THE ROLE OF *Strobilanthes crispus* ACTIVE FRACTION (F3) ON GLUCOSE METABOLISM OF MDA-MB-231 BREAST CANCER CELL LINE

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by

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LIST OF SYMBOLS

α	alpha
β	Beta
γ	gamma
μ	micro
M	molar
m	milli
nm	nanometers
kDa	kilodalton
L	litre

LIST OF ABBREVIATIONS

2DG	2-deoxy-D-glucose
2-NDBG	2-[N-(7-nitrobemz-2-oxa-1,3- diazol-4-yl) amino]-2-deoxyglucose
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ADP	Adenosine diphosphate
AKT	Protein Kinase B
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP	Ammonium persulphate
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
β-Me	Beta-mercaptoethanol
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
BLBC	Basal like breast cancer
BRCA	Breast cancer gene
CAPS	3-[Cyclohexamino]-1-Propanesulfonic Acid
CIP2A	Cancerous inhibitor of protein phosphatase 2A
CSC	Cancer stem cell
CO_2	Carbon dioxide
DCM	Dicholoromethane
DG6P	Deoxyglucose-6-phosphate

DMBA	7,12-Dimethylbenz[a]anthracene
DMEM	Dulbecco’s Modified Eagle’s Medium
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EE	Early endosome
EGFR	Epidermal growth factor receptor
EGCG	Epigallocatechin-3-gallate
EMT	Epithelial mesenchymal transition
ENO1	Enolase 1
ER	Estrogen Receptor
F26BP	Fructose-2,6-bisphosphate
$FADH_2$	Flavin adenine dinucleotide
FAK	Focal adhesion kinase
FBC	Full blood count
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G1D	GLUT1 deficiency
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCPP	Good cell culture practise
GEPIA	Gene Expression Profiling Interactive Analysis
GLOBOCAN	Global Cancer Observatory

GLUT	Glucose transporter
GSK3β	Glycogen synthase kinase-3 beta
GSH	Glutathione
Hcl	Hydrochloric acid
hEGF	Human Epidermal Growth Factor
HER2	Human epidermal growth factor receptor 2
HIF1	Hypoxia inducible factor 1
HK	Hexokinase
HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IM	Immunomodulatory
JAK	Janus kinase signaling
LDHA	Lactate dehydrogenase A
LE	Late endosome
MCT	Monocarboxylate transporter
MeOH	Methanol
MES	Mesenchymal-like
mLST8	Mammalian lethal with sec-13 protein-8
MMP	Matrix metalloproteinase
MPA	Metaphosphoric acid
mSIN1	Mammalian stress-activated MAP kinase-interacting protein1
MTI-MMP	Membrane type-1 metalloproteinase
mTOR	Mammalian target of rapamycin

mTORC	Mammalian target of rapamycin complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NSCLC	Non-small cell lung cancer
NMU	N- Methyl-N-nitrosourea
NMR	Nuclear magnetic resonance
OSCC	Oral squamous cell carcinoma
OXPHOS	Oxidative phosphorylation
PI3K	Phosphoinositide 3-kinase
pAKT	phosho-AKT
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
pCR	Pathological complete response
PD-1	programmed death receptor 1
PDK1	Phosphoinositide-dependent kinase-1
PD-L1	programmed death-ligand 1
PenStrep	Penicillin–streptomycin
PFA	Paraformaldehyde
PFK	Phosphofructokinase
PFKP	Phosphofructokinase, platelet
PFKM	Phosphofructokinase, muscle
PFKL	Phosphofructokinase, liver
PFS	progression-free survival
PI3K	Phosphoinositide 3-kinase

PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PK	Pyruvate kinase
PKC	Protein Kinase C
PKM	Pyruvate kinase M1/2
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptors
PPP	Pentose phosphate pathway
PR	Progesterone Receptor
PRAS40	Proline-rich AKT substrate 40 kDa
PROTOR1/2	Proteins observed with rictor 1 and 2
PS	Phytosterol
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
RAPTOR	Regulatory-associated protein of mTOR
RE	Recycling endosome
rictor	Rapamycin-insensitive companion of mTOR
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT	Room temperature
S6K1	Ribosomal protein S6 kinase beta-1
S.cripus	*Strobilanthes crispus*
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TAM	Tamoxifen
TBS	Tris buffer saline
TCA cycle	Tricarboxylic acid cycle
TGF1	Transforming growth factor
TK	Tyrosine kinases
TIMP2	Tissue inhibitor of metalloproteinases 2
TME	Tumour microenvironment
TEMED	N,N,N'N'-Tetra-methylethylenediamine
TNBC	Triple negative breast cancer
TPA	Phorbol ester, 12-O-tetradecanoylphorbol-13-acetate
TSC 1/2	Tuberous sclerosis ½
TXNIP	Thioredoxin-interacting protein
VDAC	Voltage-dependent anion channel
VDUP-1	Vitamin D3 upregulated protein 1
VPA	Valproic acid
WASF2	Wiskott-aldrich protein family
WHO	World health organization

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PERANAN PECAHAN AKTIF (F3) *Strobilanthes crispus* TERHADAP METABOLISME GLUKOSA MDA-MB-231 TITISAN SEL KANSER PAYUDARA

