THE ROLES OF CIRCULATING NEONATAL Nav1.5 (nNav1.5) AND ITS ANTIBODIES IN CANCER PROGRESSION OF 4T1 ORTHOTOPIC MICE MODEL AND BREAST CANCER PATIENTS

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by

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LIST OF SYMBOLS

3’	3 prime ends
5’	5 prime ends
α	Alpha
β	Beta
ɣ	Gamma
%	Percentage
*	Asterisk for multiplication
:	Colon for ratio
<	Less than
>	Greater than
≤	Less and equal than
±	Plus or minus
Δ	Delta
ΔΨm	Mitochondrial membrane potential
°C	Degree Celsius
°F	Fahrenheit scale
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometre
cm	Centimetre
cm^2	Centimetre square
cm^3	Centimetre cube
Ct	Threshold cycle
Da	Dalton

g	Gram
G	Gauge
I_{Na}	Sodium ion current
k	Kilo
kg	Kilogram
L	Litre
M	Molar
mg	Milligram
ml	Millilitre
mm	Millimetre
mmHg	Millimetre of mercury
mol	Moles
N	Total sample size
n	Subset of the total sample size
ng	Nanogram
nm	Nanometre (wavelength)
P	*P*-value for statistical differences
pg	Picogram
r	Correlation coefficient
R^2	Coefficient of determination
V_m	Plasma membrane potential
xg	Relative centrifugal force

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ANG2	Angiogenesis-promoting protein genes 2
ANOVA	Analysis of variance
Anti-nNav1.5-Ab	Antibody against nNav1.5
APCs	Antigen-presenting cells
ARASC	Animal Research and Service Centre
ASR	Age-standardised incidence rate
ATCC	American Type Culture Collection
AUC	Area under the curve
BCL-2	B-cell lymphoma-2
BestARi	Breast Cancer Awareness and Research Unit
BL1	Basal-like 1
BL2	Basal-like 2
BRCA1	Breast cancer mutation 1
BRCA2	Breast cancer mutation 2
CA15-3	Cancer antigen 15-3
CAFs	Cancer-associated fibroblasts
CAIX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
CCR4	C-C motif chemokine receptor 4
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CEA	Cancer embryonic antigen
CO_2	Carbon dioxide
CPG	Clinical Practice Guidelines
CTCs	Circulating tumour cells
CTLs	Cytotoxic T-lymphoctyes
CXCR1/2	C-X-C motif chemokine receptors 1/2
D1	Domain 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DPX	Di butyl phthalate polystyrene xylene
DTCs	Disseminated tumour cells
E3Ab	Nav1.5-third extracellular region antibody
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
ER	Oestrogen receptor
ERK2	Extracellular-signal-regulated kinase 2
FBS	Foetal bovine serum
FDA	The Food and Drug Administration
FOXP3	Fork-head box P3
G-CSF	Granulocyte colony-stimulating factor
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
GLUT1	Glucose transporter 1
H+	Hydrogen ions
H+L	Heavy and light chains
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HDAC2	Histone deacetylase 2
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor-1α
HRD	Homologous recombination deficiency
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
IARC	International Agency on Cancer Research
IDC	Invasive ductal carcinoma
IFN-ɣ	Interferon-gamma
IgG	Immunoglobulin G
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-17	Interleukin-17
IL-19	Interleukin-19

IL-1α	Interleukin-1-alpha
IL-1β	Interleukin-1-beta
IL-20Rβ	Interleukin-20 receptor β chain
IL-2Rα	Interleukin-2 receptor subunit alpha
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IL-8	Interleukin-8
ILC	Invasive lobular carcinoma
IM	Immunomodulatory
INFORMM	Institute for Research in Molecular Medicine
IQR	Interquartile range
JAK	Janus tyrosine kinases
JEPeM	Human Research Ethics Committee of USM
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LAR	Luminal androgen receptor
M	Mesenchymal
MCP-1/CCL2	Monocyte chemoattractant protein-1/ Chemokine ligand 2
MDSCs	Myeloid-derived suppressor cells
MEKi	MAPK kinase inhibitors
MET	Mesenchymal-to-epithelial transition
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
miRNAs	MicroRNAs
MMP-2	Matrix metalloproteinases-2
MMP-9	Matrix metalloproteinases-9
MMPs	Matrix metalloproteinases
MNCR	Malaysian National Cancer Registry
mRNA	Messenger RNA
MSA5	Makmal Sains Asas 5
MSL	Mesenchymal stem-like
MVD	Microvessel density
MYCODE	Malaysian Code of Practice for the Care and Use of Animals for Scientific Purposes

Na+	Sodium ions
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCX	Sodium-calcium exchanger
NHE-1	Sodium/hydrogen exchanger 1
NK	Natural killer cells
nNav1.5	Neonatal Nav1.5
OD	Optical density
Omega-3	Docosahexaenoic acid
P	Passage
P-	Pooled negative sera
P+	Pooled positive sera
PBS	Phosphate buffer saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death-ligand 1
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
qPCR	Real-time polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
REST	Repressor element silencing transcription factor
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPMI	Roswell Park Memorial Institute
S3-4	Segments 3-4
SD	Standard deviation
SIK1	Salt inducible kinase 1
siRNA	Small interfering RNA
SNAI1	Zinc finger protein 1
SNP	Single nucleotide polymorphism
STAT3/5	Signal transducer and activator of transcription 3/5
TAA	Tumour-associated antigen

TAE	Tris-acetate-EDTA
TGF-β1	Transforming growth factor-beta 1
Th-1	T-helper type 1
Th-2	T-helper type 2
TILs	Tumour-infiltrating lymphocytes
TMB	Tetramethylbenzidine
TNBC	Triple-negative breast cancer
TNF-α	Tumour necrosis factor-alpha
TNM	Tumour, node and metastasis
TP53	Tumour protein p53
TSP-1	Thrombospondin-1
UICC	International Union for Cancer Control
USM	Universiti Sains Malaysia
USM IACUC	USM Institutional Animal Care and Use Committee
VEGF	Vascular endothelial growth factor
VEGFRs	Vascular endothelial growth factor receptors
VGSCs	Voltage-gated sodium channels
VHL	Von Hippel-Lindau
WHO	World Health Organisation
YB-1	Y-box binding protein-1

LIST OF APPENDICES

PERANAN PENGEDARAN NEONATAL Nav1.5 (nNav1.5) DAN ANTIBODINYA DALAM PERKEMBANGAN KANSER MODEL TIKUS ORTOTOPIK 4T1 DAN PESAKIT KANSER PAYUDARA

