INVESTIGATING THE EXPRESSION OF SOLUBLE PD-L1 (sPD-L1) OF BREAST CANCER PATIENTS USING ELISA IN HOSPITAL USM, KELANTAN

NUR AMIRA BINTI KHAIRIL ANWAR

UNIVERSITI SAINS MALAYSIA

2024

INVESTIGATING THE EXPRESSION OF SOLUBLE PD-L1 (sPD-L1)OF BREAST CANCER PATIENTS USING ELISA IN HOSPITAL USM, KELANTAN.

by

NUR AMIRA BINTI KHAIRIL ANWAR

Thesis submitted in fulfilment of the requirements
for the Degree of
Master of Science

APRIL 2024

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious, the Most Merciful. All praises to Allah SWT for giving me the opportunity to explore the knowledge and continuous endurance to accomplish this degree. First and foremost, I would like to thank my supervisor, Dr Noor Fatmawati Mokhtar for all the help, advice and guidance throughout my study. This work would not have been possible without the financial support of USM Graduate Student Financial Assistance (GRA-ASSIST) during my study as well as the Malaysian Ministry of Education through the Higher Institution Centre of Excellence (HICoE) Program (No.311/CIPPM/4401005) and Agilent Technologies (No.304/CIPPM/6150161/A146) for funding this research. I am extremely grateful to my parents and my grandmother, Aini binti Md Yusof, for constantly supporting her stubborn granddaughter's dream emotionally and financially. I would like to extend my sincere gratitude to all my postgraduate friends for sharing technical thoughts, extensive knowledge and tremendous encouragement. I would like also to thank all the laboratory staffs and office staffs from INFORMM especially Mawaddah and Amy Amilda for their helpful contributions during sample collection, their insight and assistance for this study. I would also like to thank Breast Cancer Awareness and Research Unit (BestARi) medical officers, nurses and staffs for their sincere cooperation during patient recruitment and giving me access to the patient's data. I would also like to thank En Rashid, Dr Ahmad and Harishini for their help during patient recruitment and patients' blood sample collection. I would also like to thank Sister Kalthom Hashim from Klinik Pakar Perubatan, HUSM who helped with healthy volunteers' recruitment. Lastly, my appreciation goes to all the patients and volunteers who willingly participated in this study to contribute for the betterment of precision medicine in cancer treatment.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF EQUATIONS

Page

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

℃	Degree Celsius
%	Percentage
<	Less than
>	Greater than
≥	Greater than or equal to
±	Plus-minus
=	Equal to
/	Division or ‘or’
”	Inch
e.g	exempli gratia (for example)
g	gram
h	hour
p	p-value
L	Litre
M	Molar concentration
G	Size of the hole in the needle
cm	Centimetre
mm	Millimetre
µm	Micrometre
mM	Millimolar
cm	Centimetre
mL	millilitre
nm	Nanometre
µL	Microlitre
OD	Optical density

(w/v)	Weight to volume
(v/v)	Volume to volume
ng/mL	Nanogram per millilitre
µL/well	Microlitre per well
MΩ.cm	Resistivity units of megohm-centimeters
RCF	Relative centrifugal force
min	Minute
et al.	Et alia (and others)
pH	Exponential of the concentration of hydrogen ion
CI	Confidence interval
%CV	Coefficient of variance percentage
LoD	Limit of detection
LoB	Limit of blank
SD	Standard deviation
PD-L1	Programmed cell death ligand 1
sPD-L1	Soluble programmed cell death ligand 1
mPD-L1	Membranous programmed cell death ligand 1
ELISA	Enzyme linked immunosorbent assay
IHC	Immunohistochemistry
ICI	Immune checkpoint inhibitor
mAb	Monoclonal antibody
USM	Universiti Sains Malaysia
BestARi	Breast Cancer Awareness and Research Unit
DM	Diabetes mellitus
HPT	Hypertension
JEPeM	Human Research Ethics Committee
SPSS	Statistical Package for the Social Sciences

MyScan	Malaysian study on cancer survival
MNCRR	Malaysia National Cancer Registry Report
OS	Overall survival
PFS	Progression-free survival
EFS	Event-free survival

LIST OF APPENDICES

MENGKAJI TAHAP EKSPRESI PD-L1 TERLARUT (SPD-L1) DALAM KALANGAN PESAKIT BARAH PAYUDARA MENGGUNAKAN KAEDAH ELISA DI HOSPITAL USM, KELANTAN.

ABSTRAK

Peningkatan tahap serum dan plasma PD-L1 terlarut (*soluble PD-L1* (sPD-L1)) dalam kes barah telah banyak dilaporkan. Walaubagaimanapun, data berkaitan sPD-L1 dalam kes barah payudara adalah terhad terutamanya dalam kalangan wanita Asia terutamanya etnik Melayu. Tiga objektif utama kajian ini adalah: (1) merekrut pesakit barah payudara di Hospital Universiti Sains Malaysia (HUSM) dan mengkaji kelangsungan hidup keseluruhan (OS) dengan sifat klinikopatologi dan garis dasar pesakit, (2) membangunkan asai imunoserap terangkai enzim (ELISA) yang sensitif dan spesifik menggunakan klon antibodi monoklonal (mAb) komersial, 22C3 (Dako) dan 28-8 (Abcam) untuk mengesan dan mengukur tahap sPD-L1 di dalam darah periferal dan akhir sekali (3) mengukur tahap sPD-L1 menggunakan ELISA yang dibina diikuti dengan analisis hubungkaitnya dan OS dengan ciri klinikal dalam kalangan pesakit barah payudara di HUSM. Spesimen darah diambil daripada tiga kohort pesakit barah payudara: 92 malignan, 16 benigna, dan 23 individu sihat. Kajian ini menunjukkan bahawa pesakit yang menghidapi subjenis molekular barah payudara *triple negative breast cancer* (TNBC) mempunyai OS lebih rendah berbanding pesakit yang bukan TNBC (53 bulan (± 5.4 bulan) berbanding 272.7 bulan (± 7.5 bulan), p= 0.029, ujian log pangkat). Begitu juga dengan pesakit yang dikesan menghidapi tumor lanjutan ketika diagnosis mempunyai prognosis yang lebih teruk ($p<0.001$, ujian log pangkat). Menggunakan 22C3 sebagai antibodi tangkapan, dan 28-8 sebagai antibodi pengesanan, ELISA berapit telah berjaya dibangunkan untuk mengesan dan menilai tahap sPD-L1