ABSTRAK

MDA-MB-231 ialah sel kanser payudara tiga kali ganda negatif (TNBC) yang mewakili kanser payudara dengan prognosis yang tipis dan pilihan rawatan yang terhad. Memandangkan sel TNBC mempamerkan peningkatan glikolisis, metabolisme kanser boleh menjadi sasaran yang baik untuk terapi antikanser. *Strobilanthes crispus*/Pokok Pecah Beling *(S.crispus)*, tumbuhan yang berasal dari negara Asia tropika, terkenal dengan khasiat perubatannya. Ekstrak *S.crispus* telah terbukti menunjukkan potensi sebagai antikanser. Tujuan kajian ini adalah untuk menentukan kesan pecahan aktif *S. crispus* (F3) dan komponen bioaktifnya (lutein, β-sitosterol, dan stigmasterol) ke atas metabolisme glukosa sel MDA-MB-231. Esei MTT menunjukkan aktiviti anti-proliferatif F3 dan sebatian bioaktifnya dengan nilai IC_{50} sebanyak 100 μg/mL (F3), 20 μM (lutein), 25 μM (β-sitosterol), dan 90 μM (stigmasterol) dalam sel MDA-MB-231 selepas 48 jam. Nilai IC_{50} setiap sebatian kemudiannya digunakan dalam esei berikutnya. Spektrofotometer pendarfluor digunakan untuk mengukur aktiviti pengambilan glukosa dan kepekatan laktat dalam sel MDA-MB-231 yang dirawat. Penemuan kami menunjukkan bahawa F3 dan sebatian bioaktifnya mengurangkan pengambilan glukosa ke dalam sel MDA-MB-231, kecuali stigmasterol. Walau bagaimanapun, berbanding dengan kawalan, kepekatan laktat berkurangan secara signifikan dalam semua sel MDA-MB-231 yang dirawat. Menariknya, kehadiran F3 dan sebatian bioaktifnya tidak mempunyai kesan

ke atas aktiviti glikolitik dalam titisan sel payudara bukan malignan (MCF10A), menunjukkan bahawa rawatan itu selamat pada sel normal. Ekspresi dan lokasi Glukosa Transporter 1 (GLUT1) kemudiannya ditentukan menggunakan blot Western dan mikroskop pendarfluor. Walaupun pengambilan glukosa telah dihalang, tiada penurunan dalam ekspresi GLUT1 dalam sel yang dirawat. Sebaliknya, F3, lutein, dan β-sitosterol menghalang translokasi GLUT1 dari sitoplasma ke membran sel. Penemuan ini disokong oleh pengurangan aktiviti Jalur Protein Kinase C (PKC) selepas rawatan yang sama diukur dengan spektrofotometer, dan peningkatan ekspresi protein penginteraksi-thioredoxin (TXNIP) seperti yang dikesan oleh blot Western. Laluan PI3K/AKT/mTOR/HIF1α diketahui mengawal metabolisme dalam sel-sel kanser. Oleh itu, kesan F3 dan sebatian bioaktifnya pada ekspresi protein AKT, pAKT, mTOR, dan HIF1α ditentukan menggunakan analisis sitometri aliran, dan pengurangan protein tersebut diperhatikan dalam semua sel MDA-MB-231 yang dirawat. Aktiviti anti-metastatik juga diaruh oleh F3 dan sebatian bioaktifnya dalam TNBC, seperti yang ditunjukkan oleh penurunan dalam kedua-dua migrasi (diuji menggunakan asai migrasi transwell) dan ekspresi fibronektin. Kesimpulannya, aktiviti anti-glikolitik dan anti-metastatik dalam sel MDA-MB-231 yang dicetuskan oleh F3 dikaitkan dengan perencatan laluan isyarat AKT/mTOR/HIF1α oleh sebatian bioaktifnya.

THE ROLE OF *Strobilanthes crispus* ACTIVE FRACTION (F3) ON GLUCOSE METABOLISM OF MDA-MB-231 BREAST CANCER CELL LINE

ABSTRACT

MDA-MB-231 is a triple negative breast cancer cell (TNBC) that represents breast cancer with a poor prognosis and limited treatment options. Since TNBCs exhibit increased glycolysis, cancer metabolism may become a great target for its anticancer therapy. *Strobilanthes crispus (S.crispus)*, a plant native to tropical Asian countries, is well-known for its medicinal properties. *S.crispus* extract has been shown to have promising anticancer properties. The purpose of this study is to determine the effect of *S. crispus* active fraction (F3) and its bioactive compounds (lutein, β-sitosterol, and stigmasterol) on glucose metabolism of MDA-MB-231 cells. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay showed anti-proliferative activities of F3 and its bioactive compounds with IC_{50} values of 100 µg/mL (F3), 20 µM (lutein), 25 µM (β-sitosterol), and 90 µM (stigmasterol) in MDA-MB-231 cells after 48 h. The IC_{50} value of each compound was then used in subsequent assays. A fluorescence spectrophotometer was used to quantify glucose uptake activity and lactate concentration in treated MDA-MB-231 cells. Our findings demonstrated that F3 and its bioactive compounds reduced the uptake of glucose into MDA-MB-231 cells, except for stigmasterol. However in comparison with controls, lactate concentration was significantly reduced in all treated MDA-MB-231 cells. Interestingly, the presence of F3 and its bioactive compounds had no effect on the glycolytic activities of non-malignant breast cell line (MCF10A), indicating that the

treatments were safe for normal cells. The expression and localisation of GLUT1 were then determined using Western blot and fluorescence microscopy. Despite the inhibition of glucose uptake, no decrease in GLUT1 expression was observed in the treated cells. Instead, F3, lutein, and β-sitosterol inhibited GLUT1 localisation from the cytoplasm to the cell membrane. The findings were supported by reduced PKC activity after the same treatments measured by spectrophotometer, and increased TXNIP protein expression as detected by Western blot. The PI3K/AKT/mTOR/HIF1α pathway is known to regulate metabolism in cancer cells. Hence, the effects of F3 and its bioactive compounds on the expression of AKT, pAKT, mTOR and HIF1α proteins were determined using flow cytometry analysis, and downregulation of those proteins was observed in all treated MDA-MB-231 cells. Anti-metastatic activity was also induced by F3 and its bioactive compounds in TNBCs, as demonstrated by a decrease in both migration (assayed using transwell migration assay) and fibronectin expression. As a conclusion, anti-glycolytic and anti-metastatic activities in MDA-MB-231 cells induced by F3 are attributed to the inhbition of the AKT/mTOR/HIF1α signalling pathway by its bioactive compounds.