ABSTRAK

Kajian ini bertujuan untuk menyiasat peranan pengedaran antigen neonatal Nav1.5 (nNav1.5) dan antibodi semula jadi yang dihasilkan terhadap nNav1.5 (anti-nNav1.5-Ab) dalam keseluruhan darah dan serum model tikus orthotopik 4T1 serta pesakit kanser payudara. Penyelidikan praklinikal melibatkan tiga kumpulan tikus: kumpulan 1 kawalan (*n*=20), kumpulan 2 model kanser payudara ortotopik 4T1 (*n*=17), dan kumpulan 3 kawalan positif (*n*=3). Sampel darah, organ sasaran dan tumor 4T1 telah dikumpulkan. Tindakbalas rantaian polimerase masa nyata (qPCR) dijalankan untuk mengesan ekspresi antigen nNav1.5 dalam keseluruhan darah dan asai imunosorben untaian enzim (ELISA) digunakan untuk mengesan ekspresi anti-nNav1.5- Ab dalam serum. Tambahan lagi, asai metastasis klonogenik paru-paru, histologi dan analisis sitokin telah dijalankan. Kajian klinikal melibatkan 128 peserta: peserta yang sihat (*n*=64) dan pesakit kanser payudara (*n*=64). Butiran sosiodemografi peserta telah dianalisis. Pesakit kanser payudara dibahagikan berdasarkan status rawatan dan peringkat. Sejumlah 6 ml darah telah diambil, juga untuk analisis, qPCR dan ELISA masing-masing bagi mengesan pengedaran nNav1.5 dan anti-nNav1.5-Ab. Analisis sitokin juga dijalankan dalam kajian klinikal. Kajian praklinikal telah mendedahkan kehadiran metastasis (melalui asai klonogenik dan histologi) and pengedaran antigen nNav1.5 dalam tikus ortotopik 4T1. Tikus ortotopik 4T1 menunjukkan keserapan yang lebih tinggi secara signifikan berbanding kumpulan

kawalan (*P*<.001). Terdapat hubungan negatif yang signifikan antara pengesanan antigen nNav1.5 dan anti-nNav1.5-Ab (*P*<.05, r=-0.549). Dalam analisis sitokin, terdapat korelasi positif yang signifikan antara anti-nNav1.5-Ab, IL-6 (*P*<.05, r=0.643) dan VEGF (*P*<.01, r=0.735). Analisis sosiodemografi menunjukkan perbezaan umur yang signifikan antara peserta yang sihat dan pesakit kanser payudara (*P*<.001). Kajian klinikal menunjukkan ekspresi terhad pengedaran antigen nNav1.5, hanya dalam lima pesakit prarawatan. Ekspresi anti-nNav1.5-Ab telah dikesan dalam peserta yang sihat dan pesakit kanser payudara, namun keserapan anti-nNav1.5-Ab adalah lebih tinggi secara signifikan dalam pesakit kanser payudara (*P*<.001). Kumpulan prarawatan menunjukkan ekspresi anti-nNav1.5-Ab tertinggi secara signifikan berbanding kumpulan kawalan dan rawatan berterusan (*P*<.001). Terdapat korelasi positif yang signifikan antara anti-nNav1.5-Ab dan IL-6 (*P*<.05, r =0.726) dalam kumpulan prarawatan, diikuti dengan korelasi negatif yang signifikan antara anti-nNav1.5-Ab dan VEGF (*P*<.01, r=-0.842) dalam kumpulan rawatan berterusan. Pesakit peringkat lanjutan menunjukkan ekspresi anti-nNav1.5-Ab yang lebih tingi secara signifikan berbanding pesakit invasif-awal (*P*<.05). Kehadiran anti-nNav1.5-Ab menunjukkan keimunogenan nNav1.5. Anti-nNav1.5-Ab boleh berfungsi sebagai penanda pengawasan imun untuk metastasis kanser payudara.

Kata kunci: Nav1.5; nNav1.5; kanser payudara; metastasis; keimunogenan

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ABSTRACT

The study has aimed to investigate the roles of circulating neonatal Nav1.5 (nNav1.5) and natural antibodies produced against nNav1.5 (anti-nNav1.5-Ab) in the whole blood and serum of 4T1 orthotopic mice model and breast cancer patients. The preclinical research involved three mice groups: control (*n*=20), 4T1 orthotopic breast cancer model (*n*=17), and positive control (*n*=3). Blood samples, target organs and 4T1 tumours were collected. Real-time polymerase chain reaction (qPCR) was conducted to detect the expression of nNav1.5 antigen in the whole blood and an in-house indirect enzyme-linked immunosorbent assay (ELISA) was used to detect the expression of anti-nNav1.5-Ab in the serum. Additionally, lung metastasis clonogenic assay, histology and cytokine analysis were conducted. The clinical study involved 128 participants: healthy participants (*n*=64) and breast cancer patients (*n*=64). The sociodemographic details of the participants were analysed. The breast cancer patients were divided based on their treatment status and stages. A total of 6 ml of blood was withdrawn, and similarly, qPCR and ELISA were conducted to detect the circulating nNav1.5 and anti-nNav1.5-Ab, respectively. The cytokine analysis was also conducted in the clinical study. The preclinical study revealed the occurrence of metastasis (via clonogenic assay and histology) and circulating nNav1.5 antigen in 4T1 orthotopic mice. The 4T1 orthotopic mice showed significantly higher absorbance of anti-nNav1.5-Ab than the control group (*P*<.001). There was a significant negative

correlation between the expression of the nNav1.5 antigen and the absorbance of anti-nNav1.5-Ab (P<.05, r=-0.549). In the cytokine analyses, there were significant positive correlations between anti-nNav1.5-Ab, IL-6 (P<.05, r=0.643) and VEGF (P<.01, r=0.735). Sociodemographic analysis revealed a significant age difference between healthy participants and breast cancer patients (P<.001). The clinical study showed restricted expression of circulating nNav1.5 antigen, only in five pretreatment patients. The expression of anti-nNav1.5-Ab was detected in both healthy and breast cancer patients, but the absorbance of anti-nNav1.5-Ab was significantly higher in breast cancer patients (P<.001). The pretreatment group portrayed significantly the highest expression of anti-nNav1.5-Ab as compared to the control and ongoing treatment group (P<.001). There was a significant positive correlation between anti-nNav1.5-Ab and IL-6 (P<.05, r=0.726) in the pretreatment group, followed by a significant negative correlation between anti-nNav1.5-Ab and VEGF (P<.01, r=-0.842) in the ongoing treatment group. Advanced stage patients exhibited significantly higher expression of anti-nNav1.5-Ab compared to early-invasive patients (P<.05). The presence of anti-nNav1.5-Ab highlights the immunogenicity of nNav1.5. Anti-nNav1.5-Ab could serve as an immunosurveillance marker for breast cancer metastasis.

Key words: Nav1.5; nNav1.5; breast cancer; metastasis; immunogenicity

CHAPTER 1

INTRODUCTION

1.1 Background of study

Breast cancer is the most common type of cancer that occurs among women. In clinical settings, breast cancer can be classified based on stages and molecular subtypes. Generally, there are four stages of breast cancer which are early-invasive stages (stages I and II) and advanced stages (stages III and IV) (Ministry of Health Malaysia and Academy of Medicine Malaysia, 2019). Among the molecular subtypes of breast cancer include Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2) enriched and triple-negative breast cancer (TNBC) (Malhotra *et al*., 2010). According to the Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) 2020 report released by the International Agency on Cancer Research (IARC), breast cancer exhibited the highest percentage of incidence among women worldwide, followed by colorectum and lung cancer (Sung *et al*., 2021).

The malignancy occurs when breast cells begin to grow extensively without being complied with their natural cell cycle. The breast tumour cells spread to other parts of the body via the lymphatic systems and blood circulation (Tyagi *et al.,* 2017; Karlsson *et al*., 2017; Arneth, 2019) and such progression is known as metastasis. Invasion and metastasis have been included as part of the original version of the hallmarks of cancer which was proposed by well-known authors Hanahan and Weinberg in the year 2000.

