit, Miltenyi Biotech, Germany).

BCSC enrichment culture and tumorsphere assay

BCSCs ($1x10^4$) were cultured in stem cell enrichment media (serum free DMEM: F12K (1:1) media with 1% B27™ supplement (50X), heparin (40ng/mL), human-rEGF (20ng/mL) and human-rbFGF (20ng/mL) and plated on ultra-low adherent plate (Corning, New York, USA) and maintained for 7 days. Tumorspheres were micrographed and their count from 5 random fields was documented. Their area was calculated using ImageJ software. The tumorspheres formed were dissociated with trypsin and were centrifuged to produce single cell suspensions which were further analysed.

RT PCR

Total RNA content of single cell suspensions was extracted by Trizol

(Ambion, Thermo Fisher Scientific, MA, USA). cDNA was synthesized according to manufacturer's protocol from it using Revert Aid First Strand cDNA Synthesis Kit. Reverse transcriptase PCR was performed using 2X Go Taq Green Mix. Electrophoresis was done using 1.5% agarose gels and stained with ethidium bromide.

Western Blot

Total protein concentration of the lysates so obtained was determined by Bradford assay. 30-50µg of the protein lysates was separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane using BioRad Gel Transfer system and bands were developed using ECL Kit (Advansta, CA, USA). Band intensity was quantified using Image Lab 6.2 software (Bio-Rad, California, USA).

Flow cytometry

Briefly cells were stained with fluorescently tagged antibodies (WNT, FRIZZLED, VEGF, VEGFR1, VEGFR2, E-CADHERIN and VIMENTIN) and incubated in dark at 4°C for 30 minutes. For intracellular molecules, cells were treated simultaneously with 0.2% saponin (permeabilization buffer). Cells were finally washed, fixed with 1% paraformaldehyde and data was acquired using BD LSRFortessa X-20 Cell Analyzer (Becton Dickinson, New Jersey, USA).

siRNA mediated silencing *in-vitro*

siRNA for human-WNT and human-VEGFR2 were constructed according to the manufacturer's protocol *in-vitro* using the Ambion Silencer^{R} siRNA construction kit (Life Technologies, USA). Gene specific siRNA and scramble control siRNA (Sigma-Aldrich) were added to *in-vitro* setup to a final concentration of 50nM to the 2 hour-serum starved cells in presence of lipofectamine-2000 reagent (Invitrogen, USA). Silencing was performed as described in section 3.3.32 of Materials and methods. Sequence of primers utilized are mentioned below

***WNTsi Sense*:**

5'AAGCAGGCTCTGGGCAGCTACCCTGTCTC-3'

***WNTsi Antisense*:**

5'AAGTACTGCCCAGAGCCTGCCCTGTCTC -3'

***VEGFR2si Sense*:**

5'AAGGTGCTGCTGGCCGTCGCCCCTGTCTC-3'

***VEGFR2si Antisense*:**

5'AAGGCGACGGCCAGCAGCACCCCTGTCTC-3'

Statistical Significance

Statistical significance was drawn from One-way ANOVA / Two-way ANOVA followed by Tukey's multiple comparison test. Entire statistical analysis was performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, USA). Experimental results with $p \leq 0.05$ have been considered as significant.

Results:

TEP influenced stemness and metastasis of BCSCs is mediated in the downstream by WNT-β-Catenin-VEGF-VEGFR2 cascade

Considering the fact that physical contact dependency is involved in TEP-mediated stemness and BCSC metastasis, a number of probable receptor-ligand signalling pathways were investigated for potential relevance (Fig 5.1A). Post co-culture, the CSCs were initially analyzed using RT-PCR for the conventional pathways governing stemness and metastasis. The results demonstrated a boost in *wnt* expression but no changes in *notch1* or *notch 4* in the TEP-BCSC cohorts of both MCF-7 and MDA-MB-231(Fig 5.1B).

At the protein level too, this elevation in the expression of WNT and its receptor FRIZZLED was observed (Fig 5.1C). The cytoplasmic to nuclear translocation of β-Catenin further reinforced our findings (Fig 5.1D).

Moreover, we discovered multiple studies elucidating the role of VEGF in controlling stemness and metastasis of cancer cells. In accordance with this, BCSC's *vegf* and surface *vegf* receptor status was examined. Compared to the non-treated control CSCs, the TEP influenced cohort had higher mRNA expression of *vegf* and its corresponding receptor *vegfr2* (Fig 5.1B). Nonetheless, *vegfr1* remained almost unchanged throughout the groups (Fig 5.1B).

Similar observation was also noted at the protein level (Fig 5.1C). Additionally, String analysis strengthened our observation by disclosing direct link between the targeted molecules (Fig 5.1E).

Taken together, these findings confirmed that the influence of TEPs on BCSCs is mediated via WNT- β-Catenin-VEGF-VEGFR2 in the downstream to promote stemness and metastasis in BC subtypes.

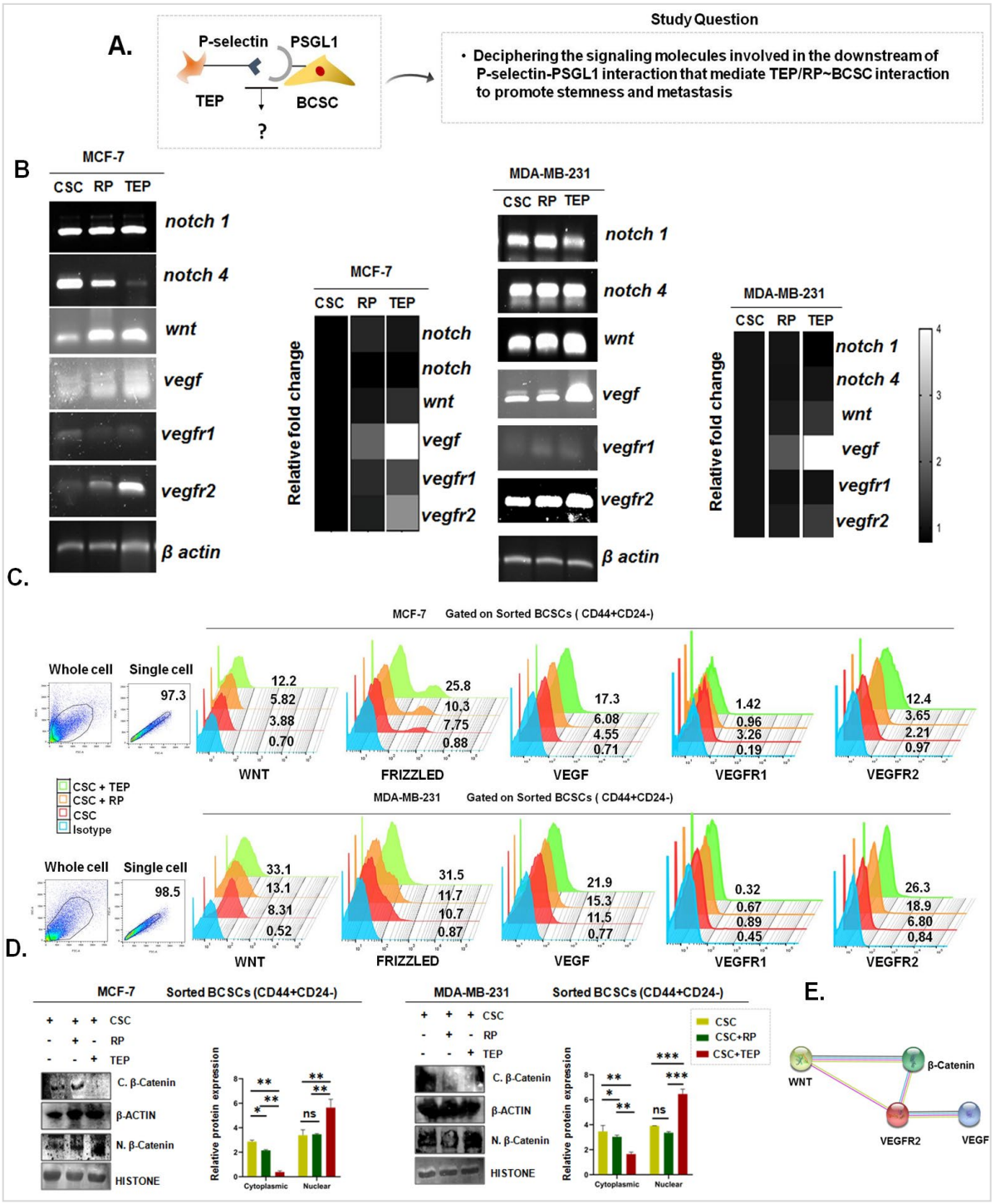


Figure 5.1: TEP influenced stemness and metastasis of BCSCs is mediated in the downstream by WNT-β-Catenin-VEGF-VEGFR2 cascade: A. Schematic representation of receptor-ligand interaction between BCSCs and TEP/RP followed by study question. **B.** mRNA expression of *notch1, notch4, wnt, vegf, vegfr1, vegfr2* in CSC, CSC+RP, CSC+TEP of MCF-7 and MDA-MB-231 by RT-PCR is represented keeping β actin as control (n=6). Relative fold change in expression levels of the genes is provided by heatmaps. Darker colour denotes lower expression while lighter colour signifies higher expression respectively. **C.** Representative flow-cytometric histograms depicting expression of proteins WNT, FRIZZLED, VEGF, VEGFR1 and VEGFR2 on the surface of CSC, CSC+RP,

CSC+TEP of MCF-7 and MDA-MB-231. *Inset*: blue, red, orange and green column denoting Isotype, CSC, CSC+RP, CSC+TEP respectively **D.** Illustrative western blots for cytoplasmic and nuclear fractionation of β-Catenin keeping β-ACTIN and HISTONE as respective controls across all the groups in both MCF-7 and MDA-MB-231.Bar diagram (mean±SD) depicting relative protein expression is presented. Statistical significance inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=3). *Inset*: yellow, green and red column denoting CSC, CSC+RP, CSC+TEP. **E.** String analysis illustrating link between WNT-β-Catenin-VEGF-VEGFR2 is provided. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

siRNA mediated knockdown of WNT and VEGFR2 reduced TEP mediated stemness of BCSCs

To further verify the impact of this cascade, WNT and VEGFR2 were knocked out both individually as well as concomitantly *in-vitro* by siRNA mediated silencing in BCSCs of both MCF-7 and MDAMB-231.Following the incubation period, co-culturing was executed using TEP and RP and cells were propagated in 3D CSC enrichment media for formation of primary tumorspheres.

Inhibition of WNT and VEGFR2 affected tumorsphere count significantly, but the greatest reduction was observed when both WNT and VEGFR2 were silenced concomitantly. Surprisingly, in RP-BCSC cohort with dual knockdown of WNT and VEGFR2 exhibited the highest reduction in tumorsphere count amongst all the experimental groups in both MCF-7 and MDA-MB-231(Fig 5.2 A,B).

To further verify the involvement of P-selectin-PSGL1-WNT-VEGFR2 in regulating stemness, String data base was explored. The interactome thus generated, provided direct link between the major stemness regulating transcription factors with the signalling cascade (Fig 5.2C).

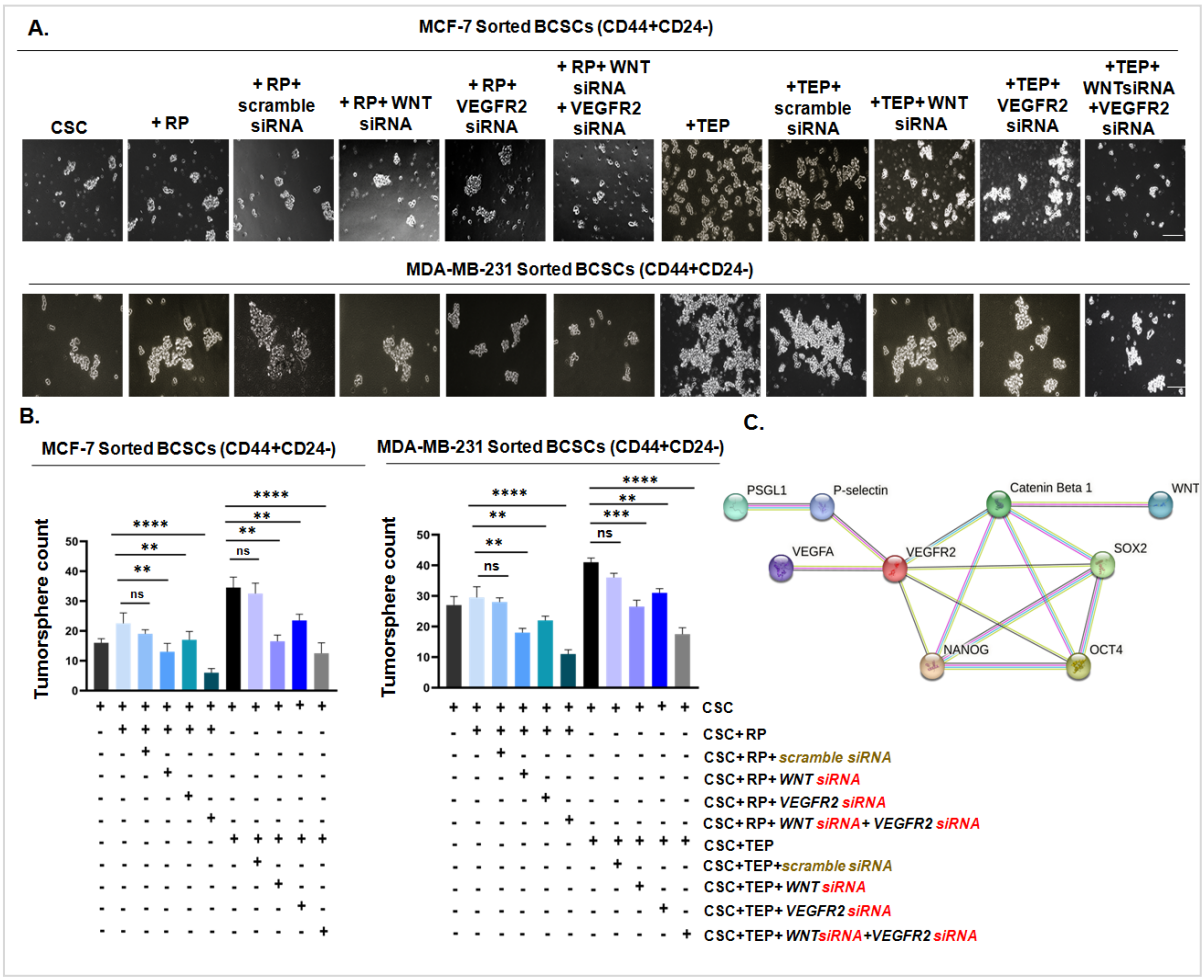


Figure 5.2: siRNA mediated knockdown of WNT and VEGFR2 reduced TEP mediated stemness of BCSCs: A. Representative micrographs of mammospheres at 10X magnification across all experimental groups in presence and absence of WNT siRNA and VEGFR2 siRNA in MCF-7 and MDA-MB-231.**B.** Bar graphs (mean±SD) depicting tumorsphere count in each of the groups of MCF-7 and MDA-MB-231 is presented with statistical significance inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=3).**C.** Representative interactome generated from String analysis displaying the link between the molecules of the signalling cascade with the major stemness controlling transcription factors.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

siRNA mediated knockdown of WNT and VEGFR2 reduced TEP mediated metastasis of BCSCs

Along with stemness, impact of WNT and VEGFR2 knockdown on metastasis was also investigated. Flow cytometric analysis of BCSCs revealed a similar trend like stemness in the percentage of metastatic CSCs of both the sub-types (Fig 5.3A,B). Here too, inhibition of WNT and VEGFR2 both individually and concomitantly reduced the percentage of metastatic TEP

influenced BCSCs ($CD44^{+}/CD24^{-}$/E-$CADHERIN^{low}/VIMENTIN^{high}$).

Further, the highest reduction was noted in RP-BCSC cohort, where the activity of P-selectin on TEPs has been blocked by aspirin along with dual knockdown of WNT and VEGFR2 in both MCF-7 and MDA-MB-231. This confirmed the direct link between P-selectin-PSGL1 axis with WNT-VEGFR2 cascade. Additionally, String database interactome proved the interconnection between the signalling cascade and the transcription factors and proteins regulating EMT and metastasis (Fig 5.3C).Cumulatively, our findings validate the involvement of WNT- β-Catenin-VEGF-VEGFR2 signalling pathway in the downstream of TEP-P-selectin-BCSC-PSGL1 interaction to mediate stemness, metastasis and overall aggressiveness in luminal-A and TNBC BC subtypes.