CHAPTER 1

INTRODUCTION

1.1 Breast cancer

Breast cancer has been identified by the World Health Organization (WHO) as the form of the disease that affects the greatest number of people worldwide. In 2020, there were approximately 2.26 million newly diagnosed cases of breast cancer recorded globally (Wilkinson and Gathani, 2022), but the mortality rate of breast cancer for both sexes is 11.1 % lower than that of lung cancer (Figure 1.1A). This might be due to the advanced screening technology (mammography) and improved therapy, which have resulted in a 1.8 - 3.4 % annual decline in mortality rates since 1990 (Hendrick et al., 2019). However, as shown in Figure 1.1B, breast cancer remains the top cause of mortality among women globally, claiming over 600,000 lives in 2020 (Sung et al., 2021; Arnold et al., 2022), with metastasis typically accounting for 90 % of deaths (Dillekas et al., 2019).

It is well known that women in developing countries have a higher rate of breast cancer. Nonetheless, rates are increasing in nearly every region worldwide. According to GLOBOCAN 2020, the most commonly reported new cases of cancer in Malaysia are breast cancer, followed by colorectal and lung cancer. Moreover, breast cancer has the highest mortality rate in Malaysia, accounting for 18.4 % of all cancer patients (Tan et al., 2023).

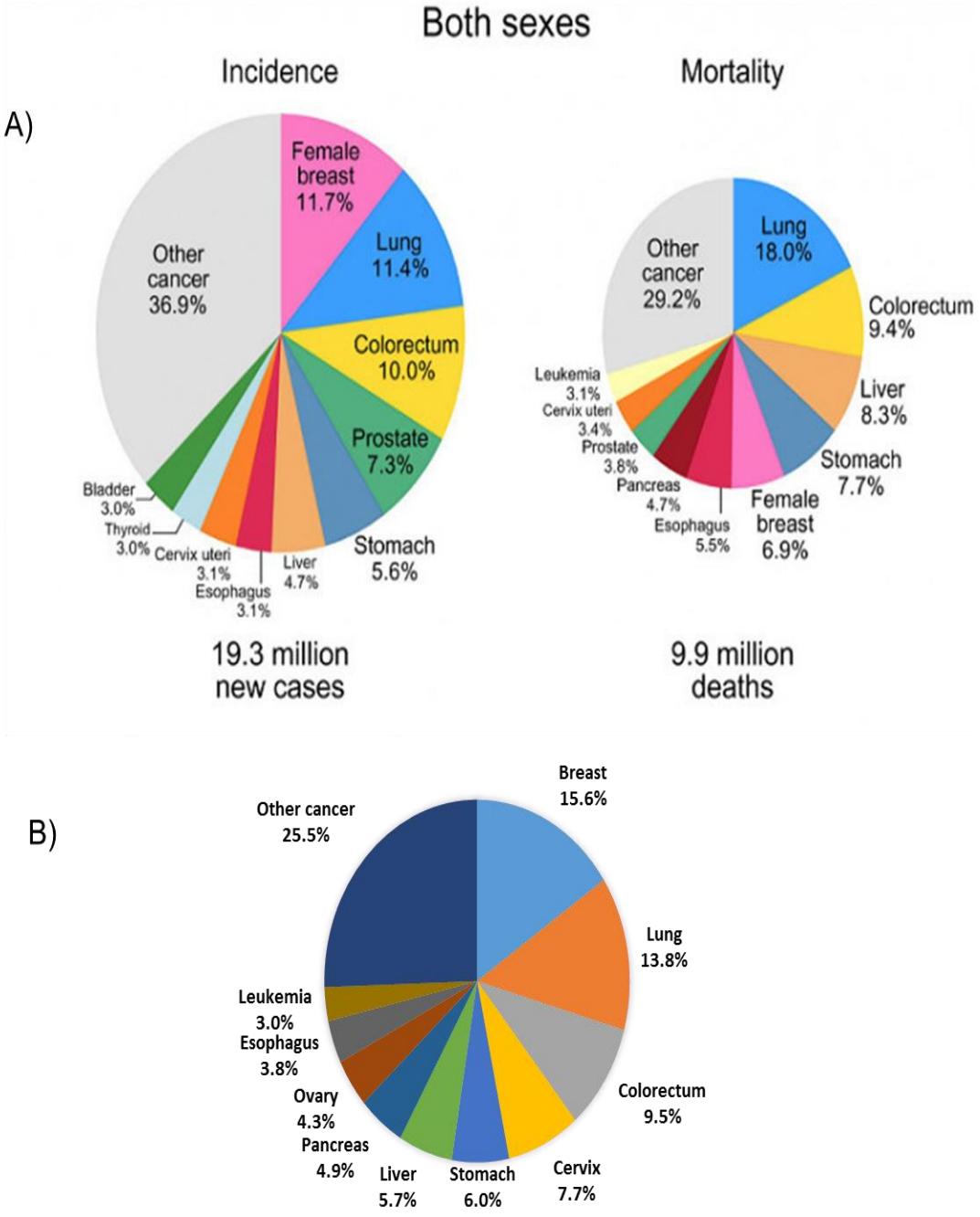

Figure 1.1: Global cancer statistics 2020 (extracted from Sung et al. 2021).

A) Distribution of new cancer incidence and death for both male and female in 2020.
B) Distribution of death for female in 2020. Source from GLOBOCAN 2020.