The advanced stage of breast cancer (stage IV) is more likely to exhibit signs of metastasis (van Uden *et al*., 2019). Metastasis is a complex mechanism undergone by cancer cells to invade various sites such as organs and tissues. In general, the metastasis cascade encompasses three main steps: invasion, intravasation, and extravasation

(Winkler *et al*., 2020). It is initiated by the dissociation of cancer cells from the primary tumour site, followed by the breaking of the extracellular membrane (invasion) that allows the cancer cells to invade the bloodstream or lymphatic vessels (intravasation) thus forming secondary tumour sites once the cancerous cells exit the circulation (extravasation) (Vasilaki *et al*., 2021).

Ion transport is well known to occur within the epithelial to conduct survival processes like secretion, solute, and water transport. Most cancers (80-90%) are carcinomas which means that they originate from normal epithelial cells (National Cancer Institute, 2021). Ion transports are typically associated with excitable cells, whereby electrical impulses are necessary to perform their acquitted tasks (Mao *et al*., 2019). Initially, it is unsure whether the aberrant expression of ion transports such as sodium channels within cancer cells contributes to the initiation and development of cancer or otherwise. Over time, the study gap has been answered via discoveries and postulations that suggest the existence of an association between the presence of ion transport and cancer metastasis (Brackenbury, 2012; Djamgoz *et al*., 2014). This is based on the fact that ion transports are generally controlled by hormones and growth factors which are also known as critical contributors to the mainstream mechanism of cancer progression (Subramani *et al*., 2017; Obradović *et al*., 2019; Murphy *et al*., 2020).

The metastatic potential of cancer cells increases with the degree of aggressiveness of the cells. The presence of voltage-gated sodium channels (VGSCs) has been associated with the occurrence of tumourigenesis and cancer progression (metastasis) (Djamgoz and Onkal, 2013; Djamgoz *et al*., 2014; Mao *et al*., 2019). VGSCs are heteromeric membrane protein complexes. The structure of VGSC is composed of one pore-forming alpha (α) and smaller beta (β)-subunits. In total, there

are nine α-subunits (Nav1.1–Nav1.9) and four β-subunits (β1–β4) (Patel and Brackenbury, 2015). VGSCα subunits are assumed to be functionally overexpressed in various types of carcinomas (cancers of epithelial origin) such as breast (Mohammed *et al*., 2016; Dutta *et al*., 2018), small-cell lung (Onganer and Djamgoz, 2005), cervical (Diaz *et al*., 2007; Hernandez-Plata *et al*., 2012; Lopez-Charcas *et al*., 2018), ovarian (Gao *et al*., 2010; Liu *et al*., 2021) and colorectal (Benhaim *et al*., 2014; Guzel *et al*., 2019) cancers.

The expressions of Nav1.5 (encoded by *SCN5A*) and its alternative splice variant, neonatal Nav1.5 (nNav1.5) are known to potentiate metastasis in breast (Fraser *et al*., 2005; Brackenbury *et al*., 2007; Yang *et al*., 2012; Nelson *et al*., 2015), colorectal (Guzel *et al*., 2019) and ovarian (Liu *et al*., 2021) cancer types. The up-regulation of such alternative splice-variant in breast cancer portrays oncofoetal gene expression since nNav1.5 would generally be expressed only during the foetal stage of human development (Ben-Porath *et al*., 2008; Yamaci *et al*., 2017). The augmented expression of nNav1.5 in the progression of breast cancer metastasis has been highlighted in several past studies. Among the earliest discovery that led to the regarded postulation was by Chioni *et al*. (2005). The study generated an anti-peptide polyclonal antibody, named NESOpAb, which specifically recognised 'neonatal' but not 'adult' Nav1.5. The antibody was then utilised by Fraser *et al*. (2005) to portray the augmented expression of nNav1.5 in human breast cancer biopsy via immunohistochemical staining. The study added that the expression of nNav1.5 is associated with the presence of lymph node metastasis.

Brackenbury *et al*. (2007) conducted a comprehensive study to highlight the effects of blocking nNav1.5 upon the migration and invasion ability of metastatic breast cancer cells, MDA-MB-231. The attenuation of nNav1.5`s functionality in MDA-MB-

231 cells was performed based on two different approaches. One was using ribonucleic acid (RNA) interference against the nNav1.5 sequence, and the other one was using NESOpAb. From both approaches, it was reported that the migration and invasion capacity of the cells was significantly reduced as compared to the control (Brackenbury *et al*., 2007). Furthermore, the study discovered that migrating breast cancer cells have a higher fold of nNav1.5 expression on the plasma membrane than non-migrating cells. This was achieved using confocal immunocytochemistry with NESOpAb (Brackenbury *et al*., 2007). An extensive study by Yamaci *et al*. (2017) revealed that nNav1.5 is significantly overexpressed in breast cancer tissues, which agrees with the previous studies by Fraser *et al*. (2005) and Brackenbury *et al*. (2007). However, the study by Yamaci *et al*. (2017) also reported the presence of nNav1.5 immunoreactivity in normal breast tissue. This was a novel finding where such a statement has never been reported previously (Yamaci *et al*., 2017).

Another critical aspect of the paper is the postulation on the association between the absence of oestrogen receptor-α (ERα) and the over-expression of nNav1.5 (Yamaci *et al*., 2017). It was inferred that the absence of ERα might influence the functional expression of nNav1.5. This resonates with the previous findings conducted using the MDA-MB-231 cell line, which is a TNBC cell line that does not express ER, progesterone receptor (PR) and HER2 (Dai *et al*., 2018). Studies by Brackenbury *et al*. (2007) and Mokhtar *et al*. (2019) reported the elevated expression of nNav1.5 in the highly metastatic MDA-MB-231 cell line as compared to a less aggressive, triple-positive cell line, MCF-7. These findings are significant as well to associate the expression of nNav1.5 with the metastatic capacity of breast cancer cells.

Over the years, the progress in studies highlighting the role of nNav1.5 in breast cancer metastasis has been well structured, from fundamental ideas using cell lines to

animal models and human breast biopsies only. However, other biological human samples such as whole blood and serum remain unexploited to investigate the expression of this neonatal channel. In addition, these previous studies have only focused on the molecular expression of nNav1.5 in breast cancer and not the immunogenicity of the protein itself.

Breast cancer immunotherapy is a developing field that received enormous attention in recent days (Emens, 2018; Makhoul *et al*., 2018; Feng *et al*., 2020). It involves the use of potential biomarkers such as antigens and antibodies to provide an intervention for the progression of breast cancer. The Food and Drug Administration (FDA) has approved atezolizumab, in combination with paclitaxel, in the year 2019, as a potential treatment for adult patients with unresectable locally advanced or metastatic TNBC, whose tumours express programmed death-ligand 1 (PD-L1) (Narayan *et al*., 2020). Atezolizumab is a monoclonal antibody that acts as an immune checkpoint inhibitor to block the interaction between PD-L1 with programmed cell death protein 1 (PD-1) (Basile *et al.,* 2018). By considering a similar concept in the case of PD-L1, it is assumed that nNav1.5 might possess promising potential as a rational target or immune checkpoint marker in preventing breast cancer metastasis. However, to achieve this goal, the fundamental immunogenicity of nNav1.5 must be investigated beforehand.

First of its kind, the current research has attempted to study the immunogenicity of nNav1.5 with regard to breast cancer metastasis. The presence of circulating nNav1.5 antigen and antibodies against nNav1.5 could serve as prognostic biomarkers for metastatic breast cancer. The study was carried out through two crucial experiments: a preclinical study using 4T1 orthotopic breast cancer mice model and a clinical study using whole blood and serum samples from breast cancer patients.