dalam serum dan plasma, dengan had pengesanan (LoD) 0.063 ng/mL di dalam serum dan 0.078 ng/mL di dalam plasma manusia. Tahap median serum sPD-L1 dalam kohort pesakit malignan dan benigna adalah lebih tinggi berbanding kohort peserta sihat (12.50 ng/mL berbanding 13.97 ng/mL berbanding 8.75 ng/mL, $p<0.05$). Nilai optimum serum sPD-L1 untuk meramalkan perkembangan penyakit adalah 8.84 ng/mL. Tahap peningkatan serum sPD-L1 adalah berkait rapat secara signifikan ($p<0.05$) dengan umur kitaran haid pertama, etnik, penggunaan kawalan kelahiran, komorbiditi, dan status HER2. Analisis multivariat menunjukkan umur kitaran haid pertama dan kawalan kelahiran pula adalah dua faktor bebas yang memberi kesan pada tahap sPD-L1. Walaubagaimanapun, perbandingan OS di antara pesakit yang mempunyai tahap sPD-L1 tinggi berbanding rendah adalah tidak signifikan (266.3 bulan (± 9.3 bulan) berbanding 60.0 bulan (± 3.3 bulan), $p=0.647$, ujian log pangkat). Hubungkait di antara tahap sPD-L1 dalam serum dengan ekpresi tisu PD-L1 juga adalah tidak signifikan ($p= 0.275$, *U-test*). Kesimpulannya, peningkatan tahap sPD-L1 berkait rapat dengan pelbagai ciri klinikal maka kajian lebih lanjut diperlukan untuk memahami hubungkait yang terlibat antara tahap sPD-L1 dalam diagnostik dan prognostik pesakit barah payu dara.

INVESTIGATING THE EXPRESSION OF SOLUBLE PD-L1 (SPD-L1) OF BREAST CANCER PATIENTS USING ELISA IN HOSPITAL USM, KELANTAN.

ABSTRACT

There are limited data on soluble PD-L1 (sPD-L1) in breast cancer, particularly those involving Asian (Malaysian) women, despite the fact that increased serum and plasma levels of sPD-L1 have been observed in numerous malignancies. This study was designed to achieve three aims: (1) to recruit breast cancer patients at Hospital University Sains Malaysia (HUSM) and examine the overall survival (OS) with clinicopathological properties and patient baseline, (2) to develop a sensitive and specific enzyme-linked immunosorbent assay (ELISA) using commercialised PD-L1 monoclonal antibody clones (mAb), 22C3 (Dako) and 28-8 (Abcam) for sPD-L1 detection and measurement in human peripheral blood , and finally (3) measure sPD-L1 level using the developed ELISA followed by analyse its correlation and OS with clinical characteristics in breast cancer patients at HUSM. Blood specimens were obtained from three cohorts of breast cancer patient: 92 malignant, 16 benign and 23 healthy controls. Our study demonstrated that triple negative breast cancer (TNBC) molecular subtype have lower OS than the non-TNBC (53 months (SD 5.4 months) vs 272.7 months (SD 7.5 months), p= 0.029, log-rank test). Similarly, patients presenting with advanced tumour staging at diagnosis has poorer prognostic ($p<0.001$, log-rank test). Using 22C3 as the capture antibody, and clone 28-8 as the detection antibody, a sandwich ELISA was successfully developed with the limit of detection (LoD) of 0.063 ng/mL in human serum and 0.078 ng/mL in human plasma. The median serum sPD-L1 concentration of malignant and benign patient cohorts was significantly elevated

compared to the healthy cohorts (12.50 ng/mL vs 13.97 ng/mL vs 8.75 ng/mL, $p<0.05$). Optimal cut-off value of serum sPD-L1 for this study was 8.84 ng/mL. Significant association existed between elevated serum sPD-L1 levels and menarche age, ethnicity, birth control usage, comorbidity and HER2 status ($p<0.05$). Menarche age and birth control were identified as independent variables impacting sPD-L1 level by multivariate analysis. However, the OS for patients with high vs low sPD-L1 level was not significant (266.3 months (SD 9.3 months) vs 60.0 months (SD 3.3 months), $p=0.647$, log-rank test). Additionally, there was no discernible correlation between tissue PD-L1 and serum sPD-L1 levels ($p= 0.275$, U-test). Elevated blood levels of sPD-L1 were strongly related with a number of clinical traits, and this relationship justifies the need for additional research for diagnostic and prognostic of breast cancer patients.

CHAPTER 1

INTRODUCTION

On the basis of mechanisms of interaction between PD-1 and PD-L1 decreasing immune monitoring and boosting tumour growth, PD-L1 emerged as a crucial protein for tumour immune evasion (Han *et al*., 2020). According to clinical evidence, the presence of this protein in tumour tissue suggests a potential response to immune checkpoint inhibitors (ICIs), such as the PD-1 inhibitors pembrolizumab (Keytruda), cemiplimab (Libtayo), and nivolumab (Opdivo), and the PD-L1 inhibitors atezolizumab (Tecentriq), avelumab (Bavencio), and durvalumab (Imfinzi), where several immunohistochemistry (IHC)-based tissue PD-L1 companion diagnostics have been approved and tied to different ICIs (Vaddepally *et al.*, 2020; Twomey and Zhang, 2021). Currently, 4 approved companion diagnostics for PD-L1 ICIs have been approved, including: (1) *PD-L1 IHC 22C3 pharmDx* kit (Dako, North America) is for pembrolizumab (Keytruda) and cemiplimab (Libtayo) treatments in triple negative breast cancer (TNBC), non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), cervical cancer, esophageal squamous cell carcinoma (ESCC); (2) *PD-L1 IHC 28-8 pharmDx* kit (Dako, North America) is for nivolumab (Opdivo) treatments in NSCLC; (3) *Ventana PD-L1 (SP142) Assay* (Ventana Medical Systems) is for atezolizumab (Tecentriq) treatments in NSCLC and urothelial carcinoma; (4) *Ventana PD-LI (SP263) Assay* (Ventana Medical Systems) is for atezolizumab (Tecentriq) treatments in NSCLC (US Food & Drug Administration, 2023; Twomey and Zhang, 2021).

Recently, numerous solid tumours have been observed to have high levels of the soluble form of PD-L1 (sPD-L1) in peripheral blood, including plasma and serum.

(Katongole *et al*., 2022; Lu *et al*., 2022; Mahoney *et al*., 2022; Shiraishi *et al*., 2022; B. Han *et al*., 2021; Cho *et al*., 2020; Ding *et al*., 2020; Buderath *et al*., 2019; Y. Li *et al*., 2019a).