1.2 Risk factors of breast cancer

Breast cancer is 100 times more likely to affect women than men (Naeem et al., 2019). However, studies found that men had a lower overall survival rate for breast cancer than women (Scomersi et al., 2021). Breast cancer is a complex disease that can be caused by a variety of factors. Despite the fact that the disease exists in every country, the disease's incidence, mortality, and survival rates vary significantly by region (Azamjah et al., 2020). These variations can be attributed to a number of factors, including population structure, lifestyle, genetic factors, and environmental factors (Hortobagyi et al., 2005; Coughlin, 2019). Malaysia has 3 major ethnic groups: Malay, Chinese and Indian, which constitute the largest ethnic groups in Asia. Race is one of the important intrinsic factors that can raise a woman's chance of developing breast cancer (Kamińska et al., 2015). This is because, lifestyle and reproductive characteristics may be influenced by its multi-cultural and multi-religious setting. According to Malaysia National Cancer Registry reports for 2012-2016, the prevalance of breast cancer is highest among Chinese, followed by Indian and Malay (Azizah et al., 2019).

Aside from sex and race, the following risk factors can increase the probability of developing breast cancer:

a) Age:

Because the disease is more common in older people, ageing is a significant contributor to the chance of developing breast cancer. In Malaysia, for example,

women over the age of 50 have a higher incidence of cancer as reported in Malaysia National Cancer Registry reports (2012-2016) (Azizah et al., 2019).

b) Family history:

A family history is responsible for one-fourth of all cases of breast cancer (Brewer et al., 2017). First-degree family history of breast cancer refers to women who have mothers, sisters, or daughters who have the disease (Tan et al., 2018). The mutation in BReast CAncer genes like BRCA1 and BRCA2 contribute to inherited susceptibility to breast cancer, which can be passed down from mother to daughter (Sun et al., 2017b). The majority of studies revealed a substantial link between family history and a higher risk of developing breast cancer (Pharoah et al., 1997; Razif et al., 2011; Reiner et al., 2018). However, study by Tan et al. (2018) revealed, there is a small correlation in Malaysia between first-degree family history and an elevated risk of breast cancer.

c) Estrogen:

It is widely known that having a high endogenous and extrogenous estrogen level is a risk factor that contributes to a higher incidence of breast cancer. In pre-menopausal women, the ovary is the organ responsible for naturally producing endogenous estrogen. Meanwhile, oral contraceptives and hormone replacement therapy (HRT), are currently the two most common forms of estrogen derived from outside the body. Although HRT can increase the risk of breast cancer, women who have not used oral contraceptives for more than 10 years have no increased risk of breast cancer (Sun et

al., 2017b). There is a report that shows increased estrogen levels in post-menopausal women are also strongly correlated with an increased risk of breast cancer (Kamińska et al., 2015) .

d) Reproductive factors:

Breast cancer risks can be increased by factors related to reproduction, such as having an early menarche, a late age of first pregnancy, a late menopause, and a low parity (the number of times a woman has given birth). Each year of early menarche delay or additional birth can reduce the risk of breast cancer by 5 % or 10 %, respectively. This happened because early menarche results in prolonged exposure times to estrogen activity (Hsieh et al., 1990). Meanwhile, when the menopause is delayed for a year, the risk of breast cancer increases by 3 % (Dall and Britt, 2017; Horn and Vatten, 2017; Sun et al., 2017b).

e) Lifestyle:

Excessive alcohol consumption, as well as a high dietary fat intake, can both increase the risk of breast cancer. Estrogen levels in blood increased with consumption of alcohol and can trigger the activation of estrogen receptor. According to studies, drinking between 35 and 44 g of alcohol daily increases the risk of breast cancer by 32 % (Jung et al., 2016, Sun et al., 2017b). High fat intake, particularly saturated fat, has been linked to an increased risk of mortality and a poor prognosis for patients who have breast cancer (Makarem et al., 2013; Xiao et al., 2019). Increasing data shows that smoking also increases the risk of developing breast cancer, especially when started at a young age (Jones et al., 2017; Zeinomar et al., 2019).

1.3 Histological types of breast cancer

Cancer can arise from any kind of cells in the body as an outcome of an abnormal proliferation rate. As a result, there are now more than a hundred different types of cancer that vary in their behaviours and responses to treatments (Cooper and Hausman, 2000). For breast cancer cells, the variation depends on the types of tissues from which they arise. Breasts are composed of 15-20 lobes of glandular tissues and stromal tissues (supporting tissues). Glandular tissues are a home for milk-production glands (lobules) and the ducts for the passage of milk. Meanwhile, the stromal tissues, which are made up of fatty and fibrous connective tissues, surround both lobules and ducts (Figure 1.2). Breast cancer develops when there is an uncontrolled growth of breast epithelial cells that line the milk duct (ductal cancers) or lobules (lobular cancers) (Love and Barsky, 1996; Sainsbury et al., 2000).

Ductal and lobules cancers can be divided into non-invasive breast cancer and invasive breast cancer.

a) Cancer cells that do not invade the surrounding fatty and connective tissues of the breast are considered to be non-invasive. They are usually referred as ductal carcinoma *in situ* (typical type of non-invasive breast cancer) and lobular carcinoma *in situ*. Although less frequent than ductal carcinoma *in situ*, lobular carcinoma *in situ* can serve as a marker for increased breast cancer risk. *In situ* is a term to describe non-spreading cancer cells.

b) Invasive breast cancers are cancer cells that manage to invade the surrounding fatty and connective tissues after penetrating the wall of ducts or lobules. Infiltrating ductal carcinoma (IDC), another name for invasive ductal carcinoma, is the most frequently diagnosed form of breast cancer, with an 80 % occurrence rate. Whereas, invasive lobular carcinoma or infiltrating lobular carcinoma (ILC) only accounted for 10-15 % of breast cancer cases (Arps et al., 2013).