1.2 Problem statement

The fundamental knowledge on the natural immunogenicity of nNav1.5 remains unanswered. Based on laboratory procedures, the production of polyclonal antibodies, NESOpAb against nNav1.5 has been designed based on the sequence of six amino acid differences that allow the characterisation of Nav1.5 and its neonatal isoform (Chioni *et al*., 2005). Nav1.5 D1:S3 splicing is unique where the 5`-exon form has 31 nucleotide differences compared to the 3`-exon form, thus resulting in the seven amino acid substitutions (Figure 1.1).

'Adult' Nav1.5 (3`)	Y T T E F V D L G N V S A L R T F R V L R A L K T I S V I S
nNav1.5 (5`)	Y **V S** E **N I K** L G N **L** S A L R T F R V L R A L K T I S V I **P**

Figure 1.1 Deduced amino acid sequences of Nav1.5 exon 6 alternatives, with changed amino acids highlighted in red fonts.

The NH2-VSENIKLGNLSALRC-amide (termed as 'NESO') was synthesised and introduced into a rabbit model to produce NESOpAb (Chioni *et al*., 2005). However, no attempt has been made to detect naturally occurring antibodies against nNav1.5 produced by the immune system itself in relation to breast cancer metastasis. The detection of antibodies against nNav1.5 (hereafter referred to as anti-nNav1.5-Ab), with respect to breast cancer metastasis could explain the immunogenicity of nNav1.5.

To date, the limited number of studies conducted on nNav1.5 have shown dependency on the use of *in vitro* studies and breast cancer tissues or biopsies. The retrieval of breast cancer tissues is highly invasive and expensive. Therefore, a more practical choice of biological samples is necessary as a replacement for breast cancer tissues to study the regulation of nNav1.5 expression in breast cancer metastasis.

Therefore, we decided to use whole blood and serum samples in this study as reliable alternatives for evaluating nNav1.5 expression and its antibodies. Since metastasising breast cancer cells may carry nNav1.5 antigen via the lymphatic and blood circulation system, it is relevant to measure the presence of circulating nNav1.5 antigen in whole blood samples and detect the presence of anti-nNav1.5-Ab in the serum of breast cancer patients. The novelty of this study is preserved, in the sense, that there has not been any study published on the detection of circulating nNav1.5 antigen and anti-nNav1.5-Ab in whole blood and serum samples, respectively.

1.3 Scope of study

This study has attempted to decipher the immunogenicity of nNav1.5 pertaining to breast cancer metastasis. nNav1.5, the alternative splice variant of Nav1.5, has been known to promote metastasis in breast cancer via the influx of sodium ions (Na+) (Gillet *et al*., 2009; Brisson *et al*., 2011). An important element of the study was the use of alternative biological samples such as whole blood and serum to replace breast cancer tissues to study the presence of nNav1.5 and its antibodies.

The validation of the presence of anti-nNav1.5-Ab with respect to breast cancer metastasis is crucial as a building block to decode the immunogenicity of nNav1.5. The study was carried out in two settings. The first setting was a preclinical study that involved the development of 4T1 orthotopic breast cancer mice model by introducing 4T1 cells to the mammary fat pad of BALB/c mice. The use of an *in vivo* model grants the advantage of observing the extent of metastasis as nNav1.5 is strongly associated with breast cancer metastasis. The presence of nNav1.5 within the 4T1 murine mammary cancer cells (TNBC cell line) was previously validated by Mokhtar *et al*. (2019), making it ideal to be incorporated in the present study. The whole prospect of

the *in vivo* model served as a good starting point to investigate the expression of circulating nNav1.5 and anti-nNav1.5-Ab in the whole blood and serum, respectively.

The second setting was a clinical study that involved the participation of breast cancer patients and healthy participants. Whole blood and serum were withdrawn from healthy participants (without breast cancer or any form of cancer) and breast cancer patients with various stages of diagnosis, subtypes, and treatment status. The main reason behind these two settings was the hypothetical factor that the blood samples retrieved from both *in vivo* models and breast cancer patients would demonstrate the presence of nNav1.5 antigen and anti-nNav1.5-Ab depending on the advancement of breast cancer metastasis. The prominent difference between the preclinical and clinical studies was the level of breast cancer metastasis.

In terms of translational approach, the 4T1 orthotopic mice model has several characteristics that make it a suitable replicate as an experimental *in vivo* model to represent the human mammary cancer. Firstly, the 4T1 tumour cells are easily transplanted into the mammary gland so that the primary tumour grows in the anatomically correct site (Pulaski and Ostrand-Rosenberg, 2001). Secondly, the 4T1 metastatic disease develops spontaneously from the primary tumour, similar to the human breast cancer (Tao *et al*., 2008; Yang *et al*., 2020). Thirdly, the progressive spread of 4T1 metastases to the draining lymph nodes and other organs is comparable to that of the human mammary cancer (Pulaski and Ostrand-Rosenberg, 1998; Yang *et al*., 2020). Fourthly, the 4T1 tumour share notable features of the human TNBC (Schrörs *et al*., 2020).

The progression of metastasis that could be achieved by the animal model has been predicted based on the metastatic capacity of the 4T1 cell line and the establishment of the model in previous studies. However, the level of metastasis varies

significantly among breast cancer patients due to the influence of breast cancer stages, subtypes, and treatment status. Since the expression of nNav1.5 upregulates breast cancer metastasis, it would be useful to understand the influence of these factors on the expression of the protein in the whole blood and its antibody in the serum. The valuable findings on the detections of nNav1.5 antigen and anti-nNav1.5-Ab from both settings were aided by the validation of the metastatic microenvironment through inspection of other parameters.

In the preclinical study, other laboratory parameters inspected for metastasis validation in the 4T1 orthotopic mice were lung metastasis clonogenic assay, histopathology and quantification of cytokines (interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF)). In the clinical study, additional laboratory analyses pertaining to metastasis profiling included the quantifications of cytokines ((I) chemokine ligand 2 (CCL2), (II) VEGF, (III) IL-6, (IV) interleukin-10 (IL-10), (V) interleukin-8 (IL-8) and (VI) tumour necrosis factor-alpha (TNF-α)) and quantification of interleukin-2 receptor subunit alpha (IL-2Rα) in the serum of participants.

1.4 Objectives of study

1.4.1 General objectives

The aim of the study was to investigate the roles of circulating nNav1.5 antigen in the whole blood and anti-nNav1.5-Ab in the serum of 4T1 orthotopic mice model and breast cancer patients.

1.4.2 Specific objectives

1.4.2(a) Specific objectives for the preclinical study

1. To develop the 4T1 orthotopic breast cancer mice model using murine mammary carcinoma-single cell line and evaluate breast cancer metastasis within the 4T1 orthotopic mice model, via the difference in relative organ weight, lung metastasis clonogenic assay, histopathological analysis, and quantification of cytokines (IL-6 and VEGF).
2. To investigate the expression of circulating nNav1.5 antigen in the whole blood of the 4T1 orthotopic mice model, using real-time polymerase chain reaction (qPCR) analysis.
3. To investigate the expression of circulating anti-nNav1.5-Ab in the serum of the 4T1 orthotopic mice model, using an in-house indirect enzyme-linked immunosorbent assay (ELISA).
4. To determine the relationship between the expressions of circulating nNav1.5 antigen and anti-nNav1.5-Ab in the 4T1 orthotopic mice model.
5. To determine the number of metastatic foci in the target organs of the 4T1 orthotopic mice model and correlate it with the expression of anti-nNav1.5-Ab.