Similar to tissue PD-L1, increased sPD-L1 also associates with poor clinical outcome in various cancer types including esophageal squamous cell carcinoma (Shiraishi *et al.*, 2022b), breast cancer (X. Li *et al*., 2022; B. Han *et al*., 2021; Yazdanpanah *et al*., 2021; Y. Li *et al*., 2019a), HNSCC (Theodoraki *et al.*, 2018), large B-cell lymphoma (Cho *et al.*, 2020), melanoma (Zhou *et al.*, 2017), pancreatic (Park *et al.*, 2019) and ovarian cancers (Buderath *et al.*, 2019). Interestingly, investigations also show that high tissue PD-L1 expression is highly correlated with high sPD-L1 levels in certain of these malignancies. (Shiraishi *et al.*, 2022a). Nevertheless, tissue PD-L1 remains the gold standard for companion diagnostics. The one potential usage of sPD-L1 which is currently being explored is to utilize it as biomarker to monitor treatment response towards PD-L1 immunotherapy or any other cancer treatments. sPD-L1 offers better insight on treatment response, particularly for those receiving ICIs, simply because it is the same biomarker tested during predictive tissue PD-L1-IHC. This is in contrast to other serum cancer biomarkers like carcinoembryonic antigen (CEA), cancer antigen 15-3 (CA 15-3), and cancer antigen 27.29 (CA 27.29), which are tested in breast cancer to monitor treatment response (Anoop *et al.*, 2022).

As of now, there are many commercialised sPD-L1 ELISA kits in the market for research purposes such as *Human PD-L1 Simple Step ELISA Kit #ab214565* (Abcam, UK), *PDCD1LG1 ELISA kit* (USCN Life Science, Wuhan, China) and other self-developed sPD-L1 ELISA. Like IHC-PD-L1 companion diagnostics, each ELISA-PD-L1 kit uses different antibody clone of commercialised anti-PD-1/PD-L1: pembrolizumab (Keytruda) and cemiplimab (Libtayo) uses clone 22C3 (Dako North

America, Inc.), nivolumab (Opdivo) uses clone 28-8 (Dako North America, Inc.), atezolizumab (Tecentriq) uses either clone SP142 (Ventana Medical Systems) or SP263 (Ventana Medical Systems) for detection (US Food & Drug Administration, 2023; Twomey and Zhang, 2021). Therefore, each kit has a different limit of detection and cut-off value for sPD-L1 level in liquid biopsy, thus giving an unstandardized reading of sPD-L1. Furthermore, different cancer types, different molecular subtypes or different patient populations would also demonstrate different cut-off values for sPD-L1. For instance, the plasma cut-off for sPD-L1 in 208 Chinese patients with breast cancer is 8.774ng/mL (Han *et al.*, 2021) whereas in 66 TNBC patients from China has cut-off value of serum sPD-L1 227.7 pg/mL (Li *et al.*, 2019). In other studies, 20 NSCLC patients from Italy had serum cut-off values of 27.22pg/mL while 115 patients from Serbia had plasma sPD-L1 cut-off of 250 ng/L (Jovanovic *et al.*, 2019; Castello *et al.*, 2020).

This study focused on developing ELISA-based sPD-L1 assay utilizing FDA-approved antibodies, 22C3 and 28-8 and applied the developed assay in the measurement of sPD-L1 level in Malaysian breast cancer patients at Hospital Universiti Sains Malaysia, Kelantan. From the data collected, sPD-L1 levels in these patients were analysed for its association with the patient's baseline and clinicopathological features.

1.1 Objectives of study

This study was designed to develop an ELISA-based sPD-L1 assay and measure sPD-L1 level in the peripheral blood of breast cancer patients in Kelantan, Malaysia, at Hospital Universiti Sains Malaysia, Kelantan.

The specific objectives are as follow:

1. To recruit breast cancer patients from BestARi, HUSM and analyse patient's clinicopathological properties correlation with overall survival (OS),
2. To develop and optimize sandwich ELISA using commercialized PD-L1 mAb clones, 22C3 (Dako) and 28-8 (Abcam) for sPD-L1 detection and measurement in human peripheral blood,
3. To measure sPD-L1 level using the developed ELISA followed by analyse its correlation and overall survival (OS) with clinical characteristics in breast cancer patients at HUSM.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Breast cancer

The term "breast cancer" refers to a particular type of cancer that develops from breast tissue, most frequently from the lobules that feed the milk ducts with milk or the inner lining of the milk ducts (Łukasiewicz *et al.*, 2021). It is the most prevalent cancer and the main reason for cancer-related deaths in women around the world (Sung *et al.*, 2021). According to the Global Cancer Statistic (2020), International Agency for Research on Cancer (IARC), there has been a surge in new cases of female breast cancer (2.3 million new cases (11.7%)) surpassing lung cancer (11.4%)

According to the Global Cancer Statistic (2020), published by the International Agency for Research on Cancer (IARC), there has been an increase in female breast cancer diagnoses (2.3 million new cases, or 11.7%), which now outnumber lung cancer (11.4%) (Sung *et al.*, 2021). Together with lung and colorectal cancers, breast cancer is currently one of the top three cancer types and accounts for one third of cancer incidence and mortality globally (Sung *et al*., 2021; Bray *et al*., 2018). In the most industrialized parts of the world, the relative incidence of breast cancer is greater (>80 per 100,000, for example, Belgium had the highest incidence rate of breast cancer with 113.2 per 100,000) (Sung *et al.*, 2021). Even when incidence of breast cancer in the low- and middle-income countries is not as high as the developed regions, however, women living here has lower survival rate of breast cancer (>15.0 in 100 000, e.g., Barbados had the highest mortality rate with 42.2 per 100 000) (Sung *et al.*, 2021). Higher incidence of breast cancer is associated with complex interaction between various modifiable and non-modifiable risk factors. Non-modifiable risk factors include female

gender, increasing age, family history of breast cancer, reproductive factors (such as early menarche age and late age at menopause) and high breast density. Modifiable risks include reproductive factors (nulliparity, lack of breastfeeding and older age at first live birth), hormonal factors (usage of oral contraceptives and hormonal replacement therapy), lifestyle (obesity, tobacco use and harmful use of alcohol) and history of radiation exposure (Ministry of Health Malaysia, 2019; Admoun and Mayrovitz, 2022). Asian women, particularly those who reside in low-middle income nations, are more likely to die from cancer due to the high incidence of advanced stage cancer and the absence of proper diagnosis and therapies (Rivera-Franco and Leon-Rodriguez, 2018).

2.2 Breast cancer epidemiology in Malaysia

In Malaysia, the incidence of breast cancer is recorded at 8418 (17.2%) among females and second most cause of death by cancer with 3503 (11.9%) deaths in 2020, (Sung *et al*., 2021; International Agency for Research in Cancer, 2020). According to report from the Malaysia National Cancer Registry Report (MNCRR) 2012-2016, there were around 34 women diagnosed with breast cancer for every 100,000 people between 2012 and 2016, up from roughly 31 women between 2007 and 2011 (National Cancer Institute, 2019). Breast cancer incidence grew from 17.7% percent in the previous 5-year cancer report (2007-2011) to 19.0% in the most recent 5-year cancer report (2012-2016), being breast cancer Malaysia's most prevalent cancer. The peak age-specific incidence rate of breast cancer increased from 55-59 years old in 2007-2011 to 60-64 years old in 2012-2016, according to the most recent 5-year cancer report (National Cancer Institute, 2019). Given that Malaysia is a multiethnic country with three major ethnic groups—Malaysians, Chinese, and Indians—the Chinese had the highest incidence of instances, with around 41 women per 100,000 women, followed by Indians

(about 38 women per 100,000), and Malays (about 32 per 100,000) (National Cancer Institute, 2019). Among women's cancer type, breast is the second highest 5-year relative survival after corpus uteri (70.6%). The Malaysian study on cancer survival also known as MyScan has reported the overall 5-year relative survival rate was 66.8% which is comparatively lower to neighboring country Singapore with 80.3% survival rate.