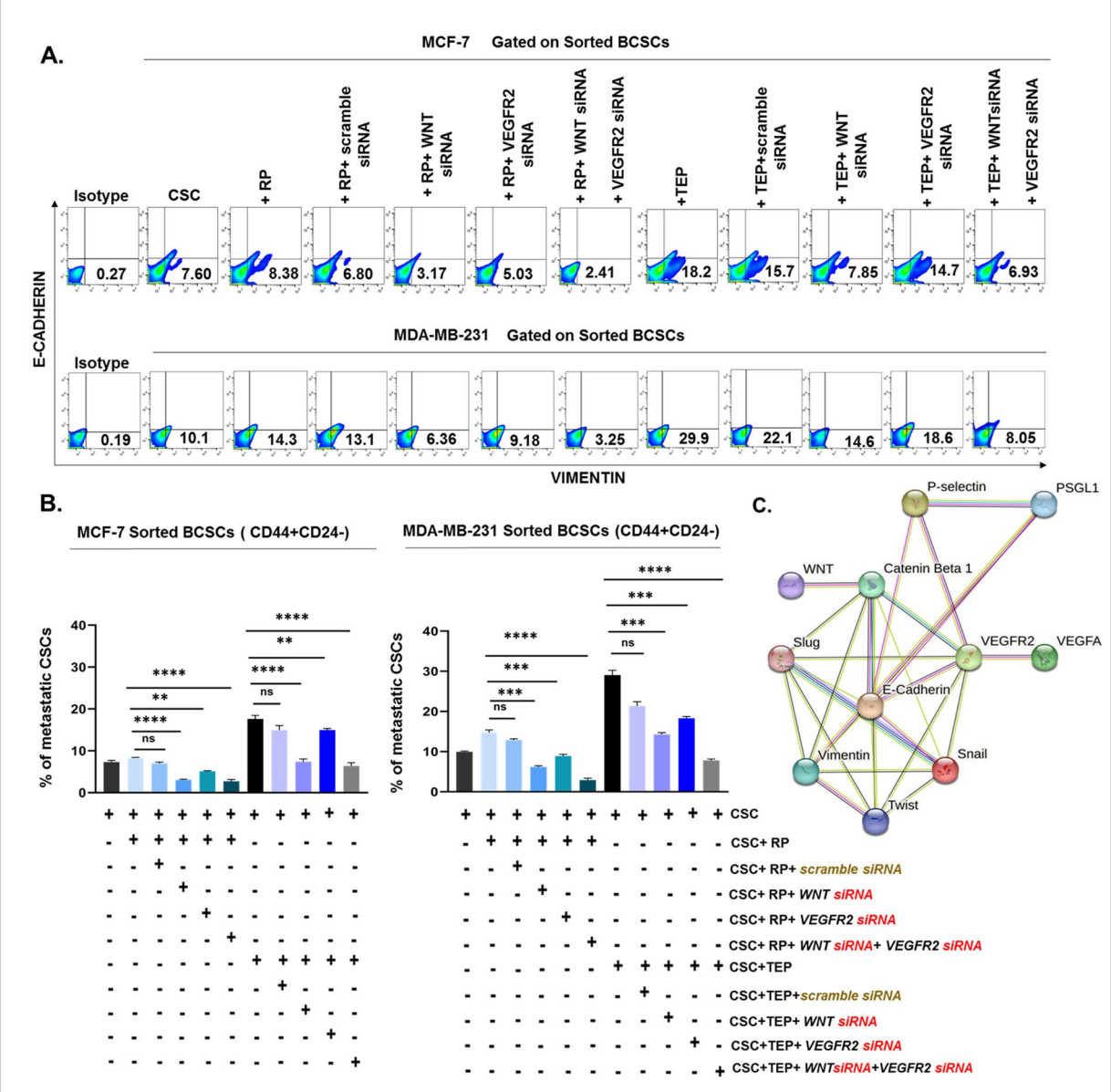


Figure 5.3: siRNA mediated knockdown of WNT and VEGFR2 reduced TEP mediated metastasis of BCSCs: A. Pseudo-colourflow cytometric plots depicting changes in $CD44^{+}24^{-}$ $VIMENTIN^{high}E\text{-}CADHERIN^{low}$ metastatic BCSC frequency across all experimental cohorts of MCF-7 and MDA-MB-231 in presence and absence of WNT siRNA and VEGFR2 siRNA. **B.** In bar graphs (mean±SD) % of metastatic BCSCs across the groups of MCF-7 and MDA-MB-231 is presented. Statistical significance inferred from one-way ANOVA followed by Tukey's multiple comparison test (n=3) **C.** Interactome from String database analysis showcasing the link between the components of the proposed signalling cascade with the major transcription factors and proteins regulating EMT and metastasis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Discussion:

Oncogenic transformation is promoted by the abnormal activation or repression of a complex signalling network that regulates the homeostasis of healthy stem cells. These abnormalities confer the tumor cells with stemness by causing CSCs to differentiate and self-renew. For survival and maintenance of their stemness, CSCs depend on these signalling pathways just like their normal counterparts.

Our study uncovers a novel mechanistic axis wherein the interaction between P-selectin on tumor-educated platelets (TEPs) and PSGL-1 on breast cancer stem cells (BCSCs) activates a signalling cascade involving WNT/β-catenin and VEGF/VEGFR2 pathways. This crosstalk enhances stemness and metastatic potential in BCSCs. Furthermore, siRNA-mediated silencing of WNT and VEGFR2 pathways reinforces the pivotal role of this axis in BCSC biology. The WNT/β-catenin pathway is well-established in regulating stemness, proliferation and chemoresistance in BCSCs [13] [14]. Activation of this pathway promotes the expression of stemness markers such as CD44, NANOG, OCT4 and SOX2 contributing to the maintenance of the BCSC phenotype. Our findings align with these observations, demonstrating that the P-selectin-PSGL-1 interaction initiates WNT/β-catenin signalling and thereby enhances BCSCs stemness. High WNT expression on BCSCs under TEPs influence may help regulate their stemness and other characteristics by encouraging the expression of NANOG-OCT4-SOX2. Furthermore, it promotes the migration of these CSCs by triggering TWIST expression. Increased TWIST levels have the ability to directly block transcription of the E-CADHERIN promoter, increasing the mobility of CSCs. Additionally; TWIST can also suppress E-CADHERIN in conjunction with SNAIL protein. Further, nuclear β-Catenin can also stimulate autocrine VEGF production on BCSCs. Additionally; the VEGF/VEGFR2 axis plays a crucial role in angiogenesis

and has been implicated in maintaining cancer stem cell properties. VEGF signalling not only promotes vascularization but also directly influences CSC proliferation and self-renewal.

In our study, activation of VEGF/VEGFR2 signalling along with WNT/β-catenin further amplifies BCSC stemness and metastatic capabilities.

The interplay between WNT/β-catenin and VEGF/VEGFR2 pathways suggests a synergistic mechanism that reinforces BCSC properties. This crosstalk has been observed in other cancers, where WNT signalling regulates angiogenic factors, including VEGF, contributing to tumor progression.

Our data support this model, indicating that the P-selectin–PSGL-1 interaction serves as an upstream activator of this synergistic signalling network in BCSCs.

Importantly, our study demonstrates that dual knockdown of WNT and VEGFR2 pathways significantly reduces cancer stemness and metastatic potential when compared to individual pathway inhibition.

This confirms the potential synergistic interaction between WNT and VEGFR2 signalling in maintaining cancer stem cell properties and facilitating metastatic dissemination. Furthermore, the most profound reduction in both stemness and metastasis was observed when this dual knockdown was combined with the inhibition of P-selectin-PSGL-1 interactions using aspirin. Aspirin's ability to interfere with this interaction may reduce the physical support tumor cells receive during hematogenous spread, effectively complementing the molecular suppression of stemness and angiogenic support through WNT and VEGFR2 knockdown. The triple-intervention approach thus appears to target distinct yet cooperative mechanisms driving cancer progression: (i) WNT signalling, for maintaining the stem-like state of tumor cells, (ii) VEGFR2-mediated angiogenesis and support of the tumor microenvironment and (iii) the adhesive and protective role of P-selectin-PSGL1 interactions in metastasis. The additive or possibly synergistic effects of this combinatorial strategy underscore the importance of multi-targeted therapies, especially for aggressive or treatment-resistant tumors. In conclusion, our study elucidates a critical mechanism by which the P-selectin-PSGL-1 interaction activates WNT/β-catenin-VEGF/VEGFR2 signalling cascade, promoting stemness and metastasis in BCSCs. Targeting this axis offers promising avenues for therapeutic intervention.

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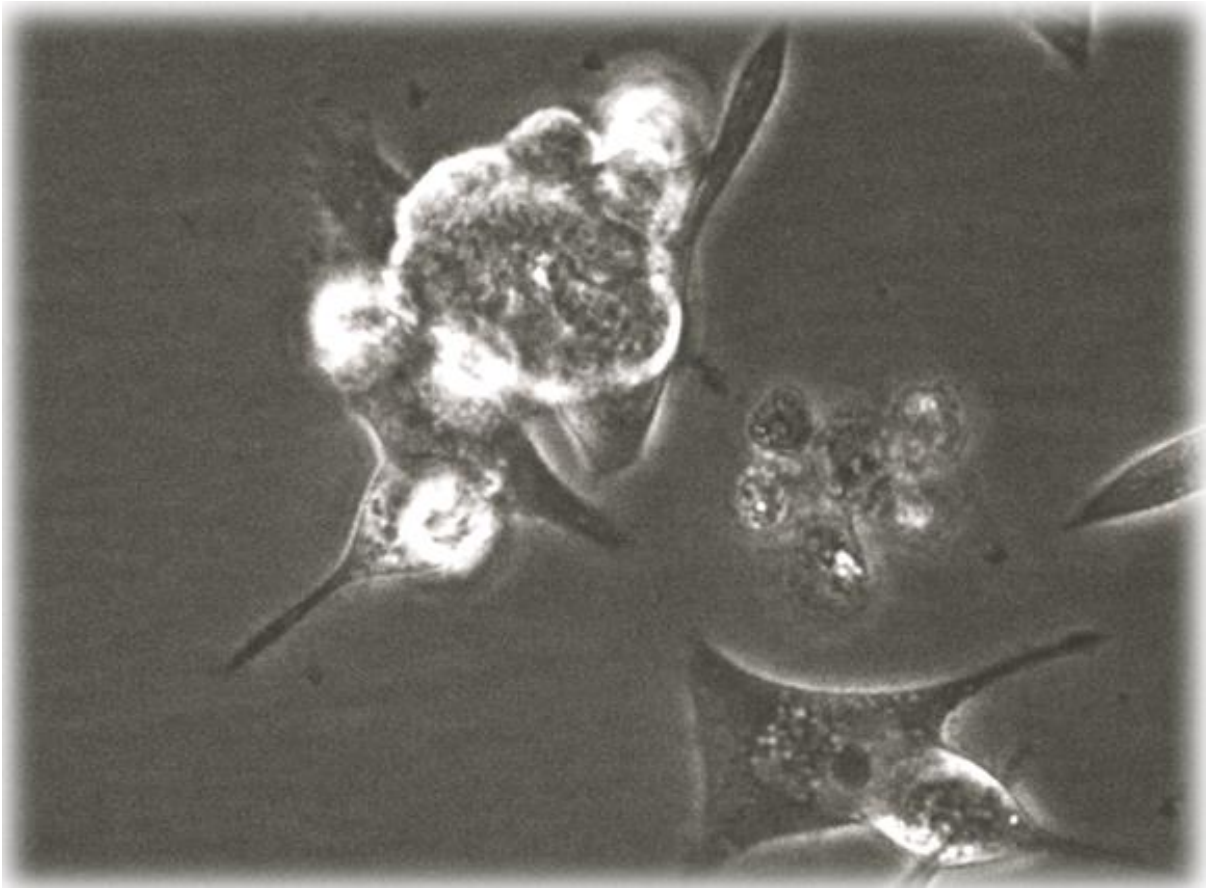
Chapter 6

Platelet-Poor Plasma Disrupts Breast Cancer Stem Cells Quiescence, Reduces Stemness and Sensitizes to Therapy



Phase-contrast microscopy image of mammospheres of MDA-MB-231 treated with platelet poor plasma at 40X magnification

Platelet-Poor Plasma Disrupts Breast Cancer Stem Cells Quiescence, Reduces Stemness and Sensitizes to Therapy

Abstract:

Tumor-educated platelets (TEPs) in breast cancer are emerging as critical modulators of tumor behaviour. In our study, we found that TEPs are morphologically and functionally distinct from platelets in healthy individuals. Importantly, they physically interact with breast cancer stem cells (BCSCs), promoting stemness traits and enhance metastatic capabilities. Pharmacological inhibition of TEPs using aspirin reversed these aggressive phenotypes, indicating a potential therapeutic strategy. While previous studies have reported that platelet-rich plasma (PRP) can inhibit breast cancer proliferation, the effect of platelet-poor plasma (PPP) on BCSCs remained unexplored. We observed that PPP significantly hindered BCSCs sphere formation, colony formation and wound healing capacity, suggesting impaired self-renewal, proliferation and migration abilities. On the molecular level, PPP treatment led to a notable down-regulation of key stemness markers (*nanog, oct4, sox2*) and drug resistance genes (*abcb1, abcc1*) indicating a loss of both stem-like properties and chemoresistance. Furthermore, PPP-treated BCSCs shifted from a quiescent G0/G1 phase into a more proliferative S phase, with increased Ki-67 expression, rendering them potentially more susceptible to conventional therapies that target dividing cells. These findings suggest that PPP-based treatments could force BCSCs out of dormancy, diminish their drug resistanceand sensitize them to chemotherapy or targeted agents. Combining PPP or agents mimicking its effects with standard treatments might effectively eliminate the BCSC population, thereby reducing recurrence, metastasis and therapy failure. This strategy could represent an important advancement in overcoming one of the major hurdles in breast cancer management resistance driven by the BCSCs.

Introduction:

Breast cancer remains one of the leading causes of cancer-related mortality among women worldwide, largely due to metastatic relapse and therapeutic resistance (Siegel et al., 2024) [1]. Growing body of evidence suggests that platelets, beyond their classical role in haemostasis, actively contribute to tumor progression and metastasis (Gay &Felding- Habermann, 2011) [2]. Tumor-educated platelets (TEPs), which are platelets modified through interactions

with tumor-derived signals, exhibit distinct characteristics which are quite contrasting to platelets from healthy individuals (Best et al., 2015) [3]. In our study, we have found that TEPs of luminal-A and TNBC BC patients are morphologically and functionally distinct from platelets in healthy individuals. Importantly, they physically interact with breast cancer stem cells (BCSCs), promoting stemness traits and enhancing metastatic capabilities.

In parallel, platelet-rich plasma (PRP), typically rich in growth factors, has paradoxically been reported to inhibit breast cancer cell proliferation under certain conditions (Xiong et al., 2021) [4]. While PRP's influence has been partially elucidated, the role of platelet-poor plasma (PPP) in modulating breast cancer biology, particularly in BCSC populations, remains unexplored. Given that PPP lacks most platelet-derived pro-metastatic mediators while retaining key plasma proteins, it presents a unique opportunity to investigate the effects of platelet depletion on BCSC behaviour.

In this study, we examined the impact of PPP on BCSCs. Our findings reveal that PPP treatment markedly hinders BCSC functional capacities, including sphere formation, colony formation and wound healing potentialities. Molecular analysis demonstrated significant downregulation of stemness-associated transcription factors like *nanog, oct4 and sox2*.Concurrently, we observed a significant reduction in the expression of multidrug resistance genes such as *abcb1* (P-glycoprotein) and *abcc1* (MRP1), which are pivotal for BCSCs ability to efflux chemotherapeutic agents and survive treatment. The suppression of these transporters suggests that PPP may directly impair the mechanisms underlying drug resistance in breast CSCs.

Furthermore, cell-cycle profiling indicated a shift of BCSCs from a quiescent G_0/G_1 phase toward active S phase, accompanied by elevated Ki67 expression. This finding is particularly important, as BCSCs often remain in a dormant or quiescent state, rendering them resistant to conventional chemotherapeutic agents that primarily target dividing cells. By driving quiescent BCSCs into the cell cycle, PPP potentially primes them for increased sensitivity to cytotoxic therapies.

The awakening of quiescent BCSCs could render them more susceptible to conventional-chemotherapies,
overcoming one of the major barriers to successful cancer treatment. In light of these findings, PPP emerges as a potential

therapeutic strategy that could be integrated with existing chemotherapy protocols. Its ability to simultaneously weaken stemness, reverse drug resistance and induce cell cycle re-entry positions PPP as a multifaceted agent capable of disrupting the BCSC-driven mechanisms underlying breast cancer progression, metastasis and relapse. Further *in-vivo* studies and clinical investigations will be essential to validate these observations and to fully harness the therapeutic potential of PPP in breast cancer management.

Objectives:

- ❖ To determine the impact of PPP on BCSC functional properties, including tumorsphere formation, colony formation and migration.
- ❖ To assess changes in the expression of key stemness-related markers (e.g., *nanog, oct4, sox2*) in BCSCs following treatment with PPP.
- ❖ To evaluate the effect of platelet poor plasma (PPP) on the viability and proliferation of cancer stem cells.
- ❖ To investigate whether PPP modulates the sensitivity of BCSCs to chemotherapeutic agents by targeting the major drug efflux transporters like *abcb1, abcc1*.