6. To identify the relationship between the concentration of cytokines (IL-6 and VEGF) and the expression of anti-nNav1.5-Ab in the serum of the 4T1 orthotopic mice model.

1.4.2(b) Specific objectives for the clinical study

1. To describe the sociodemographic details of the participants which include age, race, family history, marital status, breast cancer stages, breast cancer subtypes, menopausal status, education level, employment and other comorbidities.
2. To investigate the expressions of nNav1.5 antigen and anti-nNav1.5-Ab in the whole blood and serum of breast cancer patients, using qPCR analysis and in-house indirect ELISA, respectively.
3. To compare the expressions of nNav1.5 antigen and anti-nNav1.5-Ab in control (healthy participants), pretreatment and ongoing treatment breast cancer patients` groups.
4. To quantitate the concentrations of cytokines (CCL2, VEGF, IL-6, IL-10, IL-8 and TNF-α) and IL-2Rα using magnetic Luminex assay and commercial sandwich ELISA, respectively and determine their relationships with the expression of anti-nNav1.5-Ab in control, pretreatment and ongoing treatment groups.
5. To compare the expression of anti-nNav1.5-Ab in pretreatment breast cancer patients diagnosed with luminal A/B, HER2 enriched and TNBC subtypes.
6. To compare the expression of anti-nNav1.5-Ab in pretreatment breast cancer patients diagnosed with early-invasive and advanced stages.

1.5 Rationale of the study

The detection of anti-nNav1.5-Ab in the serum highlights the immunogenicity of nNav1.5, which has not been carried out by previous studies. The ability of nNav1.5 in eliciting an immune response is beneficial in promoting both nNav1.5 and anti-nNav1.5-Ab as potential prognostic markers to detect the progression of breast cancer metastasis. The recognition of circulating nNav1.5 antigen and its spontaneous antibody in the whole blood (liquid biopsy) may suggest a less invasive and convenient approach when such practice is implemented within the healthcare system. Additionally, this approach could shorten the duration and cost required for a diagnosis and is suitable for screening purposes as well. The concept of utilising liquid biopsy is a good alternative compared to the implementation of tissue biopsy which is a less cost-effective and highly invasive technique (Fici, 2019).

In terms of prognosis, the positive presence of nNav1.5 and anti-nNav1.5-Ab may serve as immunosurveillance markers to indicate the untreated progression of breast cancer metastasis. The different levels of anti-nNav1.5-Ab could also act as an indicator to monitor treatment efficacy which reflects the presence of interrupted metastasis. The positive outcome on the immunogenicity of nNav1.5 may ignite new ideas on the modulation of the concerned immunological pathways towards the application of passive immunotherapy in combatting breast cancer metastasis.

Immunotherapy is an emerging field that incorporates the element of immunology to eliminate breast cancer metastasis. Previously, an epitope of Nav1.5 was used to design monoclonal antibodies to reduce ovarian tumour volume (Gao *et al.*, 2019). By highlighting the immunogenicity of nNav1.5, we may be able to investigate the immunotherapeutic prospects of treating breast cancer patients who exhibit the nNav1.5 antigen. With the increasing percentage of breast cancer incidence

in Malaysia and Asia, other treatment options should be explored rather than depending on conventional treatment methods such as chemotherapy and targeted therapy.

The idea of investigating the presence of circulating nNav1.5 and anti-nNav1.5-Ab using whole blood may attract more women in society to come forward for screening purposes. The less invasive approach would reduce the stigma surrounding painful clinical procedures involved to confirm the presence of breast cancer. A biopsy is commonly conducted after physical examination as the gold standard to examine the presence of the disease.

In terms of the contribution to the country, the present study could assist in both the prevention and treatment of metastatic breast cancer, thus reducing mortality risk due to breast cancer amongst female citizens of Malaysia. Since the immunogenicity of nNav1.5 has not been explored on any international platforms, therefore, we believe that this study could uphold and strengthen the potential of Malaysia in the field of breast cancer immunotherapy. Economically, the detection of anti-nNav1.5-Ab in the serum, using an in-house ELISA assay could be reformed into a rapid and convenient commercial kit. Such implementation will not only provide financial benefits to the economy of the country but also reflect the productivity of Malaysian research in contributing to life science-based applications (diagnostic and research purposes).

1.6 Theoretical framework

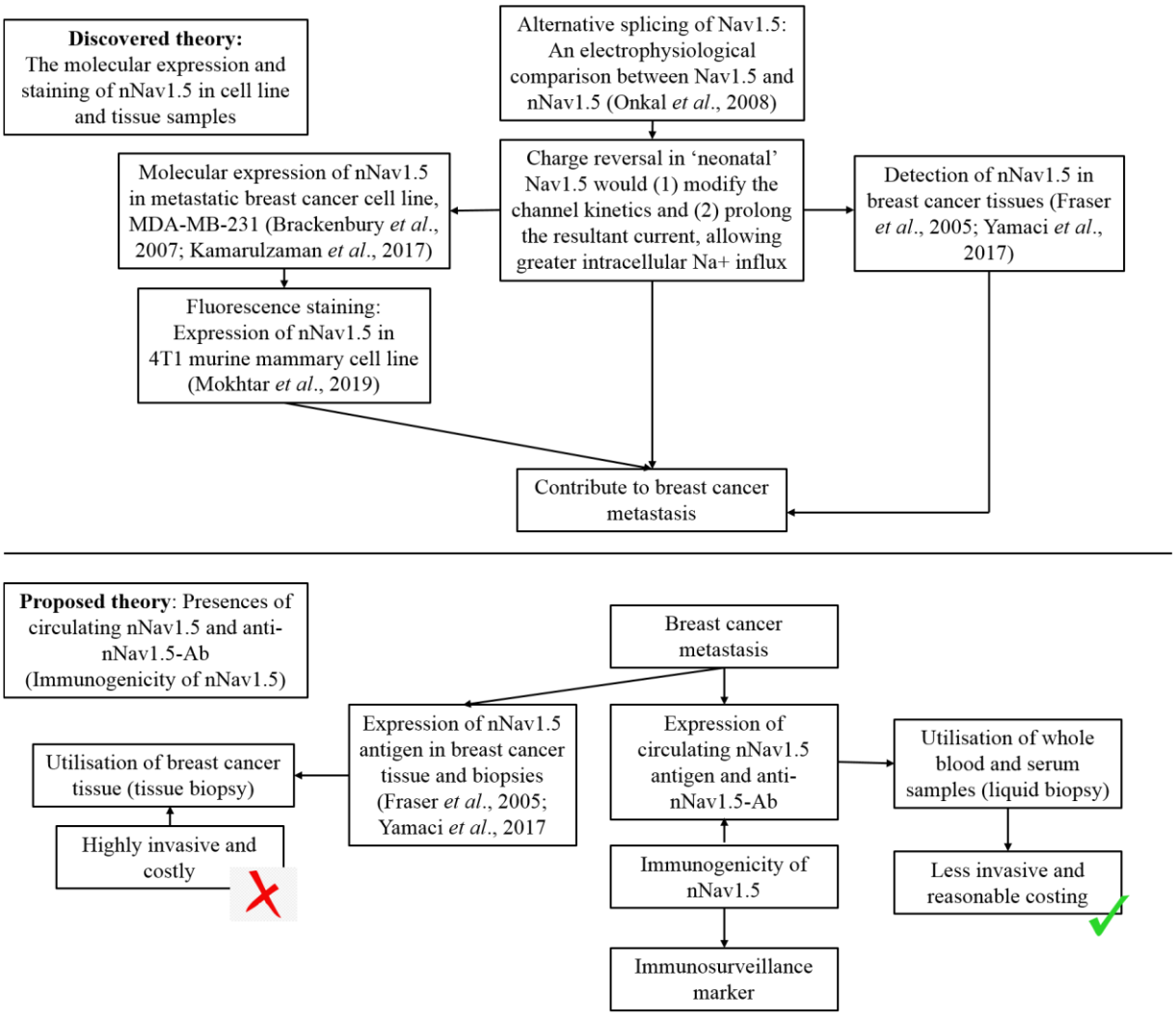

Figure 1.2 Theoretical framework of the present study (two perspectives).