Women with breast cancer typically put off receiving treatment in Malaysia, where they finally present with more advanced stages of the disease (52.2%) (M. M. Tan *et al*., 2023; Saxena *et al*., 2012) where studies have shown this delay is associated with beliefs that screening is only necessary when experiencing cancer symptoms, anxiety to attend breast cancer screening, negative attitude towards screening and treatment, false-negative diagnostic test and alternative therapy (M. M. Tan *et al*., 2023; Norsa'adah *et al*., 2011). Reports also shown the Malaysia breast cancer 5-year relative survival rates is lower in advanced stage (23.3%) as compared to other stages of breast cancer (Stage 1 87.5%, Stage II 80.7% and Stage III 59.7%) (Yip, Taib and Mohamed, 2006; National Cancer Registry Ministry of Health Malaysia, 2018; Mujar *et al.*, 2022). MyScan also reported Chinese women having the highest relative survival of 76.5% followed by Indian women (70.5%) and Malay women (57.9%). This data is obtained between the period of diagnosis 2007-2011 and followed up to 2016 in Malaysia (National Cancer Registry Ministry of Health Malaysia, 2018). Generally, the TNBC subtype has lower survival rate as compared to the other molecular subtypes (B. Han *et al*., 2021; Fallahpour *et al*., 2017) which is coherent with study by (Abdul Aziz, Md Salleh and Ankathil, 2020) with cumulative 5-year OS of 76.3%. The 5-year survival rate for breast cancer patients who chose not to receive standard cancer therapy was 43.2% (95% CI: 32.0-54.4%), which was lower than the rate for those who did (81.9%

(95% CI: 76.9-86.9%) (Joseph *et al.*, 2012). In Malaysia, breast cancer patients taking treatment at the private sector has better survival as compared with government hospital (71.6% vs. 86.8%, $p<0.001$), where most patients going to public hospital were of older age, presented with advanced stage and needing mastectomy and chemotherapy as standard treatment (Kong *et al.*, 2017). Meanwhile in New Zealand, stage at diagnosis, type of therapy and ethnicity were the contributors for survival disparities betwwen public and private healthcare facilities (Tin *et al.*, 2016).

2.3 Breast cancer treatment options

Breast cancer treatment choices are varied because it is a multidisciplinary disease, but they can be categorized into local and systemic treatments as illustrated in **Table 2.1**. The two most common local treatments are surgery and radiation. Breast surgery procedures are either breast-conserving surgery, breast reconstruction surgery (lumpectomy) or complete breast removal (mastectomy) (Schnitt, Moran and Giuliano, 2020). Radiation therapy involves giving high-energy radiation to the chest wall, the entire breast or a portion of the breast, and the nearby lymph nodes (Boyages, 2017). Systemic therapies are chemotherapy and radiotherapies, whilst a more specific breast cancer treatment such as molecular targeted therapies include endocrine therapy, anti-HER2 therapy, immunotherapy (Burguin, Diorio and Durocher, 2021; Debela *et al.*, 2021).

Chemotherapy was once believed to be the treatment of choice for all cancers, but it is no longer the sole treatment option for some diseases. Cytotoxic systemic chemotherapy is sometimes compared to "carpet bombing" in modern warfare, where the aim is to destroy the foreign invasion of cancer regardless of collateral damage and

it has not eliminated all cancer cells with the predicted level of effectiveness (Behranvand *et al.*, 2022). The main method for treating hormone receptors (ER and

Table 2.1 List of current breast cancer treatment options.

Local treatment	**Systemic treatment**
Breast conserving surgery Mastectomy or Lumpectomy	Chemotherapy (could be given as adjuvant or neoadjuvant): • Cyclophosphamide, • Taxane (paclitaxel and docetaxel) and • Anthracycline (doxorubicin, daunorubicin, epirubicin, and idarubicin) Molecular targeted therapies: Endocrine therapy (for hormonal receptor postive subtypes): • Estrogen receptor modulators (SERMs) (tamoxifen, toremifene, bazedoxifene, and raloxifene), • Selective modulators estrogen receptor degraders (SERDs)(fulvestrant and elacestrant), and • Aromatase inhibitors (AIs) (steroidal, exemestane, and non-steroidal, letrozole and anastrozole) Anti-HER2 therapy (HER2-positive subtypes): • Antibodies targeting HER2: trastuzumab (Herceptin), Pertuzumab (Perjeta) • Tyrosine Kinase Inhibitors (TKIs): lapatinib, neratinib, or pyrotinib • Antibody-drug conjugates (ADCs): Trastuzumab-emtansine (T-DM1) PARP (poly-(ADP-ribose) polymerase protein) inhibitors: olaparib, talazoparib, veliparib, and rucaparib
Radiation	Immunotherapy: • Targeted antibodies: Anti-PD-L1 (atezolizumab and durvalumab), anti-PD-1 (pembrolizumab), anti-CTLA-4 (tremelimumab) • Vaccines: Personalized peptide vaccination (PPV), PVX-410

PR) positive invasive breast cancer is endocrine therapy. The purpose of this treatment is to selectively target either the estrogen production through aromatase inhibitors or by targeting the ER directly through SERMs (selective estrogen receptors modulators) and SERDs (selective estrogen receptors degraders) (Howlader *et al.*, 2014). For hormone receptor-negative patients but HER2-positive patients, overexpression of HER2 has poorer prognosis in comparison to other molecular subtypes. Trastuzumab and other HER2-targeting drugs are therefore essential to treat breast cancer patients with HER2-positive molecular subtypes (Slamon *et al.*, 1987; Ross and Fletcher, 1998).

The immune system plays a critical role in the spread of breast cancer where tumor cells avoid the T-cell mediated immune system, depreciating the immune mechanism through deregulation of T-cell activity which calls for a more stratified treatment (Chen and Mellman, 2013; Coussens, Zitvogel and Palucka, 2013).