Materials and Methods:

Isolation of platelet poor plasma

For isolating platelet poor plasma (PPP), initially, 5ml of blood was collected in ACD anti-coagulant from healthy donors who have not consumed anti-platelet drugs for the last six months. Blood was centrifuged initially at 260g for 20 minutes to separate the platelet rich plasma. This plasma was again centrifuged for 20 minutes at 800g to separate the platelet pellet and platelet poor plasma. The PPP was separated carefully to prevent any contamination from the underlying platelet pellets. The collected PPP was aliquoted in sterile vials of 1ml each and stored at -80ºC for later use.

Cell lines and culture

MCF-7 (luminal-A) and MDA-MB-231 (TNBC) cells were cultured in MEM and RPMI complete media respectively supplemented with 10% (v/v) heat inactivated FBS, 2mM L- glutamine, 100 U/ml penicillin and 100µg/ml streptomycin in incubator at 37°C and 5% CO_2. Cells were maintained for 10-12 passages and all experiments were performed within 6 months of purchase.

Magnetic cell sorting

From the cultured cells of MCF-7 and MDA-MB-231, BCSCs were sorted by using magnetically labelled $CD44^{+}/CD24^{-}$ antibodies and cell purification was carried out according to manufacturer's protocol (MicroBead kit, Miltenyi Biotech, Germany).

Co-culture of BCSCs with PPP and enrichment in CSC enrichment media

BCSCs ($1x10^5$) were cultured in stem cell enrichment media (serum free DMEM: F12K (1:1) media with 1% B27™ supplement (50X), heparin (40ng/mL), human-rEGF (20ng/mL), human-rbFGF (20ng/mL)) were added freshlyand plated on ultra-low adherent plate (Corning, New York, USA) and maintained for 5 days in presence/absence of 50µL of PPP. The plates were micrographed and the results of 5 random fields was documented and analysed via ImageJ software.

Soft agar colony formation assay

Briefly $5x10^3$ cells from the co-culture setup were cultured on upper 0.35% soft agar layer along with 1mL culture media, keeping non-treated BCSCs as control. This layer was placed upon bottom agar bed of 0.7% agar and cell culture medium (MEM and RPMI complete media respectively for MCF-7 MDA-MB-231). The culture setup was maintained for 21 days. The colonies were finally micrographed and the number of colonies in presence or absence of PPP was quantified using ImageJ software.

RT PCR

Post co-culture samples were processed for RNA extraction and cDNA preparation according to manufacturer's protocol. Briefly, total RNA content of single cell suspensions was extracted by Trizol (Ambion, Thermo Fisher Scientific, MA, USA). cDNA was synthesized from it using Revert Aid First Strand cDNA Synthesis Kit. Reverse transcriptase PCR was performed using 2X Go Taq Green Mix. Electrophoresis was done using 1.5% agarose gels and stained with ethidium bromide.

Ki67 Proliferation assay

Experimental samples were immediately fixed in 4% PFA and incubated for 15 mins at RT to preserve the phosphorylation status. Fixed cells were then permeabilized by adding absolute methanol drop wise to the sample while vortexing it at 10-15 s at 2500 rpm at RT. The cells were then stained with fluorescently tagged Ki67 antibody and post incubation the cells were thoroughly washed to remove unbound molecules. Data was acquired using FACS Calibur

and LSR Fortessa X-20 Cell Analyzer (Becton Dickinson, New Jersey, USA)

Cell Cycle Analysis

Cells from experimental groups were harvested, fixed in chilled 70% methanol while vortexing, followed by incubation at −20◦C overnight. Following day, the cells were washed twice with PBS containing 0.1% sodium azide, and followed by a treatment of 50 µL (100 ng/mL) RNase A. Finally, the cells were stained with 200 µL (50µg/mL) propidium iodide (PI) and analysed by flow-cytometry without any delay.

ELISA

96 well plates were immobilized with antigens collected from different experimental groups. Primary antibodies for IL-6, TGFβ, VEGF and IL-10 was added to these plates and incubated overnight at 4ºC. HRP-conjugated secondary antibody was added next and incubated for 3 hours at 37ºC. Finally, TMB substrate (BD OptEIA, BD Biosciences) was added and the resultant colorimetric output was measured by Spectra-max i3X (Molecular Devices, San Jose, USA) at 450 nm and accounted via SoftMax Pro 7.1 software.

Wound healing assay

Magnetically sorted, $1x10^4$ $CD44^+/CD24^-$ BCSCs of MCF-7 and MDA-MB-231 were grown in presence/absence of PPP for 24 hours in 6 well cell culture plates. Following the incubation period, the cells were serum starved for 4 hours. A scratch or wound was drawn next using a cell scratcher and wound healing was observed by taking micrographs at different time points for 48 hours. Percentage wound closure was calculated as final area/initial area X 100%

Statistical analysis

All statistical analysis was performed using GraphPad Prism software 8.4.2. Significance was drawn from either unpaired Student T-test or One-way ANOVA / Two-way ANOVA followed by Tukey's multiple comparison. $p<0.05$ were considered as statistically significant.

Results:

Platelet poor plasma attenuates tumorigenicity of BCSCs

To investigate the effect of platelet-poor plasma (PPP) on the spheroid-forming ability of cancer stem cells (CSCs), magnetically sorted ($CD44^+24^-$) BCSCs derived from MCF-7 and MDA-MB-231 breast cancer cell lines were cultured in stem cell enrichment medium under ultra-low attachment conditions. Each well was seeded with 1×10^5 BCSCs and 50 µL of PPP was added to the treatment group at

the time of plating. Untreated BCSCs cultured under identical conditions served as the control (Fig 6.1A).

Throughout the incubation period of 5 days(0 hour – 120 hours), control BCSCs gradually formed prominent, compact and well-circumscribed tumor spheroids, consistent with their known capacity for anchorage-independent growth and self-renewal under non-adherent conditions (Fig 6.1B). In contrast, BCSCs treated with PPP exhibited a complete loss of spheroid-forming ability. Surprisingly, despite the non-adherent nature of the culture surface, PPP-treated cells displayed a strong tendency to adhere to the plate surface. These cells formed flattened, spread-out monolayers, indicating a shift away from stem-like behavior toward a more differentiated or adherent phenotype (Fig 6.1B).

Further, to assess the impact of PPP on the tumorigenic potential of BCSCs *in-vitro*, soft agar colony formation assay was performed. In untreated control groups, both MCF-7and MDA-MB-231-derived BCSCs demonstrated robust anchorage-independent growth, forming numerous large and compact colonies on soft agar plate, indicative of their strong self-renewal and tumorigenic capacities (Fig 6.1C). However, upon PPP treatment, a marked reduction in both the number and size of colonies was observed across both cell lines (Fig 6.1C). Importantly, the inhibitory effects of PPP on anchorage-independent growth were consistent across both MCF-7 and MDA-MB-231, indicating that PPP's anti-BCSC activity is not restricted to a particular molecular subtype of breast cancer but spans across both hormone receptor-positive and triple-negative breast cancers.

Additionally, treatment of BCSCs with PPP led to a significant reduction in their migratory potential, as demonstrated by the scratch wound healing assay (Fig 6.1D). In the untreated control groups, the BCSCs from both MCF-7 and MDA-MB-231 exhibited robust migration and were able to close the wound area within 48hours, demonstrating their inherent migratory ability. However, upon treatment with PPP, a marked impairment in migration was observed. The closure of the wound was significantly delayed in both cell lines, with a smaller proportion of the wound area closed at the same time points compared to the untreated controls (Fig 6.1D). Quantification of the wound area showed a significant reduction in migration in both MCF-7 and MDA-MB-231 BCSCs treated with PPP. These results suggest that PPP treatment exerts a potent inhibitory effect on the migration of BCSCs, potentially through the disruption

of key migratory pathways or processes essential for cell movement. The impaired migration observed in both MCF-7 and MDA-MB-231 BCSCs indicates that PPP may play a role in preventing the dissemination of BCSCs, a critical step in cancer metastasis.

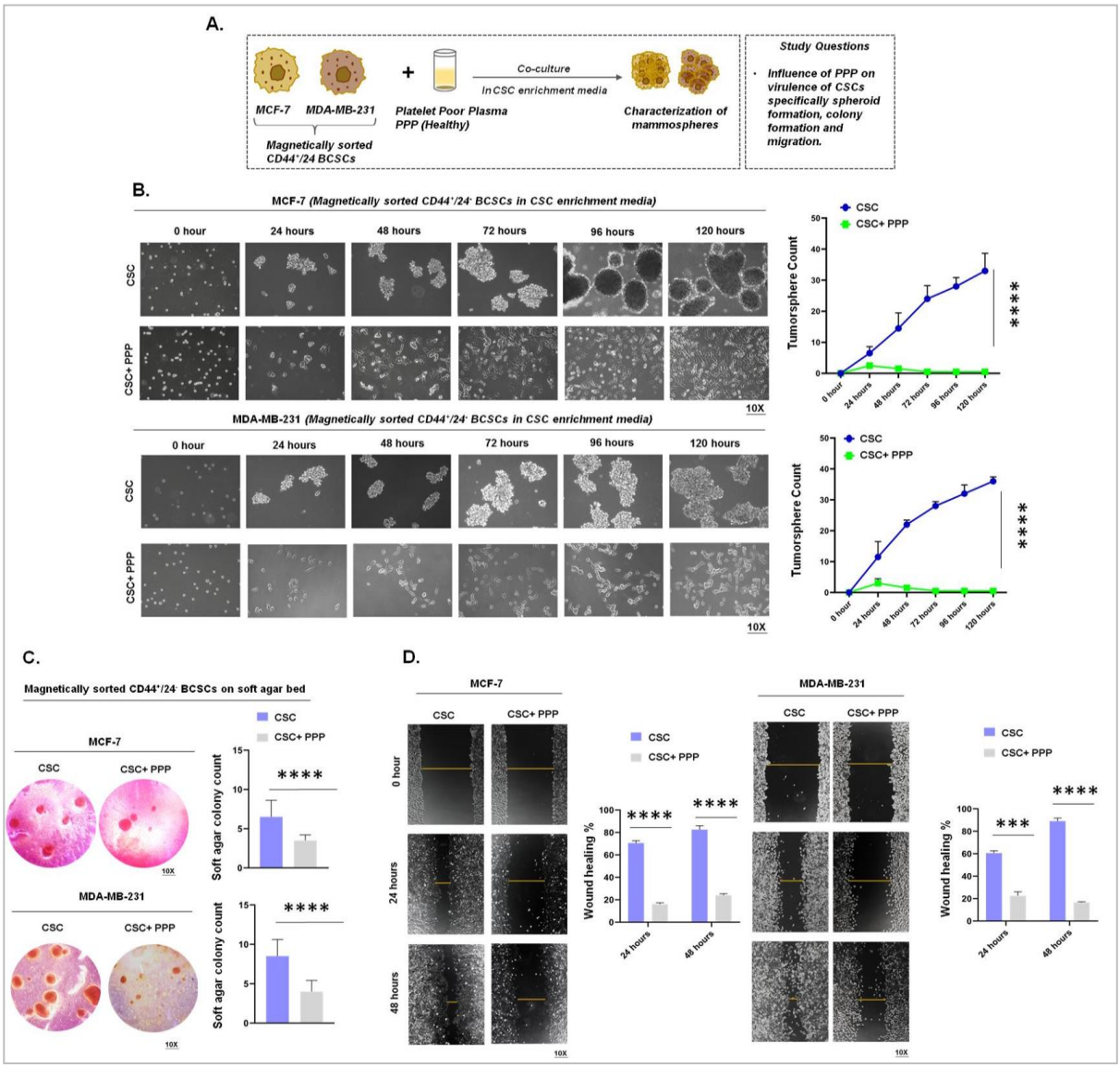


Figure 6.1: Platelet poor plasma attenuates tumorigenicity of BCSCs-A. Schematic representation of study design. **B.** Representative phase-contrast microscopy images at 10X magnification showing morphological differences between untreated magnetically sorted ($CD44^{+}24^{-}$) BCSCs and PPP-treated BCSCs of MCF-7 and MDA-MB-231 for a span of 5 days (0 hours to 120 hours) in ultra-low attachment plates. Untreated BCSCs form compact, spherical tumor spheroids, while PPP-treated BCSCs exhibit a spread, adherent morphology despite non-adherent conditions (n=10). Individual point-line graph denoting the differences in tumorsphere count in presence and absence of PPP is presented. *Inset*: Blue and Green boxes denoting CSC and CSC-PPP respectively. Statistical significance inferred from unpaired non-parametric t-test, followed by two-tailed p value. **C.** Soft agar colony images of BCSCs and PPP-treated BCSCs of MCF-7 and MDA-MB-231 is represented.

Number of colonies per field in each cohort is depicted. In bar-graph (mean±SD) with statistical significance drawn from unpaired non-parametric t-test, followed by two-tailed p value (n=3) is represented. *Inset*: Blue and Grey boxes denoting CSC and CSC-PPP respectively. **D.** Microscopic images at 10X magnification of wound healing assay at 0 hour, 24 hours and 48 hours in BCSCs and PPP-treated BCSCs of MCF-7 and MDA-MB-231 is represented. Bar diagram (mean±SD) representing percentage wound closure in all the study groups of both the cohorts. Unpaired non-parametric t-test, followed by two-tailed p value (n=3) test was performed to draw statistical significance (n=3). *Inset*: Blue and Grey boxes denoting CSC and CSC-PPP respectively. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Platelet poor plasma promotes proliferation by redrawing the BCSCs from G0/G1 quiscence phase of the cell cycle to synthesis phase

To determine the effect of platelet-poor plasma (PPP) on proliferation of MCF-7 and MDA-MB-231 CSCs, magnetically sorted ($CD44^{+}24^{-}$) BCSCs were cultured under non-adherent conditions using ultra-low attachment plates in stem cell enrichment medium (Fig 6.2A). BCSCs were treated with 50 µL of PPP at the time of seeding and untreated BCSCs served as controls. The cultures were maintained for 5 days to allow for primary tumor spheroid formation (Fig 6.2A). PPP treated BCSCs displayed strikingly different phenotype compared to untreated controls . Spheroid formation was completely abrogated and instead, cells exhibited a pronounced adherent morphology, spreading across the plate surface despite the ultra-low attachment environment. To further explore the cellular mechanisms underlying this phenotypic change, cell cycle analysis was performed. Flow cytometric quantification revealed a significant redistribution of the cell population following PPP treatment. While control BCSCs exhibited a predominant accumulation in the G_0/G_1 phase, indicative of a slow-cycling or quiescent state typical of stem cells, PPP-treated cells showed a marked increase in the S phase population (Fig 6.2B). This shift reflected towards enhanced DNA synthesis and proliferative activity.

Interestingly, the cell cycle profile of PPP-treated BCSCs closely resembled that of bulk tumor cells maintained under standard monolayer culture conditions, suggesting a reversion to a more proliferative, non-stem-like cellular state (Fig 6.2B). Consistent with the cell cycle findings, flow cytometric analysis of Ki-67, demonstrated a significant upregulation in PPP-treated BCSCs compared to untreated controls. The increase in Ki-67 expression reinforced the observation that PPP drives BCSCs out of dormancy into active cycling phase (Fig 6.2C). Collectively, these data indicate that PPP treatment not only suppresses the ability of BCSCs to form tumor spheroids

but also induces a profound shift in their biological state. The cells transit from a quiescent, spheroid-forming to an adherent, proliferative phenotype.

These findings suggest that factors present in PPP can modulate BCSC plasticity, potentially by altering pathways governing stemness, proliferation and adhesion.