Breast cancer can also develop in connective tissues, which are composed of muscles, fat, and blood vessels. However this type of cancer is very uncommon and is called a sarcoma. Examples of sarcomas that appear in the breast are phyllodes tumour and angiosarcomas. Phyllodes tumour can be either benign (non-cancerous) or malignant (cancerous). It has been reported that surgery can cure the majority of patients with benign and borderline phyllodes tumour (Demian et al., 2016). Angiosarcomas are malignancies of the inner lining of blood vessels that can develop anywhere in the body. Approximately 8 % of angiosarcomas can be found in breast tissue.

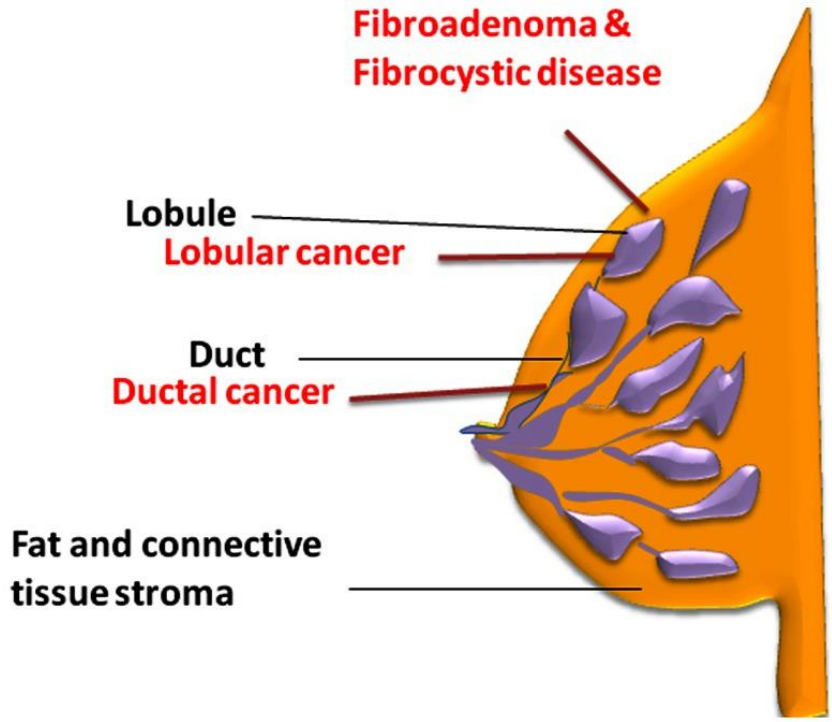

Figure 1.2: Simplified diagram of normal breast anatomy (extracted from Wu et al., 2013a).

The main components of the breast are lobules (contain milk producing glands) and ducts (tiny tubes that link all the lobules together) which are embedded in a matrix called stroma (fatty tissue and connective tissue). Most of breast cancers begin in the ducts (ductal), some in the lobules (lobular) and the rest in other tissues. Fibrocystic breast disease and fibroadenoma (a solid breast lump) are both common benign that usually develop within the breast lobules.

1.4 Molecular subtypes of breast cancer

The first gene expression profiling of primary breast cancer cells was conducted by Perou in 2000 using complementary DNA microarrays representing 8102 human genes. For the analysis, Perou used samples from 3 normal breasts, 1 fibroadenoma, 36 invasive ductal carcinomas, 1 ductal carcinoma *in situ*, and 2 invasive lobular carcinomas. The results revealed an "intrinsic" gene list that showed gene variation between cancers from different patients compared to samples from the same tumours or patients (Perou et al., 2000; Weigelt et al., 2015). This clarified the clinical, pathologic, and genomic heterogeneity of breast cancer (Perou et al., 2000; Sorlie et al., 2001; Carey et al., 2006; Parise et al., 2009). Continuosly studying breast cancer using this high throughput microarray-based gene expression profiling has allowed researchers to learn more about the molecular mechanisms behind biological traits, including the propensity for metastasis (Van't Ver et al., 2002; Wang et al., 2005; Iwamoto et al., 2019) or histological grade (Sotiriou et al., 2006), and to find signatures linked to prognosis and therapeutic response (Potti et al., 2006; Borisov et al., 2020). This can help the doctors plan the best treatments for breast cancer patients.

There are four molecular subtypes of breast cancer identified from the gene expression profiling analysis. The subtypes are categorised according to the presence of hormone receptors such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptors-2 (HER2) (Iqbal and Buch, 2016). Breast cancer cells that are ER positive require estrogen to grow, while PR positive breast cancer cells are sensitive to progesterone and grow when the receptors are activated. HER2 is another gene that can affect the development of breast cancer. HER2 growth-

promoting protein is produced by breast cancer cells that have the HER2 gene, which aids in the growth and development of the tumour. HER2 positive is a marker for an aggressive subtype of breast cancer and has been reported to present in approximately 25% of breast cancers cases. Additionally, HER2 overexpression is associated with high recurrence rates and short survival times (Mitri et al., 2012).