1.7 Study design

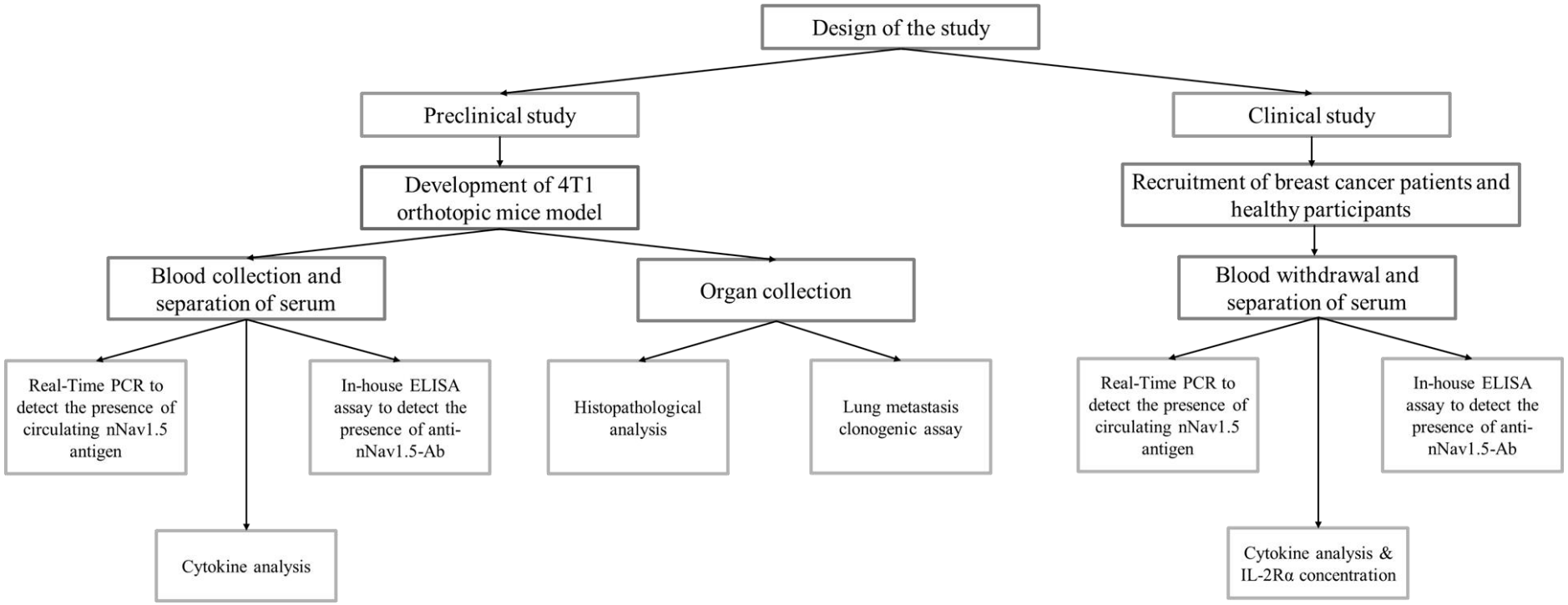

Figure 1.3 Design of the study encompassing preclinical (4T1 orthotopic mice) and clinical (samples of breast cancer patients) approaches.

CHAPTER 2

LITERATURE REVIEW

Requoting the main goals of the study which were to investigate the presences of circulating nNav1.5 antigen and its antibody, anti-nNav1.5-Ab in the blood of 4T1 orthotopic breast cancer mice model and breast cancer patients, we have identified four major literature components to establish a quadrant-axis tetralogy that serves as the main essence and driving force of the current study (Figure 2.1). These four components were a) breast cancer, b) Nav1.5 and its splice variant, nNav1.5, c) breast cancer and the immune system d) the functionality of Nav1.5 and nNav1.5 in the immune system pertaining to breast cancer. Based on each component of the tetralogy, study gaps were identified and addressed. These study gaps served as the building blocks of the current research. These study gaps will be discussed once all four components are dissected in the following subsections.