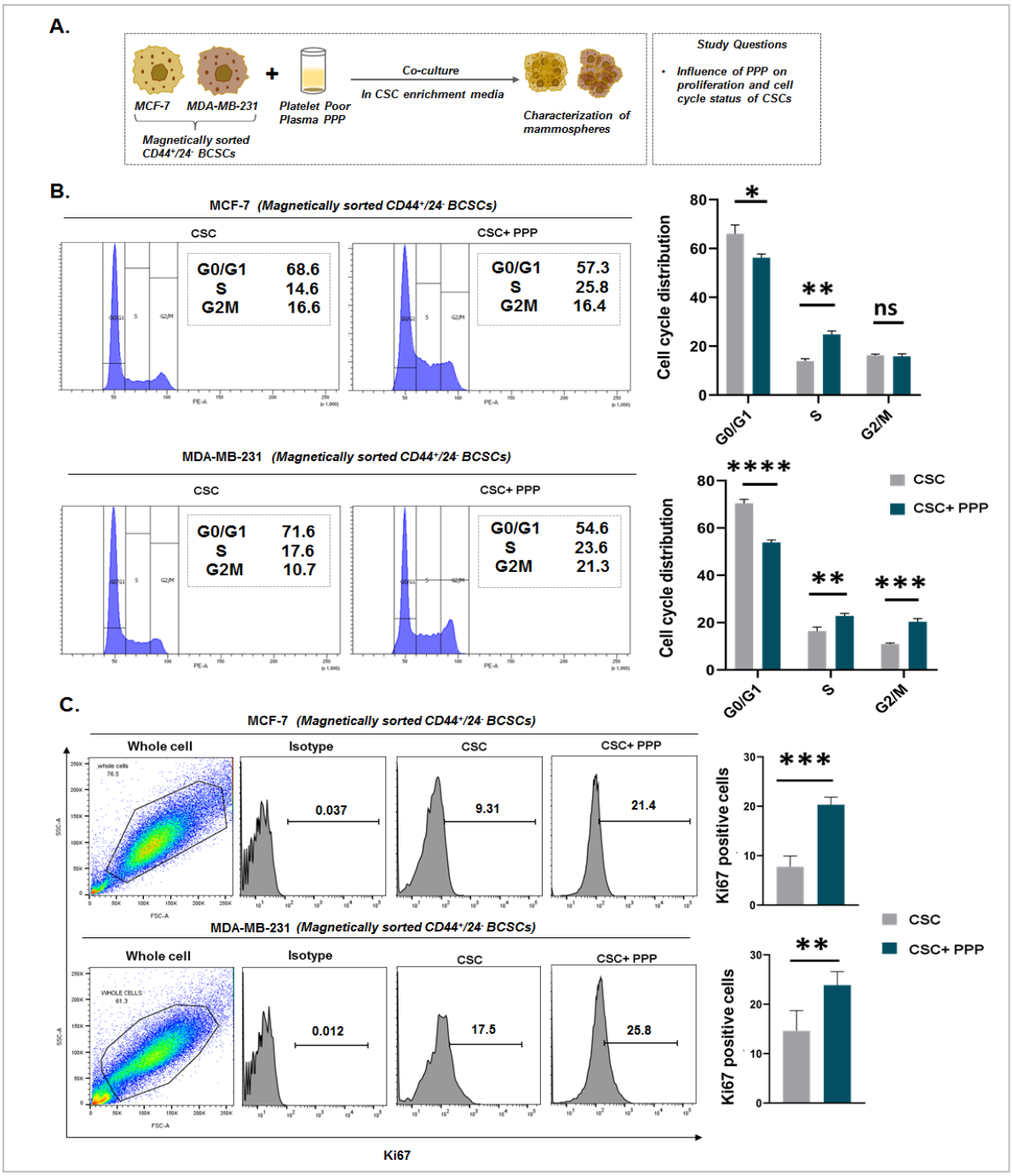


Figure 6.2: Platelet poor plasma promotes proliferation by redrawing the BCSCs from G0/G1 quiscence phase of the cell cycle to synthesis phase-A. Schematic representation of the study design. **B.**Representative histogram plots of BCSCs in G0/G1, S and G2/M phasesof the cell cycle in MCF-7 and MDA-MB-231 in presence or absence of PPP. Bar graphs(mean± SD) showcasing

percent of BCSCs in different phases of the cell cycle is represented with statistical significance inferred from two-way ANOVAfollowed by Tukey's multiple-comparison-test (n=3). **C.**Flow cytometry histogram plots depicting expression of Ki67 in BCSCs and PPP treated BCSCs of MCF-7 and MDA-MB-231 is illustrated.In bar-graph (mean±SD) with statistical significance drawn from unpaired non-parametric t-test, followed by two-tailed p value (n=3) is represented. *Inset*: Grey and Green boxes denoting CSC and CSC-PPP respectively. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant are indicated.

Platelet poor plasma reduces expression of major stemness and MDR resistance regulating genes

To gain mechanistic insights into the observed phenotypic changes, mRNA expression levels of key stemness-associated and drug resistance genes were analyzed via semi-quantitative RT-PCR. Magnetically sorted ($CD44^{+}24^{-}$) BCSCs were cultured under non-adherent conditions in stem cell enrichment medium for 5 days, with or without the addition of 50 µL of PPP at the time of seeding. Untreated BCSCs served as controls. Following the culture period, total RNA was extracted, reverse transcribed and the expression levels of key genes associated with stemness and multidrug resistance were assessed (Fig 6.3A).

RT-PCR analysis revealed that PPP treatment significantly suppressed the expression of canonical stemness-associated transcription factors, including *nanog*, *oct4* and *sox2*. These genes are well-established regulators of self-renewal and pluripotency and are critical for the maintenance of the BCSC phenotype (Fig 6.3B).

In addition to downregulating stemness markers, PPP also significantly reduced the expression of *abcb1*and *abcc1*, two members of the ATP-binding cassette (ABC) transporter family that are frequently upregulated in BCSCs and contribute to the characteristic multidrug resistance observed in many cancers (Fig 6.3B). Taken together, these findings suggested that PPP exerts a dual effect on BCSCs: it impairs their ability to maintain stemness by downregulating key pluripotency factors and it diminishes their survival advantage by reducing the expression of drug resistance genes. This molecular reprogramming may contribute to the observed morphological and behavioral shift from a spheroid-forming, stem-like phenotype to a more adherent and proliferative, yet potentially more therapy-sensitive, cellular state. Additionally, analysis of the cell culture supernatant from the co-culture of BCSCs with PPP via ELISA revealed that treatment with PPP led to a significant reduction in the levels of TGF-β in the supernatant compared to untreated BCSCs suggesting that PPP may suppress the pro-

inflammatory and pro-tumorigenic microenvironment commonly associated with BCSCs (Fig 6.3C). Interestingly, no significant changes were observed in IL-6, IL-10 or VEGF (Fig 6.3C) levels, indicating that PPP's effects might be selectively targeted towards certain signaling pathways involved in BCSC migration, self-renewal and metastasis.

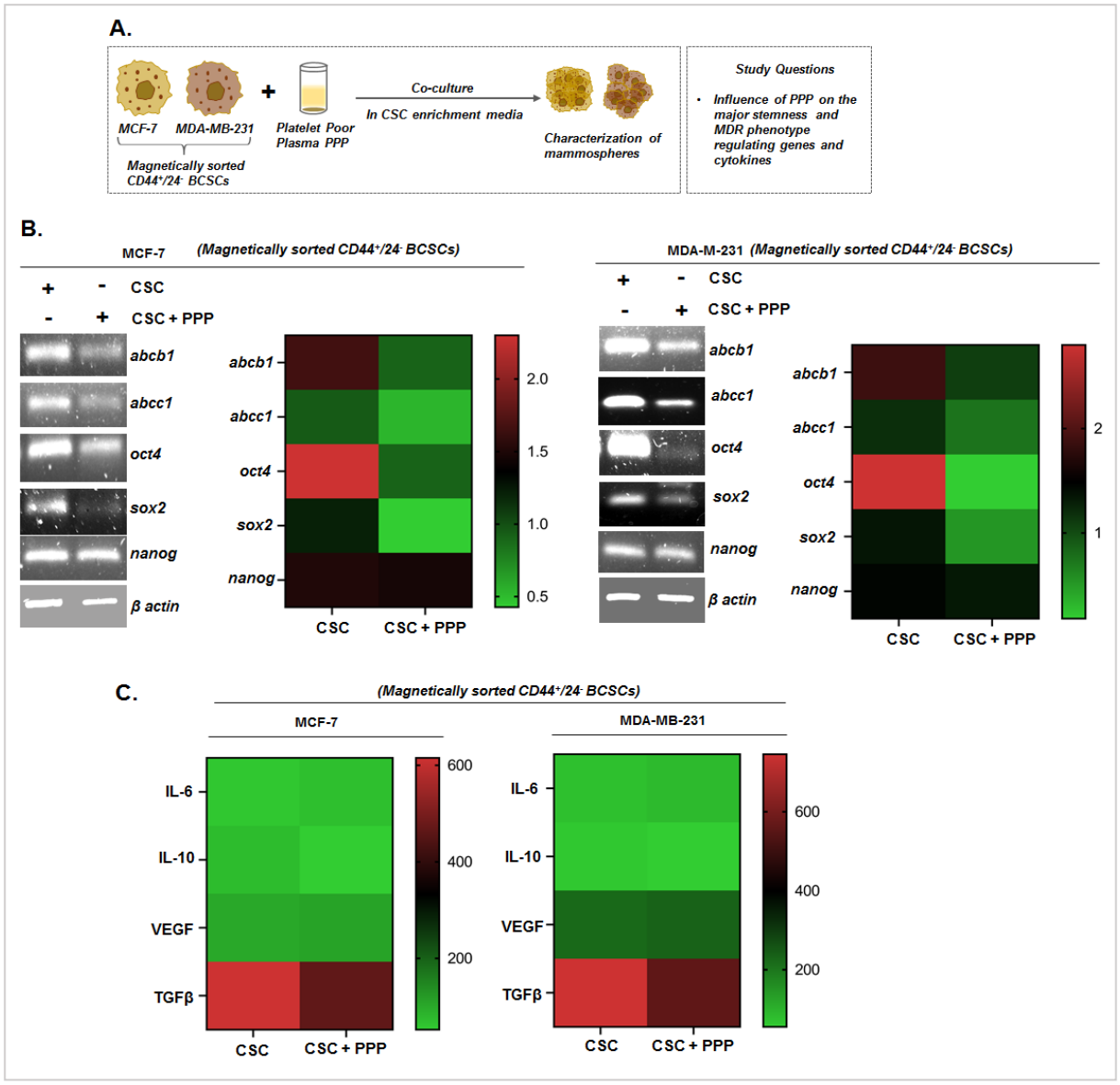


Figure 6.3: Platelet poor plasma reduces expression of major stemness and MDR resistance regulating genes-A. Schematic representation of the study design. **B.** RT-PCR analysis of the major drug resistance and stemness genes *abcb1*, *abcc1*, *oct4*, *sox2* and *nanog* is presented keeping *β-actin* as control in BCSC and BCSC+PPP cohorts of MCF-7 and MDA-MB-231. Representative heat maps denoting (mean±SD) relative gene expression in BCSC and BCSC+PPP groups of MCF-7 and MDA-MB-231 is depicted (n=3). Red and green colours denote high and low expression respectively. **C.** Assessment of possible alteration in cytokine level due to PPP administration via ELISA (Red colour

denotes overexpression while green denotes downregulation). Each box in heatmap denotes (mean± SD) for pg/ml cytokine secretion (n=3).

Discussion:

Our study reveals that platelet-poor plasma (PPP) exerts a profound inhibitory effect on BCSCs, impairing their stemness, migration and drug resistance capabilities. Importantly, PPP treatment drove a significant shift of BCSCs from a quiescent to a proliferative state, as evidenced by increased S-phase entry and Ki67 expression. These findings underscore the critical role of platelet-derived factors in maintaining BCSC stemness and therapeutic resistance and suggest that targeting the platelet–BCSC axis could serve as a novel strategy to enhance the efficacy of breast cancer treatments.

Morphologically, PPP-treated BCSCs exhibited striking alterations when cultured under stem cell enrichment conditions. Unlike untreated BCSCs, which typically form compact, spherical tumor spheroids in non-adherent plates, a hallmark of stemness; PPP-treated BCSCs failed to form spheroids and instead became adherent, even under non-adherent culture conditions. This dramatic morphological shift indicates a significant loss of self-renewal and anchorage-independent growth capacity, further reinforcing the conclusion that PPP disrupts the fundamental characteristics of BCSCs.

At the molecular level, PPP treatment resulted in notable downregulation of critical stemness-associated transcription factors: *oct4* and *sox2* and marginally in *nanog*. These factors are essential for sustaining the pluripotent, self-renewing and therapy-resistant phenotype of BCSCs and are strongly associated with poor prognosis in breast cancer. Their downregulation indicates a loss of stemness and suggests that PPP can drive BCSCs towards differentiation or functional exhaustion. This molecular reprogramming likely underlies the observed functional impairments following PPP exposure.

Functionally, PPP treatment markedly reduced the ability of BCSCs to form colonies on soft agar bed. The decline in colony numbers reflects a loss of tumor-initiating and proliferative capacities of BCSCs. Additionally, PPP significantly hindered BCSC migration, as demonstrated by reduced scratch closure in wound healing assays. Migratory ability is crucial for invasion and metastasis; thus, PPP's inhibition of wound healing suggests a suppression of BCSC-driven metastatic potential.

One of the most clinically significant observations in our study is the induction of BCSCs from dormancy to active proliferation following PPP treatment. BCSCs typically reside in a quiescent G_0/G_1 state, which protects them from chemotherapy that primarily targets rapidly dividing cells.

By redrawing BCSCs into the cell cycle, PPP treatment may sensitize these otherwise refractory populations to standard cytotoxic agents. This approach aligns with emerging therapeutic strategies aimed at "awakening" dormant tumor cells to enhance their vulnerability to chemotherapy (Sosa et al., 2014).

Therefore, combining PPP-based treatments with conventional chemotherapy could potentially overcome a major barrier to curative breast cancer therapy: the persistence of quiescent, drug-resistant BCSCs.

The observed downregulation of ABC transporters *abcb1*and *abcc1*following PPP exposure further supports the potential of this strategy. These efflux pumps are critical mediators of multidrug resistance in cancer and are particularly enriched in BCSC populations [6] [7]. Reducing their expression may not only sensitize BCSCs to chemotherapeutic agents but also limit the emergence of resistant clones during treatment. Importantly, the inhibitory effects of PPP were not limited to a particular BC subtype. We observed similar morphological, molecular and functional alterations both in luminal-A, representing a less aggressive subtype and in triple-negative breast cancer (TNBC) cells, which constitute the most aggressive and therapy-resistant form of BC. This consistency across subtypes highlights the broad applicability and potential universality of PPP's anti-BCSC effects. Further, a marked decrease in the secretion of TGF-β was noted in the cell culture supernatant of BCSCs co-cultured with PPP.TGF-β are known to play pivotal roles in regulating BCSC self-renewal, migration and resistance to chemotherapy. TGF-β promotes epithelial-to-mesenchymal transition (EMT) and metastasis [8] [9]. TGF-β signaling is also closely linked to the maintenance of stemness markers such as *nanog*, *oct4* and *sox2*, which regulates self-renewal and pluripotency [10] [11] [12]. PPP-mediated reduction in TGF-β secretion likely contributes to a decrease in the expression of these stemness factors, as evidenced by the down-regulation of *nanog, oct4* and *sox2* in treated BCSCs. This reduction in stemness markers corresponds with the impaired ability of BCSCs to form spheroids and colonies, further supporting the role of TGF-β in regulating the self-

renewal and tumorigenic potential of BCSCs. Collectively, these findings position PPP as a promising adjuvant strategy in breast cancer treatment. By simultaneously weakening stemness, reducing drug resistance, impairing migration, altering cell morphology and inducing proliferation of dormant BCSCs, PPP targets multiple pillars of BCSC-driven tumor maintenance and progression. Future investigations aimed at delineating the specific components of PPP responsible for these effects as well as validating its therapeutic potential in *in-vivo* breast cancer models needs to be performed. Moreover, combining PPP treatment with conventional chemotherapy may offer a synergistic approach to eradicate both bulk tumor cells and therapy-resistant BCSC populations, ultimately improving patient outcomes. In conclusion, our study identifies PPP as a novel and clinically actionable agent capable of disrupting the supportive platelet-derived niche for breast BCSCs, impairing their functional capacityand enhancing the therapeutic vulnerability of breast tumors.

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General Discussion

Despite advances in the field of cancer research, metastasis continues to be the leading cause of cancer related morbidity and mortality worldwide. Although, cancer survival rate has improved dramatically over time, this gain is mostly attributable to early detection and cancer growth suppression. Chemotherapy and radiation therapy are currently the main treatments for cancer metastasis. However, new anti-cancer medications, such as small molecule kinase inhibitors and monoclonal antibodies that neutralize growth factors, also affect cancer metastasis.

Metastasis is a complex process that includes several linked and sequential steps as well as numerous biochemical reactions that need further elucidation. However, the process itself bestows the tumor cells with numerous challenges including survival in the blood stream, escape of immune surveillance process and successful establishment of secondary tumor. Tumor cells bypass these hurdles by priming the different cellular components in their favour. One such companion of the tumor cells are the circulating platelets in the blood vessels. Pro-metastatic role of platelets has been reported in several malignancies including ovarian, myeloma, colon and Lewis lung carcinoma (1). In this current study, we describe how platelets contribute to breast cancer (BC) progression within two subtypes: luminal-A (the less aggressive type) and TNBC (the most aggressive type). Thrombocytosis was prevalent in both of these variants. We uncovered that a higher proportion of P-selectin^{+} platelets were present in the peripheral blood of BC hosts. Analysis of their medical records revealed that these patients did not suffer from any other health conditions at the time of diagnosis that could boost the production of platelets. This substantiated the existence of thrombocytosis and eliminated the probability of thrombocythemia.