The molecular subtypes of breast cancer can be grouped as follows :

a) **Group 1 (Luminal A):** Tumours in this category are ER and PR positive, but HER2 negative. Hormone treatment and chemotherapy are probably beneficial for women with luminal A breast cancer. According to studies, luminal A cancers have high levels of ER-activated gene expression and low levels of proliferative gene expression. In addition to this, they have a good prognosis and a low histological grade (Weigelt et al., 2010a).

b) **Group 2 (luminal B):** Tumours that fall under this group are ER positive, PR negative and HER2 positive. Chemotherapy is expected to be effective in treating luminal B breast cancers, while hormone therapy and HER2 targeted therapy may also be helpful. Luminal B tumours have a much worse prognosis than Luminal A tumours, as well as a higher histological grade and higher rates of proliferation (Weigelt et al., 2010a).

c) **Group 3 (HER2 positive):** This group includes tumours that are HER2 positive but ER and PR negative. Chemotherapy and HER2-specific treatment are likely to be beneficial for patients with HER2 breast cancer.

d) Group 4 (basal-like): This group is identified by the expression of genes, such as cytokeratins 5, 6, or 17 that are generally expressed in the basal epithelial layer of the mammary gland. Basal-like breast cancer (BLBC) also expresses the epidermal growth factor receptor (EGFR) gene, which is associated with normal basal-like myoepithelial cells located between the breast ductal and lobular systems (Rakha et al., 2008; Yao et al., 2016). BLBC has a poor prognosis and is naturally aggressive when compared to other subtypes (Toft and Cryns, 2011). Furthermore, these basal-like subtypes do not express the treatment-responsive molecular targets such as ER and HER2. Triple negative breast cancer (TNBC) gene expression profiles significantly overlap with BLBC, and between 70 – 84 % of TNBC cells are described as basal-like (Perou et al., 2000; Prat et al., 2015).

1.5 Triple Negative Breast Cancer Cells (TNBC)

TNBC is a heterogeneous group with higher grade tumour and common in younger patients (Foulkes et al., 2010). It is defined by the lack of expression of hormonal receptors (ER, PR) and the absence of HER2 overexpression (Collignon et al., 2016). TNBC also has a worse overall survival rate due to early distant recurrences that occur 3 years after diagnosis. Most TNBC cells (80-90 %) are invasive ductal carcinomas and associated with distant metastasis (Dent et al., 2007; Yuan et al., 2014). TNBCs have a greater propensity to metastasis to the lungs, the central nervous system, and the brain (Anders and Carey, 2008; Lin et al., 2008; Sihto et al., 2011). Patients with TNBC show a very poor prognosis as the tumours are more aggressive compared to other subtypes and have no targeted therapies (Chacon and Costanzo, 2008; Anders and Carey, 2009). TNBC patients (stage I-III) are usually treated with trimodality

therapy, which combines surgery, radiotherapy, and adjuvant/neoadjuvant chemotherapy. However, 50 % of them continue to experience disease recurrence, and 37 % of the patients die in the first 5 years after surgery (Pandy et al., 2019).

Basically, TNBC can be divided into 4 subtypes: basal-like, mesenchymal-like, immunomodulatory, and luminal androgen receptors.

a) **Basal-like (BL):** has increased rate of BRCA1/2 mutation, and highly activated cell cycle and DNA damage response genes (Lehmann et al., 2011; Jiang et al., 2019).
b) **Mesenchymal-like (MES):** has stem-like characteristics, and are enriched with a variety of biological processes, including cell motility, extracellular matrix interaction, epithelial-mesenchymal transition (EMT), and growth factor signalling pathways (increased phosphoinositide 3-kinase (PI3K) and Janus kinase (JAK) signalling) (Weigelt et al., 2015; Jiang et al., 2019).
c) **Immunomodulatory (IM):** has a high level of lymphocyte infiltration and high expression of genes related to immune cell activities (Jiang et al., 2019).
d) **Luminal androgen receptor:** has high androgen metabolism and very active androgen receptor signalling (AR) (Jiang et al., 2019).

1.5.1 Type of treatments for TNBC

TNBC is a growing problem worldwide as a result of early relapses and the predominance of distal metastases localised in visceral organs, which contribute to a poor overall survival rate. There is currently no specific treatment for TNBC outside

of clinical trials, necessitating the discovery of novel targeting therapies (Zakaria et al., 2019). Nowadays, patients with TNBC are treated similarly to non-TNBC patients, particularly in adjuvant and neo-adjuvant settings. Furthermore, like other subtypes of breast cancer, radiation therapy and surgery are frequently used. TNBC is typically treated with chemotherapy, either alone or in combination with surgery and/or radiotherapy. It has been reported that, early stage TNBC cells have a greater pathological complete response (pCR) rate with taxanes, anthracyclines, or platinum-based systemic therapy. However, patients with TNBC do not benefit from treatments that aimed to block hormone receptors (tamoxifen) or HER2 (Herceptin) (Bai et al., 2021).

Most commonly used cytotoxic drugs and targeted therapies for TNBC patients are (Tan and Swain, 2008):

1.5.1(a) Cytotoxic chemotherapy

1.5.1(a)(i) Microtubule stabilizer

It is one of the most effective chemotherapeutic drugs that act to stabilize microtubule and inhibit cell division (Varidaki et al., 2018). Taxane group such as paclitaxel, docetaxel and cabazitaxel have demonstrated to be more effective on TNBCs compared to receptor-positive cancers (Sparano et al., 2008; Martin et al., 2010). Another example of a microtubule stabiliser with comparable action and toxicity to those of the taxanes is ixabipelone. Additionally, it has been demonstrated to be utilised well in TNBC patients and can be coupled with Capecitabine, a

chemotherapeutic agent, to treat advanced breast cancer that exhibits taxane resistance (Pivot et al., 2009; Tkaczuk, 2011).