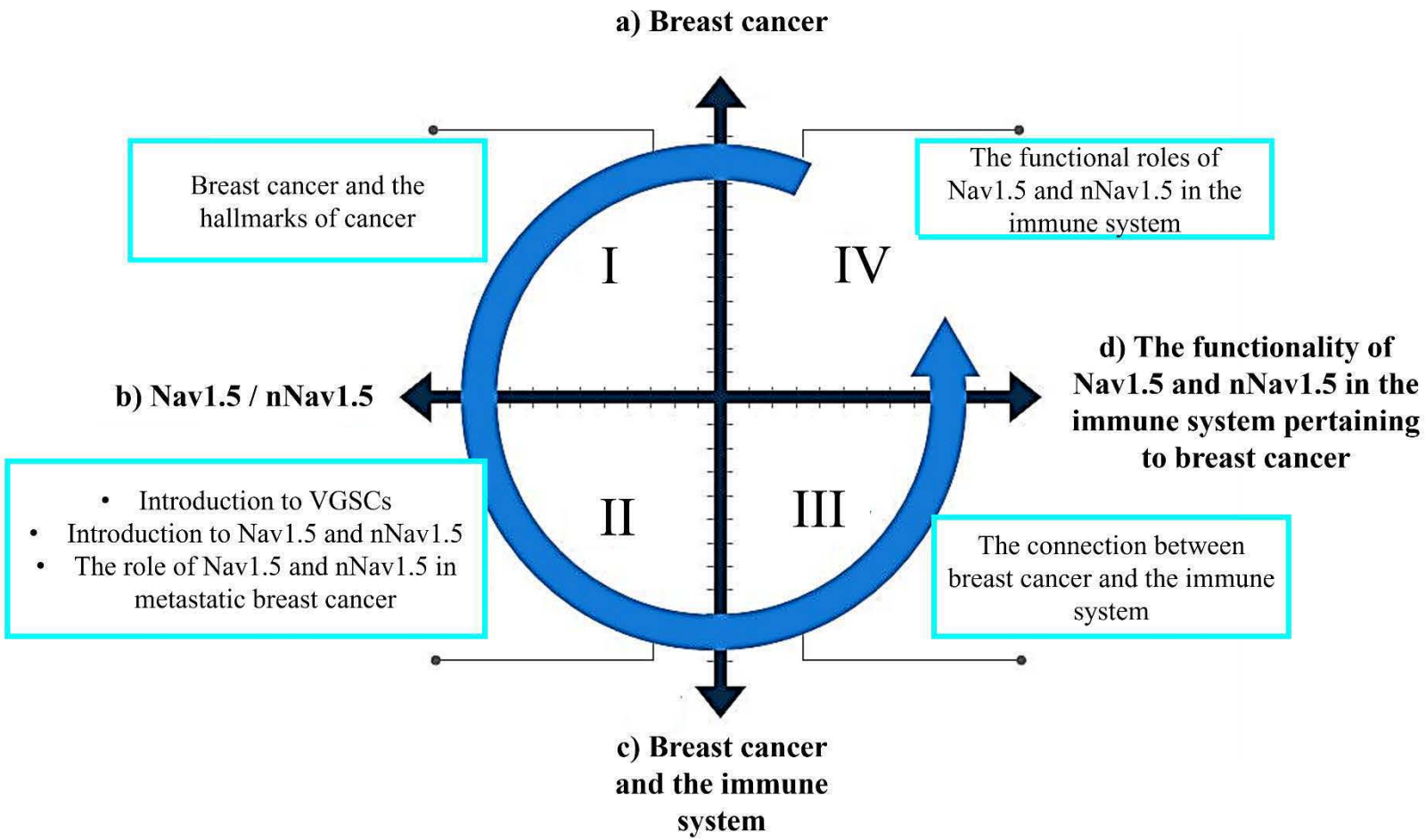

Figure 2.1 The illustration represents the four main components that serve as the fundamental building blocks of the literature review.

2.1 Breast cancer and hallmarks of cancer

2.1.1 Anatomy of female breast

The normal anatomy of the female breast includes skin, fatty tissues, fibro-glandular breast tissues (such as ducts, lobules and supporting fibrous tissue), vascular structures and ligaments. The amount of active glandular tissue is influenced by pregnancy and lactation, thus resulting in different breast sizes (Yu *et al*., 2013; Hassiotou and Geddes, 2013). Besides that, these physiological activities also influence the blood supply to the breast (Yu *et al*., 2013). Higher blood volume especially in the nipple can be observed in premenopausal women compared to postmenopausal women (Jesinger, 2014; Taroni *et al*., 2015). The arterial supply present within the breast is originated from branches of the internal thoracic, intercostal and lateral thoracic arteries (van Deventer, 2004; van Deventer and Graewe, 2016).

The venous anatomy of the breast exists parallelly alongside the arterial anatomy within the deep tissues of the breast. The superficial veins generally run to the centre and converge on the peri-areolar circular network, otherwise known as the circulus venosus of Haller (Jesinger, 2014; Borenstein and Friedman, 2020). Venous blood is drained into the internal thoracic and lateral thoracic veins. The main highlight of the breast lymphatic system is the axillary lymph node. Most of the lymph from the breast tissue goes through the pectoral or anterior lymph nodes and drains into the axillary lymph nodes (Uren *et al*., 2012; Jesinger, 2014). Deep lymphatic channels communicate with the superficial cutaneous lymphatic plexus, focusing mainly on the nipple in the subareolar plexus (Jesinger, 2014).

2.1.2 Breast cancer facts and statistics

In general, breast cancer refers to carcinoma that originates from breast tissues. Breast cancer can occur in different parts of the breast, including the ducts and lobes. The most common types of breast cancer are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Barroso-Sousa and Metzger-Filho, 2016; Cserni, 2020; Chamalidou *et al*., 2021). Breast cancer occurs regardless of gender. However, most are diagnosed among females (IARC, 2020). The development of breast cancer depends on the activation or inactivation of several types of genes. The alterations of the genes are required to promote the malignant characteristics of breast cancer. These genes may alter the normal cell cycle, which leads to the unregulated proliferation of cancerous cells (Lesicka *et al*., 2018; Wang *et al*., 2018).

Atypical ductal hyperplasia and cancer *in situ* are precursor stages that eventually develop into invasive tumours if there is no prevention introduced. The detection of metastases is crucial in determining the stages of breast cancer and appropriate treatment selection (Ingvarsson, 2001; Scully *et al*., 2012). The presence and absence of ER have played an important role in distinguishing the subtypes of breast cancer and the most effective treatment regimen, along with the expression of PR and HER2. Subtypes with the absence of all three receptors are known to have the worst prognosis such as in the case of TNBC subtypes. As the receptors are not expressed in TNBC, the hormonal and molecular targeted therapies are less effective than in other subtypes (Griffiths and Olin, 2012).

2.1.2(a) The risk factors of breast cancer

Breast cancer can be caused by various factors which include significant family history (Jannot *et al*., 2017), genetic predisposition (Shaukat *et al*., 2013), reproductive risk (Anderson *et al*., 2014), lifestyle (Kruk, 2014), and external environmental factors

(Terry *et al*., 2019). The lifetime risk of breast cancer in tumour protein p53 (TP53) mutation carriers is higher than those harbouring a breast cancer mutation 1 (BRCA1) or BRCA2. Lalloo and Evans (2012) reported that BRCA1 and BRCA2 mutations are the most identified high penetrance germline gene mutations in hereditary breast cancer, which exposes them to a higher risk of getting breast cancer (Kwong *et al*., 2020).

2.1.2(b) Symptoms of breast cancer

According to the Centers for Disease Control and Prevention (CDC) (2020), symptoms of breast cancer vary among patients, and some may not even experience any symptoms. The symptoms reported were the appearance of a new lump at the breast or armpit area, changes in the size and shape of the breast, thickening or swelling at a part of the breast, redness, and appearance of flaky skin around the nipple, pulling in of the nipple (retracted nipple), abnormal nipple discharge and the appearance of 'orange peel' skin (CDC, 2020).

2.1.2(c) Breast cancer epidemiology

Over the years, breast cancer has been one of the most commonly diagnosed forms of cancer that occurs among females worldwide. According to the latest GLOBOCAN report released by IARC (2020), breast cancer has the highest percentage of incidence (25.8%), followed by colorectal cancer (9.9%), lung cancer (8.8%) and cervical cancer (6.9%). The report added that breast cancer holds the record for the highest number of prevalent cases in 5 years (33.7%) and the number of death cases (15.6%), compared to other forms of cancer. In Malaysia, breast cancer exhibited the highest age-standardised incidence rate (ASR) accounting for up to 34.1, followed by colorectal (11.1) and cervix uteri (6.2) cancers. These figures were included in the Malaysian National Cancer Registry (MNCR) report 2012-2016 which was published

in 2019. The report added that the overall ASR per 100,000 was highest among the Chinese (40.7), followed by Indians (38.1) and Malays (31.5). A total of 13,485 cases were reported for staging purposes. Based on the staging, 47.9% of the cases were detected at the advanced stages (stage III-25.1%, stage IV-22.8%) of breast cancer. The number of advanced breast cancer cases has increased over the years, with only 43.2% reported by the MNCR report 2007-2011 (2015).

According to the annual statistics of the Breast Cancer Awareness and Research Unit (BestARi) (2020), Hospital Universiti Sains Malaysia (Hospital USM), Kubang Kerian, Kelantan, the percentage of patients diagnosed with breast cancer has increased from 4.3% of cases recorded in 2018, followed by 4.5% in 2019 and a drastic increase to 6.5% in the year 2020. Based on the statistics provided by BestARi (2020), stage IV had the highest number of cases diagnosed, compared to stages III, II, and I. Within the same year, BestARi (2020) recorded that there were 102 cases with 80% of them diagnosed as invasive breast cancer (non-special type).