Platelet's α-granules are a repository of P-selectin. They degranulate after activation, revealing P-selectin on their surface. This suggested that platelets in these two BC subgroups were activated. Furthermore, TNBC had an elevated percentage of activated TEPs than luminal-A, which indicated towards the potential involvement of TEPs in disease aggressiveness. Moreover, platelets of cancer patients formed huge aggregates and had an elaborate system of filopodia and lamellipodia, reinforcing their

activated state. Our results are consistent with preliminary reports on murine cancer models, where it has been demonstrated that excessively metastatic tumors promote platelet activation (2). Previous research reports have revealed that circulatory tumor cells interact with platelets to generate tumor educated platelets or TEPs (3-4).We further demonstrate that TEPs can infiltrate into the tumor and are an indispensable component of the breast TME. Moreover, TNBC patients exhibited higher TEP infiltration than luminal-A. This further strengthened the possible contribution of TEPs in promoting cancer aggression. We next sought to ascertain TEPs functionality within TME. We discovered that TEPs and BCSCs remained in close proximity inside TME, suggesting the likelihood of their interaction. Additionally, TEPs in TNBC demonstrated a stronger affinity towards BCSCs than luminal-A. This propinquity of TEPs towards BCSCs, especially in TNBC, confirmed that TEPs tend to form an alliance with BCSCs that dictates the overall disease outcome.

Further, analysis of the surface receptors revealed that BCSCs express PSGL1 which binds with TEPs P-selectin and this kinship facilitates tumor progression. PSGL1 is reported to have significant role in metastasis (5-6). In prostate and small cell lung cancer, malignant cells with high PSGL1 expression are reported to be capable of distant metastasis (5-6). Upon in-depth investigation of this TEP and BCSC interplay *in-vitro*, it was found that TEPs elevated BCSC population and enhanced their tumorigenenic and clonogenic potentialities. Analysis of transcription factors that regulate various attributes of CSCs disclosed that under the influence of TEPs there was significant upregulation of NANOG, OCT4 and SOX2. Also, these TEP-BCSCs over-expressed genes related to MDR phenotype such as *abcc1, abcb1 and bcrp1*. The role of TEPs in drug resistance has already been established in various tumors (7-8). Our investigation thus strengthened TEPs role in therapy resistance and disease relapse which are characteristic hallmarks of BCSCs.

The precise role of TEPs on metastasis of BC was delineated next. It was found that TEP influenced BCSCs remained in a typical mesenchymal state. The link between EMT and stemness has been demonstrated in a number of earlier studies. EMT has been connected to the development and maintenance of BCSCs, conferring them with stemness and flexibility (9-10). TEP-BCSCs also attained an elongated architecture with numerous invadopodia on their surface.

This invasive morphology strongly supported their mesenchymal nature. Matrigel invasion assay, migration assay and wound healing assay further stiffened the metastatic nature of TEP influenced BCSCs. Over-expression of VIMENTIN, TWIST, SNAIL and down-regulation of E-CADHERIN complemented our observations.

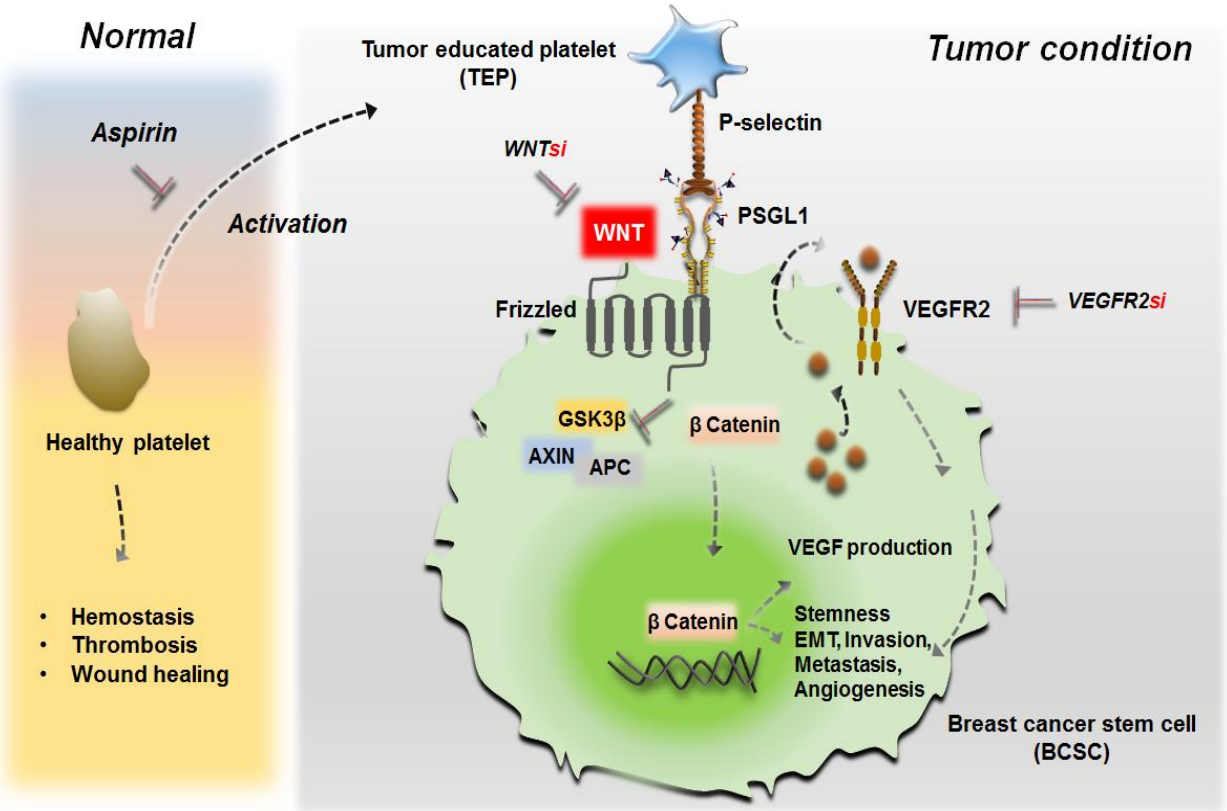
Elevation of NANOG and OCT4 might play multi-faceted role by maintaining stemness and at the same time promote invasion by down-regulating E-CADHERIN (11). Furthermore, by up-regulating the expression of VIMENTIN and suppressing E-CADHERIN, increased TWIST expression may promote EMT (17). Additionally, through its interaction with NANOG, it may enhance stemness and resistance to drugs by augmenting the expression of *bcrp 1*(13). With the inception of TEPs function in facilitating metastasis, the secondary site of migratory BCSCs was delineated. Elevated *cxcr4* has been linked to BC metastasis to liver, brain, lungs and lymph nodes according to literature reports (14-16). Furthermore, because of its great capacity for ECM breakdown, *mmp9* plays a critical role in metastasis and its high expression has been correlated with poor prognosis in BC, according to clinical studies (14-16). Experimental metastasis in both female Swiss and BALB/c murine models revealed that these BCSCs infiltrate through the blood vessels to initiate distant pulmonary metastasis. Our results also were supported by a similar observation where it was reported that in patients with osteosarcoma, high CXCR4 and MMP9 expression promoted lung metastasis (14-16).

With the established role of TEPs in promoting stemness and metastasis of BCSCs via physical contact, we next explored the downstream mediators of P-selectin-PSGL1 receptor ligand interactions. Interestingly, we observed that the WNT-β-Catenin and VEGF-VEGFR2 axis were elevated among the many signaling cascade components involved in controlling stem cell fate. Previous reports have mentioned about the collaborative role of WNT and NOTCH to control stemness and that their patterns of expression are analogous (18). Here, we are reporting a pioneering non-conventional signaling in which WNT-β-Catenin forms alliance with VEGF-VEGFR2 instead of NOTCH in TEP-BCSC interaction. The role of VEGF-VEGFR2 cascade in promoting stemness and metastasis of BCSCs has already been established (18-19). Our observation thus correlates with previous reports of crosstalk between WNT and VEGF in

breast cancer (19). Also, the direct role of β-Catenin in inducing VEGF production is very well reported in malignancies like colorectal cancer (20-23).

Finally, the therapeutic role of aspirin was explored. Novel anti-platelet agent aspirin is well established to block platelet activity and impede its tumorigenic effect (24-27). This is in line with our results, where aspirin reduced the activation of TEPs in BC subtypes. Aspirin treatment declined P-selectin expression on TEPs reverting them almost to resting platelet (RP) state. This blocked its interaction with PSGL1, thereby hindering the complete downstream signalling cascade, ultimately reducing stemness, tumorigenicity, MDR phenotype and metastasis of BCSCs. The most interesting observation was noted in RP-BCSC cohort, with the dual knockdown of WNT and VEGFR2 which in-turn exhibited the greatest reduction in tumorsphere count and metastasis. A substantial explanation could be that blocking P-selectin hampered the receptor-ligand interaction and further dual knockdown of WNT-VEGFR2 provided additional stress. This proves the direct link between P-selectin and WNT-VEGFR2 cascade. Thus, this study discloses the importance of tiny anucleated platelets in malignant disease progression and aggressiveness. Their relevance as therapeutic targets are supported by their capacity to interact with the vicious BCSCs to produce extremely aggressive and metastatic sub-variants via EMT. Besides, their significance in fostering disease aggression is aided by the fact that they interact with TNBC to a larger extent than luminal-A.

Anti-platelet medications may thus offer an innovative approach in BC treatment. Neutralization of the WNT-VEGFR2 axis may potentially be investigated in conjunction with it. Further, impact of TEPs on the other subtypes of BC may also be elucidated.



In contrast, our second major observation reveals that platelet poor plasma (PPP) impairs BCSC-like properties across both luminal-A and triple-negative breast cancer (TNBC) subtypes. BCSCs exposed to PPP lost their ability to form spheroids even in non-adherent conditions and instead displayed adherent phenotypes similar to their non-stem tumor cell counterparts like MCF-7 and MDA-MB-231. This phenotypic shift was accompanied by a marked down-regulation of stemness genes, particularly *oct4* and *sox2* and a marginal decrease in *nanog*.

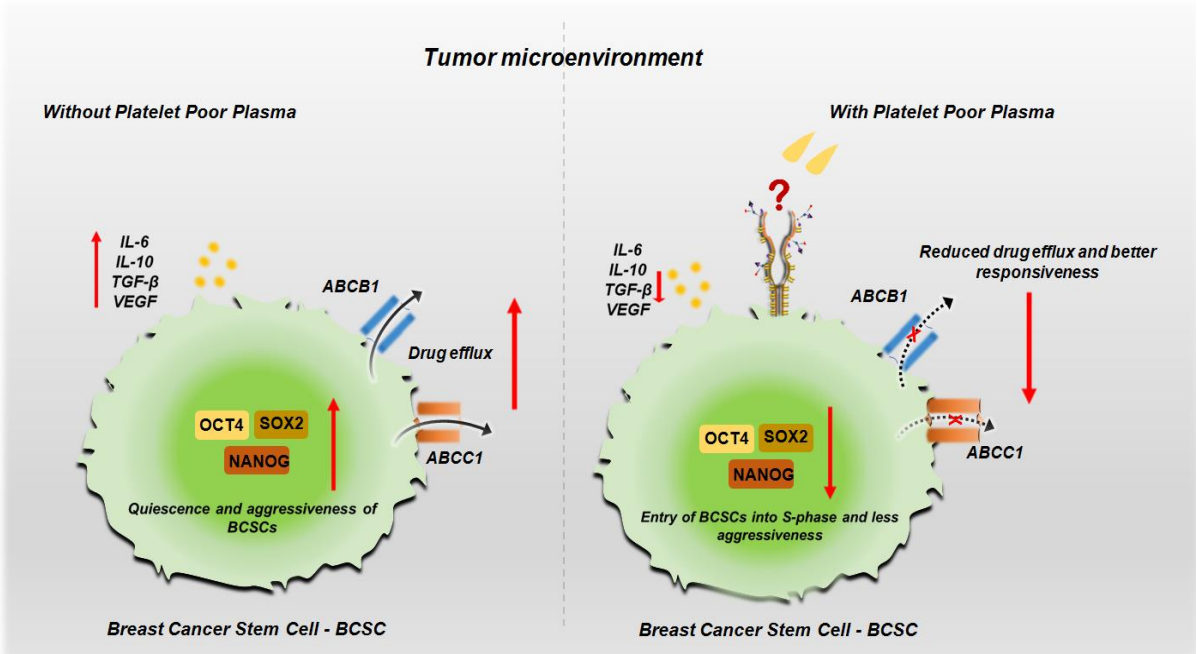
Moreover, PPP-treated BCSCs showed reduced migratory potential and diminished soft agar colony formation, indicating a profound loss of their tumor-initiating capacity.

Perhaps the most intriguing and therapeutically relevant observation is the cell cycle re-entry of BCSCs following PPP exposure. Normally residing in a quiescent G0/G1 state that renders them resistant to conventional therapies, BCSCs treated with PPP were drawn into the S-phase, marked by up-regulation of Ki-67, a proliferation marker. Concomitantly, there was a significant down-regulation of ABC transporter genes *abcb1* and *abcc1*, which are commonly associated with chemo-resistance. This suggests that PPP not only

reduces stemness but also sensitizes BCSCs to cytotoxic therapies by modulating their cell cycle dynamics and efflux capacity. From a clinical standpoint, both of our findings are highly significant. TEPs may serve as biomarkers for monitoring tumor progression. They may also be considered as therapeutic targets to suppress CSC-mediated tumor aggressiveness. PPP could be explored as a differentiation-inducing or CSC-depleting therapy. The ability of PPP to push BCSCs into the cell cycle opens a therapeutic window for sequential therapy, where PPP treatment could be followed by chemotherapy to target previously quiescent, drug-resistant BCSCs.

Unlike cytotoxic agents that aim to kill all dividing cells indiscriminately, PPP seems to reprogram the BCSC phenotype, reduce malignancy and sensitize cells to existing therapies all of which are critical attributes for durable and targeted cancer treatment. Combining PPP based therapy with standard chemotherapy or immunotherapy may offer synergistic benefits, especially in aggressive and treatment-refractory breast cancer subtypes such as TNBC.

In conclusion, our work reveals a previously underappreciated therapeutic strategy centred on modulating PPP to reprogram BCSCs and enhance treatment response. Future studies aimed at delineating the molecular pathways involved and evaluating the *in-vivo* efficacy of PPP-based interventions, either as mono-therapy or in combination with existing chemo-therapeutic regimens shall prove beneficial to the breast cancer patients.



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List of Publications

Publications

As primary author in International Peer Reviewed journals

- **Guha A**, Goswami KK, Sultana J, Ganguly N, Choudhury PR, Chakravarti M, Bhuniya A, Sarkar A, Bera S, Dhar S, Das J, Das T, Baral R, Bose A, Banerjee S. Cancer stem cell–immune cell crosstalk in breast tumor microenvironment: a determinant of therapeutic facet. **Frontiers in Immunology** (Cancer Immunity and Immunotherapy). 2023 Nov 27; 14:1245421. ISSN: 1664-3224. doi: 10.3389/fimmu.2023.1245421. Collection (Reciprocal Crosstalk Between the Tumor Microenvironment and Cancer Stem Cells). **IF: 7.3**; **Citation Index: 14**
- **Guha A,** Sultana J**,** Bhuniya A, Chakravarti M, Bera S, Sarkar A, Dhar S, Choudhury PR, Das J, Ganguly N, Guha I, Das T, Alam N, Ghosh I, Hajra S, Baral R, Bose A, Banerjee S. Tumor-Educated-Platelets interact with Breast Cancer-Stem-Cells via P-selectin- PSGL1 and ensure stemness and metastasis through WNT-β-Catenin-VEGF-VEGFR2 intra-cellular signaling: Therapeutic modulation by aspirin.