The transition from so-called trial-and-error medicine to the idea of tailored prevention, diagnosis, and treatment has been driven by precision medicine (Offit, 2011). With the help of precision medicine, treatment response and potential remission can be improved by developing therapeutic regimens that are unique to each patient's clinical, genetic, and environmental data (Schwaederle *et al.*, 2015). Today's clinical practice heavily relies on *in vitro* examination of biological samples to provide data that will help with correct diagnosis and monitoring effectiveness of treatment. The current trend in combining diagnostics tests such as IHC in conjunction with treatments is now getting a lot of attention. They're known as companion diagnostics which have been made necessary to classify patients according to how they are expected to respond to a specific treatment and how harmful it may be (Valla *et al*., 2021; Gibson *et al*., 2015; Jørgensen, 2015).

2.3.1 The discovery of PD-1/PD-L1 upregulation in cancers

The T cell mediated immune response, is highly influenced by the regulation between positive (co-stimulatory) and negative (co-inhibitory) signals that is known as immune checkpoint (Pardoll, 2012). The programmed cell death receptor-1 (PD-1, also referred to as CD279) and its ligand, programmed cell death ligand-1 (PD-L1; also referred to as CD274 and B7-H1), are single transmembrane glycoproteins belongs to the family of CD28 receptors, under physiological conditions, maintain the immune system through a state of toleration and balancing inflammatory responses (Pentcheva-Hoang, Corse and Allison, 2009; Ceeraz, Nowak and Noelle, 2013; Jung and Choi, 2013). PD-1 can be found expressed a surface of immune cells such as activated T-cells (Ishida *et al.*, 1992), where its engagement with its ligand, PD-L1, ultimately causes immunosuppression and deregulation of T cell activation (Freeman *et al.*, 2000; Topalian, Drake and Pardoll, 2015).

Structurally, PD-1 is a type I transmembrane glycoprotein. With 55-kDa and 288 amino acids protein, PD-1 has an extracellular N-terminal domain that is IgV-like, a transmembrane domain and a cytoplasmic tail C-terminal ends, with two tyrosine bases at the intracellular domain (Schildberg *et al*., 2016; Zhang *et al*., 2004; Neel *et al*., 2003). PD-1 ligand, PD-L1, is also a type 1 transmembrane glycoprotein with 290 amino acids and 33-kDa molecular weight. It is one of the B7 family of ligands having similar structure to PD-1, where PD-L1 bind to PD-1 on the IgV-like domain and addition of IgC-like domain on the extracellular region (Sanmamed and Chen, 2014) (**Figure 2.1 (A and B)**). PD-1 can exhibit co-inhibition pathway with either PD-L1 or programmed cell death ligand-2 (PD-L2; also referred to as CD273 or B7-DC). Moreover, PD-L1 also shown evidence binding to another receptor, CD80 (B7-1)

(Sugiura *et al.*, 2019) where this PD-L1/CD80 interaction on immune cells such as antigen-presenting cells (APCs) caused a deregulation in the inhibitory effect of PD-

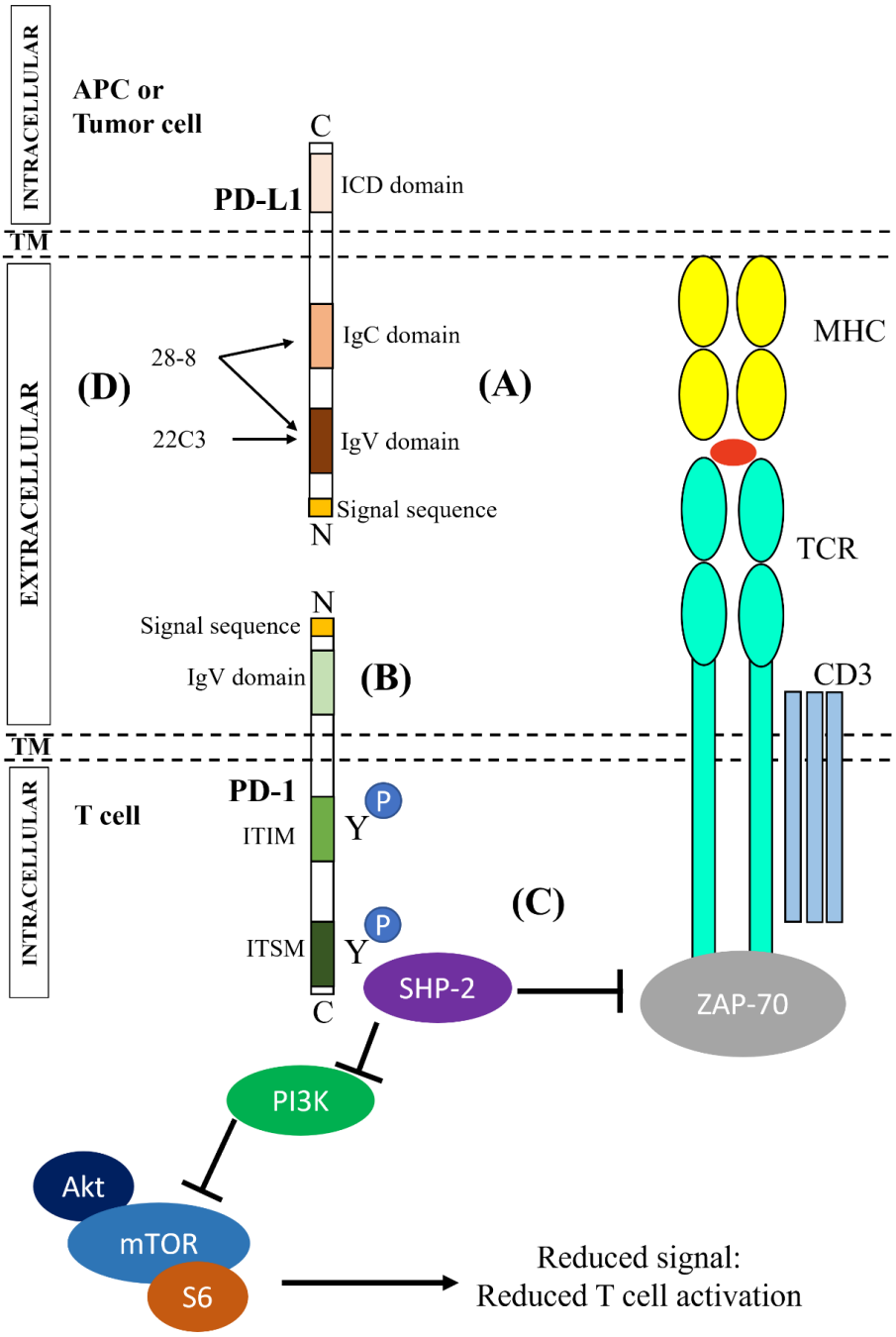

Figure 2.1 Summary of PD-1 and PD-L1 domain structure and the simplified presentation of inhibitory signalling pathway through PD-1/PD-L1 axis at T cell. (A and B) represents the domains within PD-L1 and PD-1, respectively. (C) Immune suppression pathway through PD-1/PD-L1 binding in immune cells and tumour cells. Phosphorylation of the tyrosine on the ITSM domain leads to a series of dephosphorylation to deactivate PI3K and ZAP-70 activity in T cells. (D) Binding epitopes of anti-PD-L1 clone 22C3 and 28-8 as indicated from a previous study.