2.1.3 Breast cancer classifications

2.1.3(a) Breast cancer stages

Breast cancer stages are determined based on the guidelines provided by the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (8^{th} edition) (Amin *et al*., 2017). The manual provided acts as a benchmark in classifying patients with a benign or cancerous tumour, determining patients` prognosis and the best treatment possibilities. The previous editions of the manual have only focused on the anatomical extent of the disease. The most widely used staging system among medical practitioners is the ‘tumour, node and metastasis’ (TNM) staging system which was maintained by AJCC and the International Union for Cancer Control (UICC). This system encompasses the anatomical extent of the primary tumour (T), the

involvements of regional lymph nodes (N), and the presence of distant metastases (M) (Amin *et al*., 2017).

However, the traditional approach of focusing solely on the anatomical features was diminished in the seventh (Edge and Compton, 2010) and eighth editions (Amin *et al*., 2017) of AJCC`s cancer staging manuals. Based on the eighth edition of AJCC`s cancer staging, there are three prominent categories of breast cancer which were also highlighted in the Clinical Practice Guidelines (CPG): Management of breast cancer (third edition) published by the Ministry of Health Malaysia and Academy of Medicine Malaysia (2019). These three categories were known as early breast cancer (ductal carcinoma in situ, stage I, stage IIA and stage IIB), locally advanced breast cancer (stage III) and advanced (metastatic) breast cancer (stage IV).

According to the TNM staging system (Figure 2.2), stage I does not exhibit any signs of lymph node involvement and distant metastasis. However, in stage II, the tumour size is slightly larger and metastasis may occur at the movable axillary lymph nodes. An advanced condition is observed in stage III with the presence of a tumour larger than 5 cm followed by the involvement of 4 to 9 axillary lymph nodes. Stage IV with the poorest prognosis is highlighted by the presence of a tumour that extends to the wall of the chest and the presence of distant metastasis (Figure 2.2).

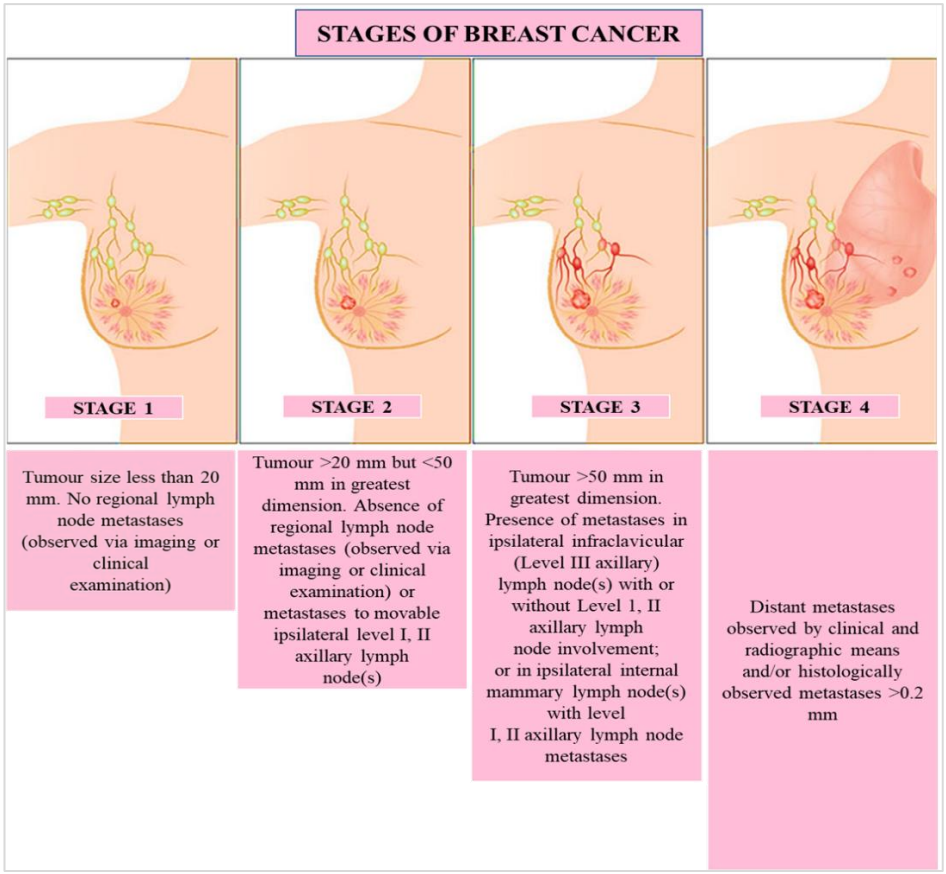

Figure 2.2 The TNM staging of breast cancer from stages I to IV.
Description: The figure was adapted from American College of Surgeons (n.d.). The information provided in the figure was retrieved from the Ministry of Health Malaysia and the Academy of Medicine Malaysia (2019).

2.1.3(b) Breast cancer subtypes

Breast cancer subtypes can be classified based on histological, molecular, and functional classifications (Malhotra *et al.*, 2010). In terms of histological classification, breast cancer cases can be categorised as either IDC or ILC (Malhotra *et al.*, 2010; do Nascimento and Otoni, 2020). Under the histopathological conduct, several characteristics are taken into strict consideration to determine whether the tumour is either more likely to be IDC or ILC. These characteristics may include cell type, number of cells, type of secretion, location of secretion, immunohistochemical analysis, and architectural features (do Nascimento and Otoni, 2020).

Molecular classification is conducted based on the intrinsic molecular subtypes of breast cancer which could be identified using gene profiling (microarray) (Sørlie *et al*., 2001; Sørlie *et al*., 2003). Initially, only six distinct subtypes of breast cancer were identified: Luminal-A, Luminal-B, HER2 enriched, basal-like, claudin-low, and normal breast-like (Malhotra *et al*., 2010).

In general, breast tumours that neither express ER, PR, nor HER2 were defined as TNBC (Zong and Pegram, 2021). TNBC subtypes frequently have a poor prognosis because conventional treatments such as hormonal therapy and HER2-targeted therapy had to be ruled out due to the absence of the corresponding receptors. Similar to the basal-like subtype, TNBC exhibits an aggressive nature compared to the other subtypes. Bertucci *et al*. (2008) highlighted that TNBC and basal-like breast cancer are not the same entity. It is essential to address that TNBC does not form a homogeneous group when analysed by gene expression profiling. In contrast, the basal-like subtype does form a homogeneous group of tumours with a similar gene expression profile related to prognosis and therapy response (Bertucci *et al*., 2008; Rakha *et al*., 2009).

The TNBC subtype was further distinguished in 2011, whereby about six different TNBC subtypes were identified. This included basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), and luminal androgen receptor (LAR) (Lehmann *et al*., 2011). An alternative classification divides TNBC into BL1 and BL2, M and LAR (Lehmann *et al*., 2016). Recently, Wang *et al*. (2019) highlighted that BL1, BL2 and IM can be further classified based on the intrinsic oncogenic changes. Despite the fact that TNBC is not a homogeneous breast cancer disease entity, a significant fraction of this subtype belongs to the basal-like tumour type (form a homogeneous group). As a result, the