Under Review- Breast Cancer Research, BMC, Springer Nature; Impact Factor - 6.1

Preprint

- **Guha A,** Sultana J, Bhuniya A, Chakravarti M, Bera S, Sarkar A, Dhar S, Roy Choudhury P, Das J, Ganguly N, Guha I, Das T, Alam N, Ghosh I, Hajra S, Baral R, Bose A, Banerjee S. Tumor-Educated-Platelets interact with Breast Cancer-Stem-Cells via P-selectin-PSGL1 and ensure stemness and metastasis through WNT-β-Catenin-VEGF-VEGFR2 intra-cellular signaling: Therapeutic modulation by aspirin. **BioRxiv. 2025-06.**

As co-author in International Peer Reviewed journals

- Bhuniya A, Sarkar A, **Guha A,** Choudhury PR, Bera S, Sultana J, Chakravarti M, Dhar S, Das J, Guha I, Ganguly N, Banerjee S, Bose A, Baral R. Tumor activated platelets induce vascular mimicry in mesenchymal stem cells and aid metastasis. **Cytokine**. 2022 Oct; 158:155998. DOI: 10.1016/j.cyto.2022.155998. Epub 2022 Aug 15. **IF: 3.488**; **Citation Index: 10**
- Neem Leaf Glycoprotein Disrupts Exhausted CD8+ T-Cell–Mediated Cancer Stem Cell Aggression. Chakravarti M, Bera S, Dhar S, Sarkar A, Choudhury PR, Ganguly N, Das J, Sultana J, **Guha A,** Biswas S, Das T, Hajra S, Banerjee S, Baral R, Bose A. **Molecular**

Cancer Research. 2024 Aug 2; 22(8): 759–778. /doi.org/10.1158/1541-7786.MCR-23-0993. **IF: 5.2**; **Citation Index: 1**

❖ Sultana J, Choudhury PR, **Guha A,** Das P, Bera S, Das J, Banerjee S. Evolving Trends in Breast Cancer Immunotherapy: Recent Updates. **Journal of Current Oncological Trends**. 2025 July. doi: 10.4103/JCOT.JCOT_19_24

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❖ **Guha A,** Sultana J, Bhuniya A, Chakravarti M, Bera S, Sarkar A, Dhar S, Das J, Ganguly N, Das T, Alam N, Ghosh I, Hajra S, Baral R, Bose A, Banerjee S. Evaluating interaction between tumor educated platelets and cancer stem cells on breast cancer subtypes. **Journal of Clinical Oncology**. 2024 Aug 12;42: 23 suppl, 5-5doi.org/10.1200/JCO.2024.42.23suppl.5**. IF: 45.2**; **Citation Index: Yet to cite**

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❖ **Guha A,** Sultana J, Bhuniya A, Chakravarti M, Bera S, Sarkar A, Dhar S, Das J, Ganguly N, Das T, Alam N, Baral R, Bose A, Banerjee S. Elucidating the role of tumor educated platelets in tumorigenesis promoting EMT and stem cell properties in breast cancer ‘‘Combating Cancer: Biology to Therapy to Drug Resistance” & An International Symposium on: Cancer & Stem Cells IACR 2022 2nd-5th March. **ISBN: 978-81-954625-2-0**

❖ **Guha A,** Sultana J, Bhuniya A, Chakravarti M, Bera S, Sarkar A, Dhar S, Das J, Ganguly N, Das T, Alam N, Baral R, Bose A, Banerjee S. Tumor Educated Platelets in crosstalk with Breast Cancer Stem Cells promotes metastasis and angiogenesis in breast

cancer subtypes. Proceedings of National Seminar on Advances in Biology Breakthroughs in Microbiology and Cancer Research 2024 19th November. **ISBN: 978-93-341-4224-2**

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❖ Sultana J, **Guha A**, Chakravarti M, Bera S, Choudhury PR, Dhar S, Sarkar A, Das J, Ganguly N, Alam N Baral R, Bose, Banerjee S. The metabolic regulator, 2-deoxy glucose (2DG), modulates cancer stem cells differentially in luminal A and TNBC breast tumor subtypes. ‘‘Combating Cancer: Biology to Therapy to Drug Resistance” & An International Symposium on: Cancer & Stem Cells IACR 2022 2nd-5th March. **ISBN: 978-81-954625-2-0**

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❖ Choudhury PR, Chakravarti M, Bera S, Sultana J, **Guha A**, Dhar S, Sarkar A, Das J, Ganguly N, Baral R, Bose, Banerjee S. Statin treatment in hypercholesterolemic mice enhances tumor growth by affecting dendritic cell associated antigen presentation pathway41st Annual International Conference of Indian Association for Cancer Research “Combating Cancer: Biology to Therapy to Drug Resistance” & An International Symposium on: Cancer & Stem Cells IACR 2022 2nd-5th March. **ISBN: 978-81-954625-2-0**

❖ Choudhury PR, Chakravarti M, Bera S, Sultana J, **Guha A**, Dhar S, Sarkar A, Das J, Ganguly N, Baral R, Bose, Banerjee S. Prolonged statin treatment alleviates hypercholesterolemia mediated dendritic cell dysfunction in breast cancerProceedings of National Seminar on Advances in Biology Breakthroughs in Microbiology and Cancer Research 2024 19th November. **ISBN:978-93-341-4224-2**

List of Seminars and Conferences

Presentations in Seminars and Conferences

List of Presentation in National/International Conferences

- Presentation as 1st author in National Conferences – **6**
- Presentation as 1st author in International Conferences – **4**
- Presentation as Co-author in National Conferences – **17**
- Presentation as Co-author in International Conferences – **1**

Presentation as 1st author-National Conferences

1. 41st Annual Conference of the Indian Association for Cancer Research, Amity University Noida (IACR); 2nd-5th March, 2022

***Oral presentation*:** Understanding the role of tumor educated platelets in promoting EMT and metastasis in breast cancer - **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Pritha Roy Choudhury, Sukanya Dhar, Anirban Sarkar, Juhina Das, Nilanjan Ganguly, Tapasi Das, Neyaz Alam, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

2. National Science Day 2022, CNCI - 'Integrated approach in S&T for sustainable future'; 28th February, 2022

***Poster presentation*:** Elucidating the interaction between platelets and breast cancer stem cells in promoting EMT and metastasis - **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Pritha Roy Choudhury, Sukanya Dhar, Anirban Sarkar, Juhina Das, Nilanjan Ganguly, Tapasi Das, Neyaz Alam, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

3. Society of Biological Chemists- SBC Kolkata Chapter, Sister Nivedita University; 9th-10th April, 2022

***Oral presentation*:** Understanding the role of tumor educated platelets in promoting aggressiveness of breast cancer - **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Pritha Roy Choudhury, Sukanya Dhar, Anirban Sarkar, Juhina Das, Nilanjan Ganguly, Tapasi Das, Neyaz Alam, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

4. Netaji Subhas Chandra Bose International Onco Summit-NIOS; 28th-29th January, 2023

***Poster presentation*:** Tumor educated platelets promote metastasis in breast cancer by enhancing the aggressiveness of cancer stem cells - **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Pritha Roy Choudhury, Sukanya Dhar, Anirban Sarkar, Juhina Das, Nilanjan Ganguly, Tapasi Das, Kuntal Kanti Goswami, Neyaz Alam, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

5. Proceedings of National Seminar on ADVANCES IN BIOLOGY - Breakthroughs in Microbiology and Cancer Research, JIS University, Kolkata; 19th November, 2024

***Poster presentation*:** Tumor Educated Platelets in Crosstalk with Breast cancer stem cells promote metastasis and angiogenesis in breast cancer subtypes - **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Pritha Roy Choudhury, Sukanya Dhar, Anirban Sarkar, Juhina Das, Nilanjan Ganguly, Tapasi Das, Kuntal Kanti Goswami, Neyaz Alam, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

6. 'Anusandhan – 2025'- Bose Institute, Kolkata; 7th-8th March, 2025

***Oral presentation*:** P-Selectin on Tumor Educated Platelets interact with PSGL1 on Cancer Stem Cells promoting EMT and Metastasis in Breast cancer: Intervention by Aspirin - **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Pritha Roy Choudhury, Sukanya Dhar, Anirban Sarkar, Juhina Das, NilanjanGanguly, Tapasi Das, Neyaz Alam, Rathindranath Baral, Anamika Bose, Saptak Banerjee.

Presentation as 1st author -International Conferences

1. 8th International Conference on Stem cells and Cancer and Cancer Proliferation, Differentiation and Apoptosis, ICSCC-2023 and Research and Innovation Pediatric Surgical Society Conference, RIPSSCON-IAPS-2023, AIIMS, New Delhi; 20th-22nd December, 2023

***Oral presentation*:** Tumor educated platelet promote metastasis in breast cancer by enhancing the aggressiveness of breast cancer stem cells - **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Pritha Roy Choudhury, Sukanya Dhar, Anirban Sarkar, Juhina Das, Nilanjan Ganguly, Tapasi Das, Kuntal Kanti Goswami, Neyaz Alam, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

2. ESMO Breast Cancer-Annual Congress, Berlin Germany; 15th -17th May, 2024

***Poster presentation*:** Tumor educated platelets promote disease advancements in breast cancer by interacting with breast cancer stem cells - **Aishwarya Guha,** Jasmine Sultana, Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Anirban Sarkar, Pritha Roy Choudhury, Nilanjan Ganguly, Juhina Das, Tapasi Das, Neyaz Alam, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

3. American Society of Clinical Oncology-ASCO Breakthrough 2024, Yokohama, Japan; 8th-10th August, 2024

***Poster presentation*:** Evaluating interaction between tumor educated platelets and cancer stem cells on breast cancer subtypes- **Aishwarya Guha,** Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Anirban Sarkar, Sukanya Dhar, Pritha Roy Choudhury, Juhina Das, Nilanjan Ganguly, Tapasi Das, Neyaz Alam, Indranil Ghosh, Srabanti Hajra, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

4. International symposium on "GENETIC DIVERSITY& DISEASE BIOLOGY 2025" JIS Institute of Advanced Studies and Research (JISIASR), JIS University; 23rd May, 2025

***Poster presentation*:** Platelet Poor Plasma-A Novel Autologous Approach to Target Breast CSCs. **Aishwarya Guha,** Jasmine Sultana, Pritha Roy Choudhury, Saurav Bera, Prodipto Das, Soumyabrata Roy, Anamika Bose, Rathindranath Baral, Saptak Banerjee.

Presentation as co-author National Conferences

1. 41st Annual Conference of the Indian Association for Cancer Research, Amity University Noida (IACR); 2nd-5th March, 2022

***Poster presentation*:** Understanding the metabolic regulation of Breast cancer stem cells and its impact on immune landscape: Modulation by 2DG - Jasmine Sultana, **Aishwarya Guha,** Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Avishek Bhuniya, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

2. 41st Annual Conference of the Indian Association for Cancer Research, Amity University Noida (IACR); 2nd-5th March, 2022

***Poster presentation*:** Statin treatment in hypercholesterolemic mice promotes tumor progression by affecting dendritic cell associated antigen presentation pathway – Pritha Roy Choudhury, Mohona Chakravarti, Saurav Bera, Jasmine Sultana, **Aishwarya Guha**, Anirban Sarkar, Sukanya Dhar, Rathindranath Baral, Anamika Bose, Saptak Banerjee.

3. National Science Day 2022, CNCI - 'Integrated approach in S&T for sustainable future'; 28th February, 2022

***Poster presentation*:** Understanding the metabolic regulation of Breast cancer stem cells and its impact on immune cells: Modulation by 2DG - Jasmine Sultana, **Aishwarya Guha,** Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Avishek Bhuniya, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

4. National Science Day 2022, CNCI - 'Integrated approach in S&T for sustainable future'; 28th February, 2022

***Poster presentation*:** Prolonged statin treatment in hypercholesterolemic mice enhances tumor growth by affecting dendritic cell associated antigen presentation pathway – Pritha Roy Choudhury, Mohona Chakravarti, Saurav Bera, Jasmine Sultana, **Aishwarya Guha**, Anirban Sarkar, Sukanya Dhar, Rathindranath Baral, Anamika Bose, Saptak Banerjee.

5. 11th General assembly of APOCP, Kolkata, 8th-10th, December 2022

***Oral presentation*:** Prolonged statin treatment in hypercholesterolemic mice promotes tumor progression by affecting dendritic cell mediated antigen presentation pathway – Pritha Roy Choudhury, Mohona Chakravarti, Saurav Bera, Jasmine Sultana, **Aishwarya Guha**, Anirban Sarkar, Sukanya Dhar, Rathindranath Baral, Anamika Bose, Saptak Banerjee.

6. International Conference Organized by Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Tata Memorial Centre (IACR); 12th-16th January, 2023

***Poster presentation*:** Role of breast cancer stem-cells in modulating CD8 + Tcells within molecular sub-types of breast cancer: Intervention by 2DG and NLGP therapy – Jasmine Sultana, **Aishwarya Guha,** Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Avishek Bhuniya, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

7. International Conference Organized by Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Tata Memorial Centre (IACR); 12th-16th January, 2023

***Poster presentation*:** Elucidating the impact of tumor educated platelets in promoting EMT, metastasis and angiogenesis by interacting with breast cancer stem cells and mesenchymal stem cells – Saptak Banerjee, **Aishwarya Guha**, Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, Rathindranath Baral, Neyaz Alam, Anamika Bose.

8. Netaji Subhas Chandra Bose International Onco Summit-NIOS; 28th-29th January, 2023

***Poster presentation*:** Neem leaf glycoprotein is effective in rectifying exhausted T cell induced breast cancer stem cell aggressiveness – Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Anirban Sarkar, Pritha Roy Choudhury, Nilanjan Ganguly, Juhina Das, Jasmine Sultana, **Aishwarya Guha**, Tapasi Das, Saptak Banerjee, Rathindranath Baral, Anamika Bose.

9. Netaji Subhas Chandra Bose International Onco Summit-NIOS; 28th-29th January, 2023

***Poster presentation*:** Role of Breast Cancer Stem-Cells in Modulating CD8+ T Cells within Molecular Subtypes of Breast Cancer: Intervention by 2DG and NLGP – Jasmine Sultana, **Aishwarya Guha,** Mohona Chakravarti, Saurav Bera, Sukany Dhar, Avishek Bhuniya, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

10. Netaji Subhas Chandra Bose International Onco Summit-NIOS; 28th-29th January, 2023

***Poster presentation*:** NLGP: An emerging therapeutic opportunity to target Breast-CSC- Mohona Chakravarti, Sukanya Dhar, Saurav Bera, Pritha Roy Choudhury, **Aishwarya Guha,** Jasmine Sultana, Anirban Sarkar, Nilanjan Ganguly, Tapasi Das, Akata Saha, Shayani Dasgupta, Saptak Banerjee, Rathindranath Baral, Anamika Bose.

11. Netaji Subhas Chandra Bose International Onco Summit- NIOS; 6th-7th January, 2024

***Poster presentation*:** Dynamics of BCSC Aggressiveness and Immune Status across Subtypes in Breast Cancer.Jasmine Sultana, **Aishwarya Guha,** Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, Nilanjan Ganguly, Kuntal

Goswami, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

12. 13th East Zonal Oncology Symposium, Saroj Gupta Cancer Institute; 10th February, 2024

***Poster presentation*:** Prolonged statin treatment in hypercholesterolemic mice restricts tumor progression through dendritic cell mediated antigen presentation pathway – Pritha Roy Choudhury, Mohona Chakravarti, Saurav Bera, Jasmine Sultana, **Aishwarya Guha**, Anirban Sarkar, Sukanya Dhar, Rathindranath Baral, Anamika Bose, Saptak Banerjee.

13. 13th East Zonal Oncology Symposium, Saroj Gupta Cancer Institute; 10th February, 2024

***Poster presentation*:** Interaction between breast cancer stem cells and immune cells in breast cancer subtypes. Jasmine Sultana, **Aishwarya Guha,** Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, Nilanjan Ganguly, Kuntal Goswami, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

14. Indian Immunological Society, IMMUNOCON-2024, Annual Meeting, Indian Institute of Science, Bengaluru; 17th-20th, October 2024

***Poster presentation*:** Elucidating intricate interaction between TAM-subtypes and CD8 T-cells within different molecular subtypes of Breast Cancer. Saptak Banerjee, Prodipto Das, Jasmine Sultana, Saurav Bera, Pritha Roy Choudhury, **Aishwarya Guha,** Mohona Chakravarti, NilanjanGanguly, Juhina Das, Sukanya Dhar, Anirban Sarkar, Rathindranath Baral, Anamika Bose.

15. Indian Immunological Society, IMMUNOCON-2024, Annual Meeting, Indian Institute of Science, Bengaluru; 17th-20th, October 2024

***Poster presentation*:** Prolonged statin treatment in hypercholesterolemic mice remedies 27-hydrocholestrol mediated dendritic cell dysfunctions in Breast Cancer Pritha Roy Choudhury, Mohona Chakravarti, Saurav Bera, Jasmine Sultana, **Aishwarya Guha**, Anirban Sarkar, Sukanya Dhar, Rathindranath Baral, Anamika Bose, Saptak Banerjee.

16. Proceedings of National Seminar on ADVANCES IN BIOLOGY - Breakthroughs in Microbiology and Cancer Research, JIS University, Kolkata; 19th November, 2024

***Poster presentation*:** Breast Cancer Stem Cells and CD8 T cells interplay within tumor microenvironment: 2DG's role in therapeutic rescue. Jasmine Sultana,

Aishwarya Guha, Mohona Chakravarti, Saurav Bera, Sukanya Dhar, Anirban Sarkar, Juhina Das, Prith aRoy Choudhury, NilanjanGanguly, Kuntal Goswami, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

17. Proceedings of National Seminar on ADVANCES IN BIOLOGY - Breakthroughs in Microbiology and Cancer Research, JIS University, Kolkata; 19th November, 2024

***Poster presentation*:** Prolonged statin treatment alleviates hypercholesterolemia mediated dendritic cell dysfunction in breast cancer. Pritha Roy Choudhury, Mohona Chakravarti, Saurav Bera, Jasmine Sultana, **Aishwarya Guha**, Anirban Sarkar, Sukanya Dhar, Rathindranath Baral, Anamika Bose, Saptak Banerjee.