1.5.1(a)(ii) Anthracyclines

This class of drug is extracted from certain types of *Streptomyces* bacteria. Examples of anthracyclines that are often used for breast cancer treatments are doxorubicin and epirubicin (Yao et al., 2017). Its mechanism of action involves the DNA base pairs intercalation, which subsequently affects DNA transcription and translation (Wang et al., 1987; Shandilya et al., 2020). Many studies have shown that TNBC is responsive to regimens comprising anthracycline (Liedtke et al., 2008). For example, a higher clinical response to doxorubicin has been observed in TNBC than in non-TNBC patients (Carey et al., 2007). TNBC also has been observed to respond better to anthracyclines compared to taxanes (Narui et al., 2019). However, frequent anthracycline treatment of cancer cells can result in chemoresistance (Krohn, 2009).

1.5.1(a)(iii) Platinum-based agents

Platinum is a common second-line anticancer drug for breast cancer treatments that show promising results in the neoadjuvant and metastatic settings (Alli et al., 2011; Zhang et al., 2015; Poggio et al., 2018). Examples of the platinum-based agents used for TNBC patients are cisplatin and carboplatin (E Valsecchi et al., 2015; Hill et al., 2019). Platinum has been suggested as a possible drug therapy for breast cancer caused by inherited BRCA1 gene mutations, which is most likely associated with TNBC (Chen et al., 2018). When BRCA1 mutations occur, the DNA repair cascade is not

regulated (Schneider et al., 2008). The platinum will cause a double strand break and a DNA lesion, which will kill cancer cells by stopping the formation of the replication fork (Hastak et al., 2010). However, the responsiveness and survival rates of TNBC patients can be increased by combining platinum with other chemotherapeutic drugs (Sirohi et al., 2015).

1.5.1(b) Targeted therapies

1.5.1(b)(i) mTOR inhibitors

The mammalian target of rapamycin (mTOR) is a kinase and key component of the PI3K-AKT-mTOR pathway, which controls the growth and proliferation of cells (Nicolini et al., 2015; Saxton and Sabatini, 2017). When compared to other subtypes, TNBC cells have higher mTOR expression, which often indicates poor prognosis (Ueng et al., 2012; Walsh et al., 2012). It has been reported that mTOR inhibitors such as rapamycin and its analogues (temsirolimus and everolimus) on TNBC patients can delay the progression of the disease and increase their chances of survival (Bahrami et al., 2018; Costa et al., 2018). However, to overcome the resistance that TNBC cells develop to the mTOR inhibitors, a combinational multi-kinase inhibitor strategy must be used (He et al., 2019).

1.5.1(b)(ii) PARP inhibitors

Poly (ADP-ribose) polymerase (PARP) is an enzyme that is essential for repairing DNA damage in cells via the base excision repair pathway (Dantzer et al., 2000).

TNBC as previously stated, has a high frequency of BRCA1 or BRCA2 mutation (Winter et al., 2016). Therefore, PARP inhibitors can induce cell death in BRCA-mutated TNBC cells due to its inability to repair DNA damage (Yao et al., 2017). Currently, olaparib and talazoparib are two approved PARP inhibitors for TNBC treatments (Barchiesi et al., 2021). Although approximately 50 % of TNBC patients show progress with treatments using PARP inhibitors, numerous mechanisms of PARP inhibition resistance have been proposed in BRCA-associated cancer (Noordermeer and van Attikum, 2019; Han et al., 2020). Several strategies are under investigation to overcome these resistance mechanism.

1.5.1(b)(iii) Angiogenesis inhibitors

Angiogenesis is a complex process that results in the formation of new blood vessels near the primary tumour, which is necessary for the growth and metastasis of cancer cells. As a result, angiogenesis inhibitors can reduce the aggressiveness of the cancer cells (Van Petten de Vasconcelos Azevedo et al., 2022). Expression of angiogenic factors may be a good indicator of how aggressive tumour cells are (Nishida et al., 2006). It has been reported that TNBC cells express more vascular endothelial growth factor (VEGF), a key mediator for angiogenesis, compared to non-TNBC cells (Linderholm et al., 2009). Bevacizumab (Avastin), an anti-VEGF monoclonal antibody has been shown to improve progression-free survival (PFS) in HER2 negative breast cancer cells when used in combination with chemotherapy (Brufsky et al., 2011).

1.5.1(b)(iv) EGFR inhibitors

The epidermal growth factor receptor (EGFR) consists of an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain (Burgess, 2008; Hynes and MacDonald, 2009). When the ligand binds to the ligand binding domain, its kinase activity will be activated, followed by autophosphorylation at multiple tyrosine residues to recruit various substrates. The activation of this receptor usually promotes proliferation, survival, and motility of the cells via activation of various signalling pathways such as Ras-Raf-MEK-ERK, PI3K-AKT-mTOR, and Src-STAT3 (Yarden and Sliwkowski, 2001). EGFR is overexpressed on TNBC surfaces and has been recognised as a factor for poor prognosis as well as decreased response to chemotherapy in TNBC cells (Nielsen et al., 2004; Banerjee et al., 2006; Reis-Filho and Tutt, 2008). Cetuximab and lapatinib are examples of anti-EGFR agents used to treat TNBC cells (Herbst and Shin, 2002; Liu et al., 2016; Tanei et al., 2016).

1.5.1(b)(v) TK inhibitors

Tyrosine kinases (TKs) are enzymes that are usually activated in cancer cells, and stimulation of the enzyme leads to the alteration of the cell cycle, angiogenesis, as well as lack of apoptosis in epithelial malignant cells (Harari, 2004; Paul and Mukhopadhyay, 2004). An example of overexpressed tyrosine kinase in TNBC cells is Src, which was reported to affect cells adhesion and migration (Hochgrafe et al., 2010). Many anti-EGFR drugs have also been shown to inhibit tyrosine kinase in TNBCs, such as erlotinib, lapatinib, dasatinib, pyrotinib, and pazopanib (Rakha et al., 2007; Di Desidero et al., 2016; Bao et al., 2017; Cai et al., 2020). Those TK inhibitors

will bind to ATP-binding sites and inhibit EGFR activity. However long-term usage of inhibitors will promote resistance (Broekman et al., 2011).