L1/PD-1 in conjunction to another immune checkpoint receptors, CD80/CTLA-4. However, this out of norms interaction binding between PD-L1 and CD80 still preserved the ability of CD80 to positively impact in immune co-stimulatory receptor CD28 (Zhao *et al.*, 2019).

Co-inhibition pathway triggered by PD-1/PD-L1 interaction has a main biological function to maintain immunological tolerance and prevent autoimmune disorders, and this serve as a crucial mechanism for immune surveillance escape by inhibiting activated T cells (Kythreotou *et al.*, 2018; Lingling *et al.*, 2020). In study PD-1 expressed T cells membrane shown to intracellularly has 2 tyrosine bases known as immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM) (Patsoukis *et al.*, 2020). PD-1 co-inhibition function depends only on the phosphorylation of tyrosine at ITSM as shown in mutational studies, which preferentially recruits (Src homology region 2-containing protein tyrosine phosphatase 2) SHP-2 phosphatase at the phosphotyrosine (Yokosuka *et al.*, 2012; Patsoukis *et al.*, 2020). ITSM-recruited SHP-2 resulted in dephosphorylation of proximal signalling molecules of TCR, zeta-chain-associated protein kinase 70 (ZAP70) and LCK, with subsequent suppression of two main pathways: CD28-related PI3K/AKT (**Figure 2.1C**) and MAPK signalling pathways as shown in (Zhu and Lang, 2017; N Patsoukis *et al*., 2012; Sheppard *et al*., 2004).

This immune checkpoint regulation is often evaded by tumor cells through irregular expression of PD-L1 on the surface membrane of tumor cells. This is a known characteristic of cancer cells to avoid the body's immune surveillance (Mortezaee, 2020; Curiel et al., 2003; Dong et al., 2002). As a result, interaction between PD-1 and PD-L1 axis together with another immune checkpoint, cytotoxic T lymphocyte antigen 4 (CTLA-4), became crucial targets of cancer immunotherapy (Gong *et al.*, 2018). Since

anti-tumor immunity is suppressed throughout the progression of cancer, immunotherapies that target the immune checkpoint, PD-1/PD-L1 and CTLA-4/CD80 signaling axis have been developed to reawaken T cells and stimulate immune-mediated tumor eradication (Khair *et al.*, 2019; Hudson *et al.*, 2020).

2.3.2 PD-1/PDL-1 paved way to immune checkpoints inhibitors

Cancer immunotherapy field saw its next revolutionary wave as a result of deeper knowledge of immune surveillance, the mechanism by which innate immune cells can eliminate cancer cells. The discovery of these immune checkpoints marked a turning point in cancer immunotherapy, and the scientific community recognised this by awarding the 2018 Nobel Prizes to Tasuku Honjo of Kyoto University for discovering PD-1 and James Allison of MD Anderson Cancer Center for discovering CTLA-4. The discovery prompted the development of immunosuppressive humanized monoclonal antibodies (mAbs) (also known as immune checkpoint inhibitors (ICIs)) that target PD-1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab, avelumab, and durvalumab) to suppress tumor immune evasion (Lee, Lee and Heo, 2019) (**Figure 2.2**). More than a thousand clinical studies are presently focusing on these inhibitors, which the US Food and Drug Administration (FDA) has approved for the cancer treatment of various cancers (Nie *et al.*, 2020).

ICIs have shown promise in the clinical setting for the treatment of colorectal cancer (Shek *et al*., 2021; Overman *et al*., 2017), lung cancer (Miao *et al*., 2022; Amrane *et al*., 2020; Ready *et al*., 2019; Hellmann *et al*., 2018) and melanoma (Si *et al.*, 2019; Rossi *et al.*, 2021). Presently, the success of immunotherapy can be seen in lung cancer patients. The OS before and after the deployment of ICIs was the subject of a

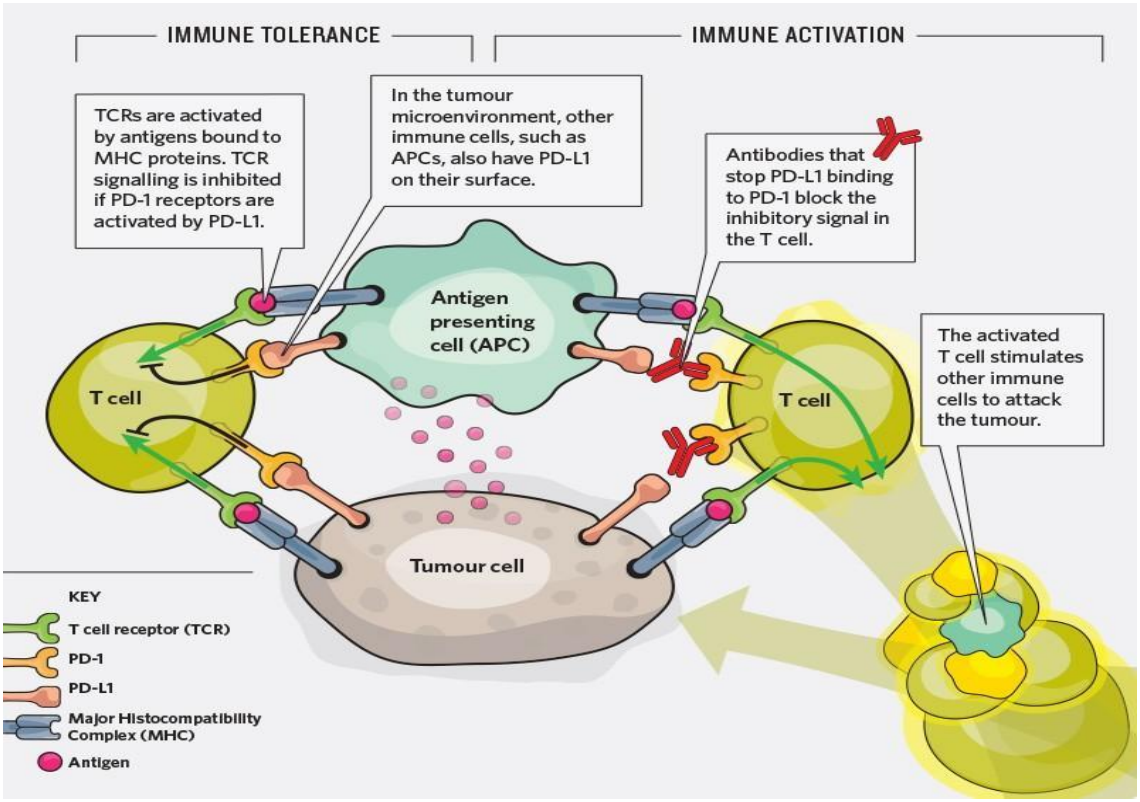

Figure 2.2 Anti-PD-1/anti-PD-L1 immunotherapy mechanism of action, where mAb blocks immune suppression signalling through the PD-1/PD-L1 axis. The figure was taken from an online website.

real-world investigation in NSCLC patients, identifying and assessing first-line ICI-treated patients treatment data has shown significant survival improvement from median OS of 7.8 months (95% CI 7.4–8.2) in pre-approval cohorts to 19.0 months (95% CI 16.0–22.0) in patients receiving ICIs cohort (Mouritzen *et al.*, 2021).