Presentation as co-author-International Conferences

1. Society for Immunotherapy of Cancer, SITC, Houston, USA, 2024

***Poster presentation*:** Intervention of intra-tumoral breast cancer stem cells and $CD8^+$ T cell dysregulation: The impact of 2-Deoxy-D-Glucose. Jasmine Sultana, **Aishwarya Guha,** MohonaChakravarti, Saurav Bera, Sukanya Dhar, Anirban Sarkar, Juhina Das, Pritha Roy Choudhury, NilanjanGanguly, Rathindranath Baral, Neyaz Alam, Anamika Bose, Saptak Banerjee.

Awards

Awards related to thesis work

- **2nd Position in Oral Competition on Ph.D. thesis related research work.**

  Awarded by - 8th International Conference on Stem Cells and Cancer Proliferation, Differentiation and Apoptosis, ICSCC-2023 and Research and Innovation Pediatric Surgical Society Conference, RIPSSCON-IAPS-2023, **Conducted by All India Institute of Medical Science, AIIMS**

- **International Travel Grant to attend and present Ph.D. thesis related research work** in European Society for Medical Oncology, ESMO Breast Cancer Conference, held at Berlin, Germany from 15th May-17th May, 2024.

  Awarded by - Science and Engineering Research Board, SERB, Government of India

- **International Travel Grant to attend and present Ph.D. thesis related research work** in American Society of Clinical Oncology, ASCO Breakthrough Conference, held at Yokohama, Japan from 8th August-10th August, 2024.

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Dated: 16-Apr-2024

To
Ms. Aishwarya Guha,
Chittaranjan National Cancer Institute , 37, S.p.mukherjee Road, Kolkata-700026, Kolkata, West Bengal-700026

Subject: Financial Assistance to Ms. Aishwarya Guha for participating in **"ESMO Breast Cancer 2024, Germany (15 May, 2024 to 17 May, 2024)"**

Sir/Madam

We are happy to inform you that your application seeking financial grant to attend the above mentioned international scientific event has been recommended for support by the Science and Engineering Research Board (SERB). **We will provide to and fro economic class air-fare by the shortest route,airport tax , visa fees & registration fees**. It is hoped that the support will give you an opportunity to interact with leading international experts in the area. The support, however, is subject to the following conditions.
1. You should not have received financial support during the last three years under this scheme.

2. The air tickets are to be booked in economy class at "**Best Available Fare**" on the date of booking. It may be noted that rescheduling/cancellation charges will not be reimbursed.

3. SERB is directed to instruct the applicant to purchase the air tickets from either of three Authorised Travel Agents viz. i) M/s Balmer Lawrie & Company Limited (BLCL) and ii) M/s Ashok Travels & Tours (ATT) and iii) Indian Railways Catering and Tourism Corporation (IRCTC) vide order no. 19024/03/2021-E.IV dated 31-12-2021 issued by Department of Expenditure, Ministry of Finance. In case of failure of adherence to this guideline, air fare will not be reimbursed.

4. The candidate availing ITS must have to submit the certificate for "**Best Available Fare**" at the time of reimbursement. In case, tickets are booked through IRCTC, it is mandatory to submit a screenshot of the related IRCTC webpage as an evidence to ensure that the tickets has been booked at the "Best Available Fare"

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July 03, 2024

Ms Aishwarya Guha
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Dept. of Immunoregulation and Immunodiagnostics
Chittaranjan National Cancer Institute,37
Shyama Prasad Mukherjee Road,Bakul Bagan Kolkata-700 026 WEST BENGAL

SUBJECT: CSIR Foreign Travel Grant

Dear Ms Guha

With reference to your application on the aforesaid subject, we are happy to inform that the Director General, CSIR has been pleased to sanction foreign travel grant to enable you to attend and present your paper at the **ASCO Breakthrough 2024, Japan** during **08 Aug 2024 to 10 Aug 2024** subject to the following conditions:-

1. The CSIR Foreign Travel Grant is limited to **Half Air Fare Only** payable in Indian Rupees only. **The journey should be strictly performed by the shortest route in excursion economy class and International Travel can be made by private airlines. Tickets must be purchased by utilizing the services of Government Authorized Travel Agents only viz. M/s Balmer Lawrie & Company, M/s Ashok Travels & Tours and IRCTC as warranted under Govt. of India orders in this regard.**

2. The grant should be claimed by filling-in the Grant-in-Aid bill (Annexure-3), Tour Report (Annexure-4) & NEFT Proforma (Annexure-5) available at https://csirhrdg.res.in/Home/Index/1/InPage/51/14 (in duplicate) along with the counter foil of original boarding passes, e-ticket and certificate of attending the conference from the organizers.

3. The grant should be claimed within two months of return from abroad. The claim bill must be forwarded through your Supervisor/Head of the Institution. Any claim received after two months will be entertained only in exceptional cases subject to submission of reasons for delay, duly forwarded through Head of the Organization. In no case, the claim will be entertained after 4 months. All the above documents should be self attested by the candidate.

4. One reprint of the research paper presented at the Conference/Symposium etc. should be sent to CSIR, invariably after its publication.

Yours sincerely,

(Dhirendra Bansal)

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Scientific Poster Presentation

Regards,

Amanda Davis Aitken

Amanda Davis Aitken, Vice President Meetings Services
American Society of Clinical Oncology, Inc.

Reprints

Reprints of Publications

Confirmatory Results

Tumor-Educated-Platelets interact with Breast Cancer-Stem-Cells via P-selectin-PSGL1 and ensure stemness and metastasis through WNT-β-Catenin-VEGF-VEGFR2 intra-cellular signaling:Therapeutic modulation by aspirin

Aishwarya Guha, Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Anirban Sarkar, Sukanya Dhar, Pritha Roy Choudhury, Juhina Das, Nilanjan Ganguly, Ipsita Guha, Tapasi Das, Neyaz Alam, Indranil Ghosh, Srabanti Hajra, Rathindranath Baral, Anamika Bose, Saptak Banerjee

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Abstract

Background Protagonistic role of platelets promote capillary infiltration of tumors for distant metastasis along with immunosurveillance. Despite existing reports highlighting role of platelets in tumorigenesis, its impact on breast cancer stem cells (BCSCs) remain underexplored. Our first ever report on murine and human system, accentuate that, tumor educated platelets (TEPs) of luminal A and TNBC subtypes are distinct from healthy counterparts, collaborating with BCSCs to generate sub-variants that elevate tumor aggressiveness.

Methods Impact of TEPs on BCSCs was evaluated from primary breast tumor and blood samples of luminal A/TNBC patients along with EC/4T1 murine breast tumor models and MCF-7/MDA-MB-231 cell lines. For downstream assays, TEPs were co-cultured with breast tumor samples or cell lines, followed by magnetic sorting of $lin^{-}CD44^{+}CD24^{-}$ BCSCs. TEP induced alterations of BCSCs were evaluated from 3D tumorsphere, colony formation, transwell migration, scratch-wound healing, matrigel invasion, *in-vitro* tube formation assays. Fluorescence-confocal microscopy, RT-PCR, flow-cytometry, western-blotting was utilized to

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Cancer stem cell–immune cell crosstalk in breast tumor microenvironment: a determinant of therapeutic facet

Aishwarya Guha[1†], Kuntal Kanti Goswami[2†], Jasmine Sultana[1], Nilanjan Ganguly[1], Pritha Roy Choudhury[1], Mohona Chakravarti[1], Avishek Bhuniya[1], Anirban Sarkar[1], Saurav Bera[1], Sukanya Dhar[1], Juhina Das[1], Tapasi Das[1], Rathindranath Baral[1], Anamika Bose[3] and Saptak Banerjee[1*]

[1]Department of Immunoregulation and Immunodiagnostics, Chittaranjan National Cancer Institute, Kolkata, India, [2]Department of Microbiology, Asutosh College, Kolkata, India, [3]Department of Pharmaceutical Technology Biotechnology National Institute of Pharmaceutical Education and Research (NIPER) Sahibzada Ajit Singh (S.A.S.) Nagar, Mohali, Punjab, India

Breast cancer (BC) is globally one of the leading killers among women. Within a breast tumor, a minor population of transformed cells accountable for drug resistance, survival, and metastasis is known as breast cancer stem cells (BCSCs). Several experimental lines of evidence have indicated that BCSCs influence the functionality of immune cells. They evade immune surveillance by altering the characteristics of immune cells and modulate the tumor landscape to an immune-suppressive type. They are proficient in switching from a quiescent phase (slowly cycling) to an actively proliferating phenotype with a high degree of plasticity. This review confers the relevance and impact of crosstalk between immune cells and BCSCs as a fate determinant for BC prognosis. It also focuses on current strategies for targeting these aberrant BCSCs that could open avenues for the treatment of breast carcinoma.

KEYWORDS

breast cancer (BC), breast cancer stem cells (BCSCs), tumor microenvironment (TME), innate immune cells, adaptive immune cells

Abbreviations: ALDH1, alcohol dehydrogenase 1; APCs, antigen-presenting cells; BC, breast cancer; BRCA, breast cancer gene; BCSCs, breast cancer stem cells; CD, cluster of differentiation; CTCs, circulating tumor cells; CSF, colony-stimulating factor; DCs, dendritic cells; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; Id1, inhibitor of DNA binding 1; IL, interleukin; iDCs, immature DCs; MDSCs, myeloid-derived suppressor cells; MFG, milk fat globule; MHC, major histocompatibility complex; MIC, MHC-I chain-related protein; MMPs, matrix metalloproteinases; M-DCs, mature DCs; NK, natural killer cells; NKG2D, NK-activating receptor; PGE2, prostaglandin E2; ROS, reactive oxygen species; RNS, reactive nitrogen species; TME, tumor microenvironment; TAMs, tumor-associated macrophages; Tregs, T regulatory cells; TILs, tumor-infiltrating lymphocytes; TINs, tumor-infiltrating neutrophils.

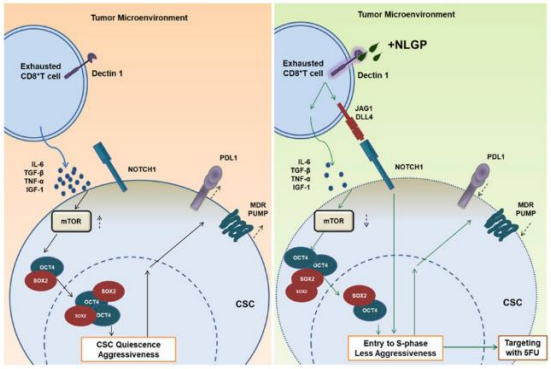
Neem Leaf Glycoprotein Disrupts Exhausted CD8$^+$ T-Cell–Mediated Cancer Stem Cell Aggression

Mohona Chakravarti[1], Saurav Bera[1], Sukanya Dhar[1], Anirban Sarkar[1], Pritha Roy Choudhury[1], Nilanjan Ganguly[1], Juhina Das[1], Jasmine Sultana[1], Aishwarya Guha[1], Souradeep Biswas[2], Tapasi Das[1], Subhadip Hajra[2], Saptak Banerjee[1], Rathindranath Baral[1], and Anamika Bose[3]

ABSTRACT

Targeting exhausted CD8$^+$ T-cell (T_{EX})–induced aggravated cancer stem cells (CSC) holds immense therapeutic potential. In this regard, immunomodulation via Neem Leaf Glycoprotein (NLGP), a plant-derived glycoprotein immunomodulator is explored. Since former reports have proven immune dependent–tumor restriction of NLGP across multiple tumor models, we hypothesized that NLGP might reprogram and rectify T_{EX} to target CSCs successfully. In this study, we report that NLGP's therapeutic administration significantly reduced T_{EX}-associated CSC virulence in *in vivo* B16-F10 melanoma tumor model. A similar trend was observed in *in vitro* generated T_{EX} and B16-F10/MCF7 coculture setups. NLGP rewired CSCs by down-regulating clonogenicity, multidrug resistance phenotypes and PDL1, OCT4, and SOX2 expression. Cell cycle analysis revealed that NLGP educated–T_{EX} efficiently pushed CSCs out of quiescent phase (G_0G_1) into synthesis phase (S), supported by hyperphosphorylation of G_0G_1–S transitory cyclins and Rb proteins. This rendered quiescent CSCs susceptible to S-phase–targeting chemotherapeutic drugs like 5-fluorouracil (5FU). Consequently, combinatorial treatment of NLGP and 5FU brought optimal CSC-targeting efficiency with an increase in apoptotic bodies and proapoptotic BID expression. Notably a strong nephron-protective effect of NLGP was also observed, which prevented 5FU-associated toxicity. Furthermore, Dectin-1–mediated NLGP uptake and subsequent alteration of Notch1 and mTOR axis were deciphered as the involved signaling network. This observation unveiled Dectin-1 as a potent immunotherapeutic drug target to counter T-cell exhaustion. Cumulatively, NLGP immunotherapy alleviated exhausted CD8$^+$ T-cell-induced CSC aggravation.

Implications: Our study recommends that NLGP immunotherapy can be utilized to counter ramifications of T-cell exhaustion and to target therapy elusive aggressive CSCs without evoking toxicity.



Introduction

Despite being the primary arsenal in anticancer response, CD8$^+$ T cells gradually lose their functionality within the tumor niche due to immune-suppressive milieu and prolonged antigenic exposure. They exhibit a marked reduction in clonal proliferation and production of cytolytic molecules and effector cytokines such as IFNγ, IL2, TNFα, and β-chemokines. At this aberrant, hypo-responsive "exhausted" state, CD8$^+$ T cells fail to regulate the tumor (1, 2). The onset of exhaustion program triggers the heightened surface expression of co-inhibitory receptors (IRs)—PD1, CTLA4, TIM3, LAG3, BTLA, SLAMF6, and TIGIT—imposing restriction on CD8$^+$ T-cell fate (3). Immunotherapy in the form of immune checkpoint inhibitor (ICI) abrogates the interactions between these IRs and their ligands (4, 5). Presently pembrolizumab, nivolumab (PD1 inhibitor), durvalumab, atezolizumab (PDL1 inhibitor), and ipilimumab (CTLA4 inhibitor) are in use, either as a monotherapy or in combination for various solid tumors and hematologic malignancies in both metastatic and adjuvant settings (6, 7).

However, despite its promising venture, the ICI efficacy rate remained suboptimal; with a 15% to 60% response rate (8). Among others, development of autoimmune toxicities called irAEs (immune-related adverse events) raises concern (9). Moreover, ICI therapy is thwarted by heterogeneity within the intended target population. Recent discoveries have revealed the presence of two cohorts—a stem cell–like progenitor exhausted (T_{PEX}; PD1$^+$TCF1$^+$) and effector cell–like terminally exhausted (T_{TEX}) population (PD1$^+$TCF1$^-$) within exhausted CD8$^+$ T-cell (T_{EX}) pool. These two epigenetically and phenotypically distinct populations respond

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Tumor activated platelets induce vascular mimicry in mesenchymal stem cells and aid metastasis

Avishek Bhuniya, Anirban Sarkar, Aishwarya Guha, Pritha Roy Choudhury, Saurav Bera, Jasmine Sultana, Mohona Chakravarti, Sukanya Dhar, Juhina Das, Ipsita Guha, Nilanjan Ganguly , Saptak Banerjee, Anamika Bose, Rathindranath Baral

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Highlights

- Platelet-activation is dependent on extent of metastasis.
- Activated-platelets promote migration of mesenchymal-stem-cells (MSCs) towards secondary site.
- MSCs form vascular-mimicry (VM) at secondary site.
- Activated platelet-MSC influenced VM expedites metastasis.

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Abstract

Extent of metastasis influences activation of platelets in tumor-microenvironment. Activated platelets potentiate mesenchymal-stem-cells (MSCs) to migrate in secondary

Review Article

Evolving Trends in Breast Cancer Immunotherapy: Recent Updates

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Abstract

Breast cancer encompasses manifold molecular subtypes and clinical heterogeneity. Distinct therapeutic regimes and the treatment responses vary according to the stage and grade of mammary malignancy. Conventional oncotherapies such as radiation, chemotherapy inflict hazardous toxicities and may lead to disease recurrence. Contemporary research advancements in the arena of immunobiology have paved the way toward efficacious augmentation of host immunity against breast tumor. Although breast tumors were initially considered to be nonimmunogenic, a plethora of clinical and experimental investigations revealed immunotherapy as an emerging alternative with substantial potential and reduced off-target toxicities. Despite looming challenges in the clinical implementation of immuno-therapeutic strategies, there are opportunities for improvement. Here, in this review, the efficacy and risks of evolving strategies of immunotherapy, including monoclonal antibodies, immuno-checkpoint inhibitors, chimeric antigen receptor T-cell therapy, oncolytic virotherapy, cytokine therapy, and breast cancer (BC) vaccines have been elaborated. It also elucidates the ongoing trials which aim for bench-to-bedside administration of immunotherapy for BC care.