In breast cancer treatment, atezolizumab (Tecentriq®), is the first anti-PD-L1 monoclonal antibody to earn FDA approval. It has demonstrated outstanding outcomes in international Phase III trials for NSCLC and TNBC, extending the range of cancer immunotherapies (US Food & Drug Administration, 2023). Combination therapy between atezolizumab and nab-paclitaxel was essentially approved for triple negative metastatic or unresectable breast cancer in the *IMpassion130* trial, which reported the first clinically significant immunotherapy advancement in November 2018 (Schmid *et al.*, 2018).

In a PD-L1 immunotherapy treatment study involving a PD-L1-positive cohort, IMpassion*130* trial showed a good progression-free survival (PFS) benefit and minor OS benefit in this cohort (absolute median benefit of 2.5 months survival) (Mavratzas *et al.*, 2019). However, atezolizumab and paclitaxel combination did not improve PFS or OS in the PD-L1-positive cohort compared to paclitaxel treatment alone in the subsequent trial, *IMpassion131*, indicating that the trial did not achieve its primary end point of randomized controlled trials for treatment in PD-L1-positive cohort (HR, 0.82; 95% CI, 0.60-1.12; p =0.20, median PFS 6.0 months with atezolizumab-paclitaxel versus 5.7 months with placebo-paclitaxel). This trail also proves no difference in survival advantage in the PD-L1–positive (HR 1.11, 95% CI 0.76-1.64, median OS 22.1 months with atezolizumab-paclitaxel versus 28.3 months with placebo-paclitaxel in the PD-L1-positive population) nor the intention to treat population (Miles *et al.*, 2021). Following consultation with the FDA, Roche, the firm responsible for the medication,

has withdrawn the indication for atezolizumab (Tecentriq®) in combination with nab-paclitaxel (Abraxane) chemotherapy as treatment for patients with TNBC whose tumours express PD-L1 (Van Wambeke and Gyawali, 2021; Virgil, 2021). The withdrawal, however, only affects Tecentriq®'s indications for treating breast cancer in the United States. Additionally, the withdrawal has no bearing on the approval of Tecentriq® for metastatic breast cancer treatment in PD-L1-positive patients in other nations (*Genentech Withdraws Breast Cancer Indication From Tecentriq*, 2023).

One of the pioneer anti-PD-1 immunotherapy drug approved by the US FDA was pembrolizumab (Keytruda ®). Pembrolizumab was administered as a sole drug in the *KEYNOTE-086* study to treat patients with metastatic TNBC. In this trial, cohort A, where pembrolizumab was used as a second or third line of therapy for TNBC, the objective response rates were 5.7%, while in cohort B, when pembrolizumab was used as first-line therapy for TNBC patients with tumors having PD-L1-positivity, they were 21.4% (Adams, Loi, *et al.*, 2019; Adams, Schmid, *et al.*, 2019).

According to interim analysis phase 3 *KEYNOTE-355* trial, patients with advanced TNBC who had tumours that stained positive for PD-L1 with a combined positive score (CPS; the number of PD-L1-staining tumour cells, lymphocytes, and macrophages divided by the total number of viable tumour cells, multiplied by 100) of 10 or higher had a longer OS when pembrolizumab was in a combination with chemotherapy than when chemotherapy was used as placebo. Among patients with CPS >10, patients who got both pembrolizumab and chemotherapy had a longer OS of 23.0 months compared to 16.1 months for those who only received chemotherapy based on a median follow-up of 44 months (HR 0.73; 95% CI, 0.55-0.95; p=0.0185) (Cortes *et al.*, 2022).

In another clinical trial, *KEYNOTE-522*, Dr. Peter Schmid of the Barts Cancer Institute presented data showing pembrolizumab introduced to neoadjuvant and adjuvant therapy resulted in a statistically significant improvement in event-free survival (EFS) of 37%. (HR, 0.63; p=.00031). Findings from the *KEYNOTE-522* study suggest the use of pembrolizumab in combination with platinum-based chemotherapy in the neoadjuvant setting, followed by adjuvant pembrolizumab following surgery, as a new standard treatment for patients with early-stage TNBC (Jacobson, 2022).

2.4 PD-L1 immunohistochemistry (IHC) companion diagnostics

Before receiving ICIs treatment, cancer patients need to be stratified through tissue histology first known as immunohistochemistry (IHC), which remain as gold standard predictive assay till this day (Koch, 2016; Gotzsche and Jorgensen, 2013; Duraiyan *et al*., 2012). Each anti-PD-1/PD-L1 is usually co-developed with the corresponding IHC predictive assay, which is recognized as the foundation of cancer precision medicine and plays a crucial role in treatment decision-making. There are currently three IHC-based companion diagnostic tests specific for PD-L1 connected to various inhibitors that were independently developed and commercialized: (I) *PD-L1 IHC 22C3 pharmDx* (Dako North America, Inc.) indicated for Keytruda® (pembrolizumab), (II) *PD-L1 IHC 28-8 pharmDx* (Dako North America, Inc.) indicated for Opdivo® (nivolumab) in combination with Yervoy® (ipilimumab, anti-CTLA-4); (III) *Ventana PD-L1 (SP142) Assay* (Ventana Medical Systems, Inc.) indicated for Tecentriq® (atezolizumab); (IV) *Ventana PD-LI (SP263) Assay* (Ventana Medical Systems, Inc) indicated for Tecentriq® (atezolizumab) (US Food & Drug Administration, 2023).

Tissue biopsy using PD-L1 IHC assay is feasible, but the accuracy of PD-L1 as a predictor for treatment stratification is limited by the heterogeneity of PD-L1 in tumour tissues, as well as the differences in performance of the detection antibody (Zhao *et al.*, 2022). The PD-L1 antibody clones used during IHC have specific epitopes to bind to PD-L1 on cancer tissue samples. A study to understand and distinguish the specific binding sites responsible for antibody binding of PD-L1 protein has been conducted on clones 22C3, 28-8, SP142, SP263 and E1L3N by binding to recombinant PD-L1 and assessed using chemical linkage of peptides on scaffolds discontinuous epitope mapping, surface plasmon resonance, hydrogen/deuterium exchange mass spectrometry, and mutational analysis (Lawson *et al.*, 2020). Their study proves that clone SP142, SP263 and E1L3N binds in the cytoplasmic domain at the C-terminus of PD-L1 protein where SP142 and SP263 have identical binding sites (284-290aa) and E13LN binding sites does overlap SP142/SP263 binding sites but not identical. Distinctive from the other clones, 22C3 and 28-8 bind epitopes on different binding sites of the extracellular region of PD-L1 within the IgC and IgV-like region, where 22C3 binds at epitopes 196-206aa and 28-8 binding epitopes at 154-168 aa and 205-215aa as annotated on **Figure 2.1 (D)**.