Keywords: Breast cancer, chimeric antigen receptor T-cell therapy, immuno-checkpoint inhibitors, immunotherapy, monoclonal antibodies

Introduction

In the preceding decade, cancer immunotherapy has emerged as one of the most substantial therapeutic advances in oncotherapy.[1] Primarily, breast tumors are considered to be poorly immunogenic and are insusceptible to immunotherapy due to a lack of infiltration of immune cells.[2] Later, several clinical trials in breast cancer (BC) patients have gained attention toward the prognostic effects of immunotherapy.[3] Transcriptomic and immunophenotyping approaches reveal the presence of varied immune cell components within the breast tumor microenvironment (TME). Pro or anti-tumor immunogenicity has shown noteworthy effects on the clinical prognosis of BC patients.[4] However, the extent of immune infiltration and immune contextures shows subtype-specific variations. The TME consists of diverse immune cell populations, comprising both suppressive and cytotoxic components. Key examples are regulatory T-cells (Tregs), cytotoxic T lymphocytes (CTLs), dendritic cells (DCs), and tumor-associated macrophages (TAMs). Among these, $CD8^+$ T cells – the chief cytotoxic subset – can directly eliminate cancer cells and are generally associated with enhanced patient survival. In contrast, higher levels of immunosuppressive cells, such as regulatory $CD4^+$ T cells (Tregs) and TAMs, are associated with poorer outcomes.[5] Despite several drawbacks, biomarkers such as programmed cell death protein ligand-1 (PD-L1) and tumor mutational burden (TMB) assist in the selection of precise therapeutics for patients.[6]

BC is the most commonly diagnosed cancer in 2024 and the primary cause of cancer death among women, highlighting the need for novel therapies. It is categorized into four primary subtypes based on HER2 and hormone receptor expression and each subtype and stage has a different course of treatment, for example, chemotherapy alone is used for triple-negative BC (TNBC), endocrine therapy is used for all HER2-/ER+/PR + tumors, and trastuzumab, used for HER2-directed

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ABSTRACT · Volume 9, Supplement 4, 103084, May 2024

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78P Tumor educated platelets promote disease advancement in breast cancer by interacting with cancer stem cells

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Affiliations & Notes Article Info

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Background

The critical role of platelets in cancer metastasis is widely proclaimed. While in circulation, they shield tumor cells to escape immunosurveillance and establish secondary tumors. During this interaction platelets become activated and are termed as tumor educated platelets (TEPs). Recent reports confirmed pivotal role of platelets within tumor microenvironment (TME) though, functional status of these TEPs remains elusive. This work emphasises the phenotypic and genotypic alterations in breast cancer stem cells (BCSCs) triggered by TEPs, which in turn lead to metastasis.

Methods

Frequency of humoral TEPs ($CD41^+/CD62P^+$) were analysed in murine tumor models along with luminal A (LumA) and triple-negative (TNBC) patients. Effects of TEPs (isolated from patients blood) on BCSCs were assessed by co-culturing BCSCs from MCF-7 and MDA-MB-231 with TEPs. Tumorsphere, clonogenicity, migration assays were done with validation in swiss mice to decipher the impact of

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Evaluating interaction between tumor educated platelets and cancer stem cells on breast cancer subtypes.

Authors: Aishwarya Guha, Jasmine Sultana, Avishek Bhuniya, Mohona Chakravarti, Saurav Bera, Anirban Sarkar, Sukanya Dhar, ... SHOW ALL ... , and Saptak Banerjee | AUTHORS INFO & AFFILIATIONS

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87 PDF

Abstract

5

Background: Thrombocytosis has been reported to be responsible for poor prognosis of numerous malignancies. This swarm of platelets acts as foe for the body but as mate for the cancer cells by providing a protective shield around them, helping them to escape immunosurveillance process. A bidirectional interaction between platelets and tumor cells promote their conversion to an activated tumor educated platelet (TEP) state. The present report reveals the critical role played by TEPs to promote metastasis in BC subtypes by interacting with cancer stem cells (CSCs). **Methods:** Status of intra-tumoral and humoral TEPs ($CD41^+/CD62P^+$) was screened in luminal A (LumA) and triple negative (TNBC) BC subgroups. The impact of TEPs on $lin^-/CD44^+/CD24^-$ CSCs of both the categories *in-vitro,* was deciphered by performing mammosphere assay, clonogeneic and migration assays, with final validation in murine system. Effect on various genes and proteins related to stemness, metastasis and angiogenesis as a result of this interaction was elucidated by RT-PCR, flow cytometry and western blotting. **Results:** High percentage of TEPs in the peripheral blood is a potential biomarker and responsible for poor prognosis in BC subtypes. Both Lum A and TNBC patients had elevated TEP frequency, compared to healthy blood. Further, patients in the pre-carcinogenic stage had platelet count of >3.5 lakhs. Additionally, screening of breast tumor sections revealed intra-tumoral enrichment of TEPs, comparatively to a greater extent in TNBC than LumA, supporting their role in disease aggression. Also, a positive correlation was noted between TEP and CSC frequencies proving their synergistic interactions. Moreover, *in-vitro*, TEP influenced CSCs exhibited enormous clonogenic and tumorigenic potentialities. Their

930 INTERVENTION ON INTRA-TUMORAL BREAST CANCER STEM CELLS AND CD8+ T CELL DYSREGULATION: THE IMPACT OF 2-DEOXY-D-GLUCOSE

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Background Intricate nature within tumors presents major obstacle in successful cancer management. A self-renewing subset of stem cells, designated as cancer stem cells (CSCs), drives this heterogeneity. Truncation of effector response from infiltrated CD8+ T-cells enhance Breast cancer stem cells (BCSC) survival.[1–3] Within tumor microenvironment (TME), $CD8^+$ T-cells undergo a hierarchical loss of proliferation and effector functions. However, the crosstalk between BCSCs and $CD8^+$ T-cells are poorly understood.

2-deoxyglucose (2DG), a glucose analogue used as a metabolic regulator, inhibits glycolysis and augments $CD8^+$ T-cell infiltration into the TME, potentially enhancing their cytotoxic activity. Given the eminence of BCSCs in metastatic cancer progression by evading therapies, we became interested to study the possible crosstalk between $CD8^+$ T-cells and BCSCs in tumor advancement within BC sub-types and intervention of 2DG in regulating the same.

Methods MACS-sorted BCSCs ($Lin^-CD44^+CD24^-$) and T-cells (Lin^+CD8^+T-cell) were isolated from patients' tumor. These $CD8^+$T cells were co-cultured with BC sub-types to study the interaction with $CD8^+$T-cells. Further the influence of 2DG was elucidated. Flow-cytometry, Western-blot, RT-PCR were performed to identify involved transcription factors (KLRG1, Granzyme B, Tox, Tim3, PD1 etc.), stem-cell markers ($CD44^+$ $CD24^-$) and $CD8^+$T-cell (PD1, KLRG1, IFNγ, GrB) within BC.

Results A positive co-relation between BCSCs and $CD8^+PD1^{high}KLRG1^{high}IFN\gamma^{low}$ T-cells was noted in TNBC cells compared to other subtypes. Exhausted $CD8^+$T-cells ($PD1^{high}TOX^+Eomes^+CXCR5^+$) collaborated with elevated BCSC level. Exposure of TNBC-BCSCs in T-cell co-culture suggested a prominent gain of exhaustion features with a significant loss of effector function within CD8+T-cells. Addition of 2DG partially retrieving $CD8^+$T-cell from their dysfunction by lowering the status of KLRG1 and PD-1. Simultaneously, 2DG helps better $CD8^+$ T- cell mediated killing by enhancing IFNγ, Granzyme-B secretion. 2DG might restrict BCSCs while boosting $CD8^+$ T-cells. Corrupted $CD8^+$ T-cells significantly enhance the tumorigenic potential of BCSCs. The modulation of BCSCs and T-cells by 2DG and its potential implications in breast cancer management was also discerned (figure 1).

Conclusions Study discloses strong association between exhausted $CD8^+$ T-cell population and CSCs in BC, particularly in TNBC indicating their possible association in tumor promotion. The exhaustion status of CD8+ T-cells might be influenced by BCSCs and overall immune infiltration of the TME. This aligns with the increase in BCSCs and corresponded to poor prognosis. 2DG alleviates dysfunctional-$CD8^+$T cell by interfering BCSC and T-cell crosstalk. Therefore, 2DG could be utilized as prospective therapeutic targets against $CD8^+$T-influenced aggressive BCSCs and it provides a new nontoxic therapeutic alternative to target BCSCs.

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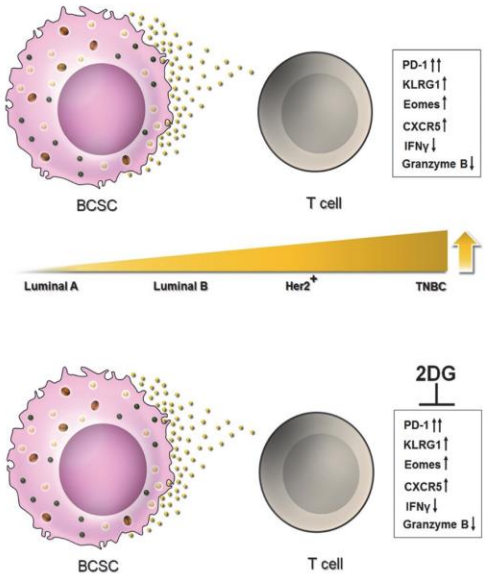
Ethics Approval All human experiments were approved by Institutional Human Ethical Committee of Chittaranjan National Cancer Institute, Kolkata, India. Approval numbers are CNCI-IEC-SB-20. All patients included gave informed consent before taking part in the study.



Abstract 930 Figure 1 Model proposing BCSC and CD8+T cell crosstalk

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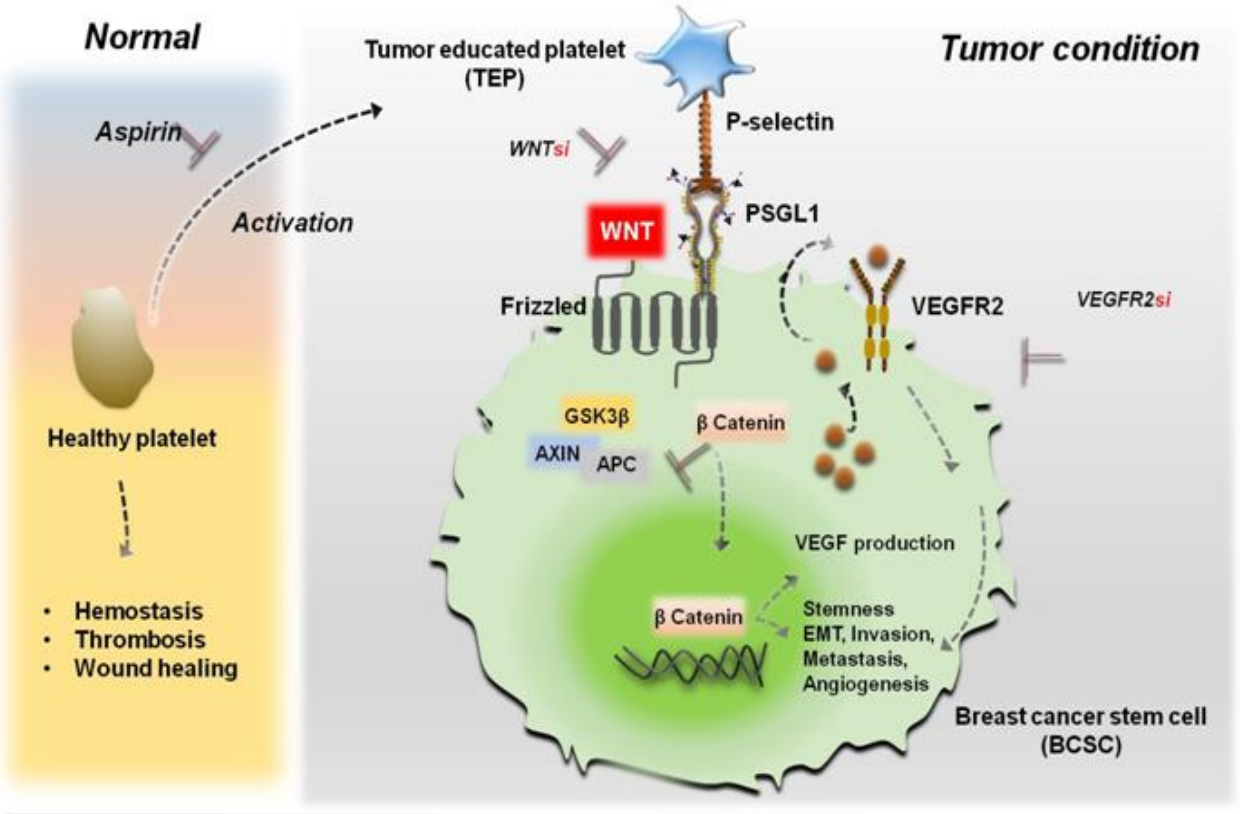
Summary


Normal
Aspirin
Activation
Healthy platelet
Hemostasis
Thrombosis
Wound healing
Tumor educated platelet (TEP)
Tumor condition
P-selectin
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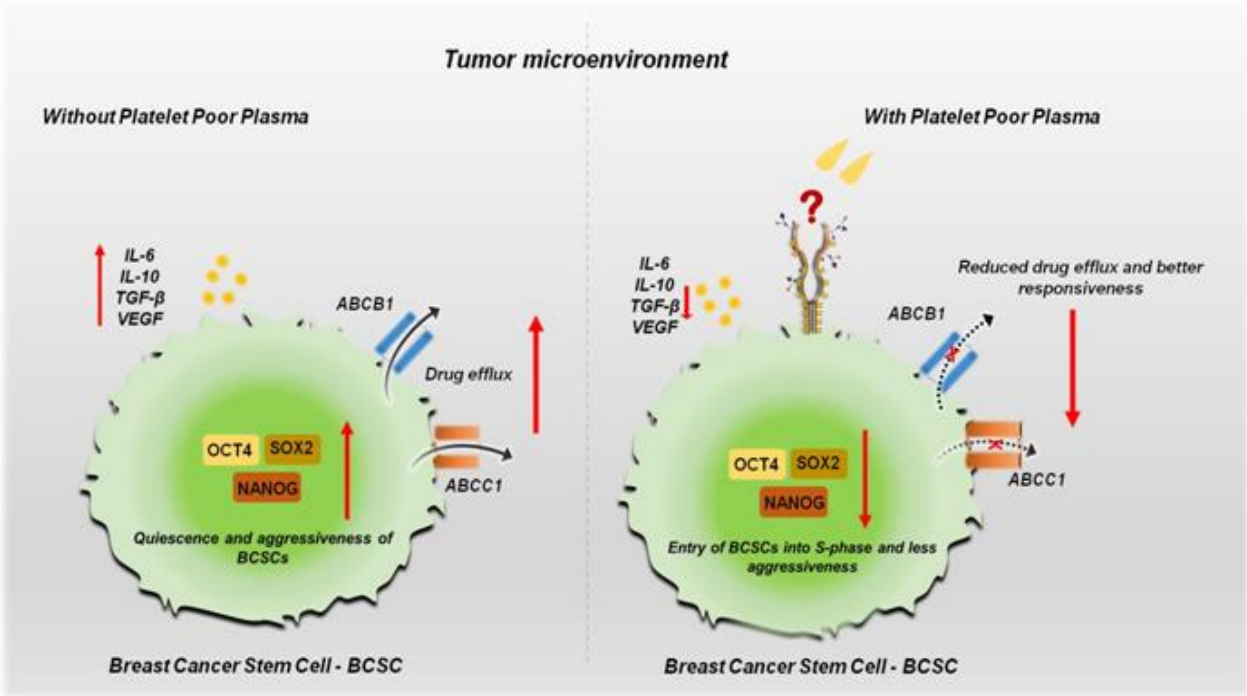
WNT
PSGL1
Frizzled
VEGFR2
VEGFR2si
GSK3β
β Catenin
AXIN
APC
VEGF production
β Catenin
Stemness
EMT, Invasion, Metastasis, Angiogenesis
Breast cancer stem cell (BCSC)


Tumor microenvironment
Without Platelet Poor Plasma
IL-6
IL-10
TGF-β
VEGF
ABCB1
Drug efflux
ABCC1
OCT4
SOX2
NANOG
Quiescence and aggressiveness of BCSCs
Breast Cancer Stem Cell - BCSC
With Platelet Poor Plasma
?
IL-6
IL-10
TGF-β
VEGF
Reduced drug efflux and better responsiveness
ABCB1
ABCC1
OCT4
SOX2
NANOG
Entry of BCSCs into S-phase and less aggressiveness
Breast Cancer Stem Cell - BCSC