2.5 The emergence of soluble PD-L1 and its clinical implications

Circulating form of PD-L1 (sPD-L1) present in some pathologies and conditions: (1) the serum and plasma of cancer patients (Liu *et al*., 2020; Y. Li *et al*., 2019; Koukourakis *et al*., 2018), (2) patients with auto-immune diseases or certain viral diseases (Jovanovic *et al.*, 2018; Du *et al.*, 2020) and (3) in pregnant women (Okuyama *et al.*, 2019). Essentially, there are two proposed ways to generate sPD-L1: either through membrane PD-L1 proteolytic cleavage PD-L1 or by alternative splicing of the

PD-L1 mRNAs and producing soluble protein devoid of the transmembrane domain (Bailly, Thuru and Quesnel, 2021).

Fibroblasts and other immune cells frequently express PD-L1 on their membranes. To promote immunosuppression in the environment, fibroblasts create more PD-L1-containing vesicles when TGF- (transforming growth factor-beta) is stimulated, enhancing immunosuppression in the environment. The expression of PD-L1, which is crucial for the regulation and upkeep of the tumor microenvironment, is impacted by the presence of tumor cells that are associated with fibroblasts (Kang *et al.*, 2020). Previous study demonstrates that sPD-L1 can be produced by proteolysis from mPD-L1 through MMP-13 (matrix metalloproteinase protein-13) specific cleavage of mPD-L1 on fibroblasts, converting it to sPD-L1. This process would limit the immune system's ability to regulate inflammation and would aggravate the inflammatory status in tissues (Dezutter-Dambuyant *et al.*, 2016). Additionally, the PD-L1 gene's alternative splicing also allows for the development of sPD-L1 (CD274). This non-proteolytic route can produce a variety of splice variants that are truncated without the transmembrane domain but have demonstrated various functions in controlling immune surveillance in various cancers (Wang *et al*., 2021; B. Gong *et al*., 2019; Ng *et al*., 2019; Zhou *et al*., 2017; Brodská *et al*., 2016; He *et al*., 2005).

Additionally, recent research has revealed that high sPD-L1 is a sign for a bad prognosis in several solid tumours (Scirocchi *et al*., 2022; B. Han *et al*., 2021; Oh *et al*., 2021). A study in Korea involving 128 patients with stage IV solid tumors (which are melanoma, non-small cell lung cancer, urothelial carcinoma and other cancers), patients with high sPD-L1 levels have poorer prognostic as compared to lower sPD-L1 levels (OS: median 7.4 months (95% CI 6.3–8.5) vs 13.3 (95% CI 9.2–17.4) months ($p = 0.005$)) (Oh *et al.*, 2021).

Prior to receiving first-line cancer therapy, sPD-L1 is an effective tumour biomarker in patients with metastatic or recurrent breast cancer, and high plasma levels of sPD-L1 are linked to poor OS and PFS. According to a survival analysis, patients with high sPD-L1 levels had significantly worse PFS and OS than those with low sPD-L1 levels (PFS: 7.2 months vs. 13.6 months, $p < 0.001$; OS: 21.4 months vs 28.0 months, $p = 0.001$). This study also proves that TNBC patients with high sPD-L1 level has significantly shorter PFS and OS, followed by HER-2 positive subtype and luminal subtype breast cancer patients (PFS: 5.1 months vs 7.2 months vs 8.0 months, $p<0.05$; OS: 17.4 months vs 21.7 months vs 21.9 months, $p<0.05$) (Han *et al.*, 2021). There are currently no data on sPD-L1 level survival analysis of breast cancer patients following cancer treatments and tumour stage or metastasis (Li *et al.*, 2019, 2022; Yazdanpanah *et al.*, 2021a).

2.6 Monitoring breast cancer prognosis through liquid biopsy

The predictive IHC assay allows for precise and customised ICIs treatment decision-making. However, monitoring the ICIs treatment response through solid biopsy cannot be applied repeatedly after ICIs treatment as the patient must endure invasive, uncomfortable, and occasionally impossible tissue extraction. Moreover, predictive IHC is a qualitative method where the interobserver variability and diagnostic efficacy of PD-L1 immune scoring are influenced by the pathologist's personality (Butter *et al.*, 2022). Therefore, in allowing assessment for patient’s prognosis, treatment response and post-treatment surveillance, cancer biomarker detection in both tissue and bloodstream has become indispensable. An ideal strategy for managing treatment response would be to assess predictive tissue tumour biomarkers expressed on tissue biopsy and then monitor the level of the same biomarkers released into the

bloodstream through liquid biopsy. However, the majority of cancer biomarkers currently discovered through tissue analysis have not been successfully validated in liquid analysis.

As a result, work is still being done to identify circulating tumour cells (CTCs) from liquid biopsies that could provide predictive information (Li *et al.*, 2021; Mandair *et al.*, 2021). Recently, a few CTCs with the potential to be markers in liquid biopsies have been identified, such as circulating tumour DNA that is DNA fragments containing tumour-specific somatic or epigenetic alteration that comprises a subset of plasma cell-free DNA originated from tumour primary and/or metastatic sites and tumour circulating antigens, e.g. soluble form of tissue tumour markers and circulating tumour-associated nucleic acids that are shed from tumours and their metastatic sites into the circulatory systems or bodily fluids of cancer patients.

Several CTCs with the potential to serve as markers in liquid biopsies have recently been discovered, including (1) circulating tumour DNA, which is a subset of plasma cell-free DNA derived from primary and/or metastatic tumour sites and contains DNA fragments with tumour-specific somatic or epigenetic alteration (Dang and Park, 2022; Adashek *et al*., 2021; Rizzo *et al*., 2020) and (2) tumour circulating antigens, such as soluble form of tissue tumour biomarkers and circulating tumour-associated nucleic acids, are substances released into the bloodstream or other bodily fluids of cancer patients by tumours and their metastatic sites.

In a study to evaluate the relationship between serum and tissue HER2/neu oncoprotein level in breast cancer patients, the study found that serum HER2 assay may complement the tissue assay by providing information lacking in tissue assay but not replacing tissue assay entirely (Shukla *et al.*, 2016). Evidently, elevated serum level of vascular endothelial growth factor (VEGF) during preoperative are substantially