1.5.1(b)(vi) PD-1/ PD-L1 inhibitors

In the tumour microenvironment (TME), tumour cells develop a variety of strategies to evade host immunity (Dunn et al., 2002). Studies on tumour immune escape have shown that a key component of the tumour escape mechanism is the immunological checkpoint mediated by the programmed death receptor 1 (PD-1) and programmed death-ligand 1 (PD-L1) in the TME (Inaguma et al., 2018; Prestipino and Zeiser, 2019; Zhang et al., 2020). Immune cells have the checkpoint protein PD-1, which can interact with the cancer cell surface protein, known as PD-L1. This interaction acts like an "off switch," preventing T cells from attacking the tumour cells (Tavares et al., 2021). Furthermore, the prognosis of TNBC cells can be impacted by the expression of PD-L1 in the tumour microenvironment. Therefore, blocking either PD1 or PD-L1 with specific antibodies can improve T-cell responses and enhance anti-tumour efficacy (Liu et al., 2021). Pembrolizumab, Avelumab, and Atezolizumab are a few examples of PD1/PD-L1 inhibitors that are utilised to treat TNBC cells. Finding shows that PD1/PD-L1 inhibitors appear to be more effective when combined with other chemotherapy (Tavares et al., 2021).

1.6 Metabolic reprogramming in cancer cells

Although chemotherapy can improve clinical outcomes for TNBC patients, recurrence rates and the risk of developing drug resistance remain significant (Carey et al., 2007;

Foulkes et al., 2010). Furthermore, relapsed and metastatic TNBCs are more aggressive and exhibit strong resistance to chemotherapy, radiotherapy, and surgery treatments (Schmid et al., 2018). Highly proliferating cancer cells modify their metabolic pathway to meet their needs for ATP and intermediates for macromolecular biosynthesis. This modification process, known as metabolic reprogramming occurs as a result of metabolic changes in cancer cells caused by oncogene mutations, dysregulation in the expression and activity of some metabolic enzymes, and changes in the flow of metabolic pathways (Hanahan and Weinberg, 2011). Furthermore, dependence on glucose and glycolysis, pharmacological stress (adaptation to oxidative stress, lipid metabolism, and bioenergetics), and an increase in polyamine synthesis all affect metabolic adaptability in cancer cells (Zhang et al.,2016; Lee et al., 2017; Zhang et al., 2017; You et al., 2019). Interestingly, metabolic reprogramming also can induce cancer cells to become resistant towards chemotherapeutic drugs (Rahman and Hasan, 2015; Cardoso et al., 2018). TNBC may develop chemotherapeutic resistance due to epithelial-mesenchymal transitions (EMT), cancer stem cells (CSC), and hypoxia (Bai et al., 2020). Thus, understanding TNBC metabolic features is critical for developing effective TNBC therapeutic targets.

1.6.1 Metabolic differences in normal and cancer cells

Glucose metabolism is a process where glucose, a simple sugar (monosacharide) found in many foods, is broken down to produce energy in the form of ATP. As shown in Figure 1.3, normal cells and cancer cells have distinct differences in their metabolic processes. In normal cell, glucose will be catabolized into 2 pyruvates and transported out from the cytoplasm into mitochondria. After that, it will be further converted into

acetyl-CoA and subsequently react with oxaloacetate to produce citrate, which will then enter the tricarboxylic acid (TCA) cycle (Lee et al., 2016a). When acetyl-CoA enters the TCA cycle, it will generate 2 carbon dioxide (CO_2), 3 NADH, 1 FADH2, and 1 ATP molecules. Therefore, a total of 4 CO_2, 6 NADH, 2 FADH2 and 2 ATP molecules are generated per 1 glucose molecule. OXPHOS is a redox process where ATP synthesis is connected to the flow of electrons through mitochondria electron transport chain. During the process, these coenzymes (NADH and FADH2) serve as electron donors, acting as carriers of electrons to the electron transport chain. Therefore, normal cells create 36 ATP molecules for every glucose molecule involving the process of glycolysis, the TCA cycle, and OXPHOS (Jóźwiak et al., 2014).

Cancer cells derive most of their energy in the form of ATP from aerobic glycolysis instead of mitochondria respiration. During the conversion of pruvate to lactate, 2 ATP molecules are produced for every glucose. Lactate is produced when the oxygen supply is limited in normal cells, but cancer cells prefer to use aerobic glycolysis even when oxygen supply is abundance (Deberadinis et al., 2008; Pahn et al., 2014). Although aerobic glycolysis is far less efficient than mitochondria OXPHOS, the cell will generate more energy due to the rapid uptake of glucose into the cell. The increased glucose uptake along with simultaneous lactate production, even when aerobic conditions are present, is referred to as the Warburg effect (Warburg, 1956; Jóźwiak et al., 2014).

Initially, metabolic changes in cancer cells were thought to be caused by mitochondrial damage which inhibited OXPHOS activity (Warburg, 1956; Ward and Thompson,

2012). However, mitochondria have been reported to be involved in the biosynthesis of molecules required for cancer cell growth and many cancer cells have been shown to produce ATP via OXPHOS (Moreno-Sánchez et al., 2007; .Wallace, 2012; Neuzil et al., 2013). TNBC cells, for example, have low OXPHOS activity, making them highly dependent on glycolysis (Pelicano et al., 2014). However, gene expression profiling revealed that when OXPHOS is high, it is associated with a higher risk of recurrence and death in TNBC cells. It also plays a role in chemotherapeutic resistance. Thus, inhibiting OXPHOS can also improve the efficacy of potential TNBC treatments (Evans et al., 2021).

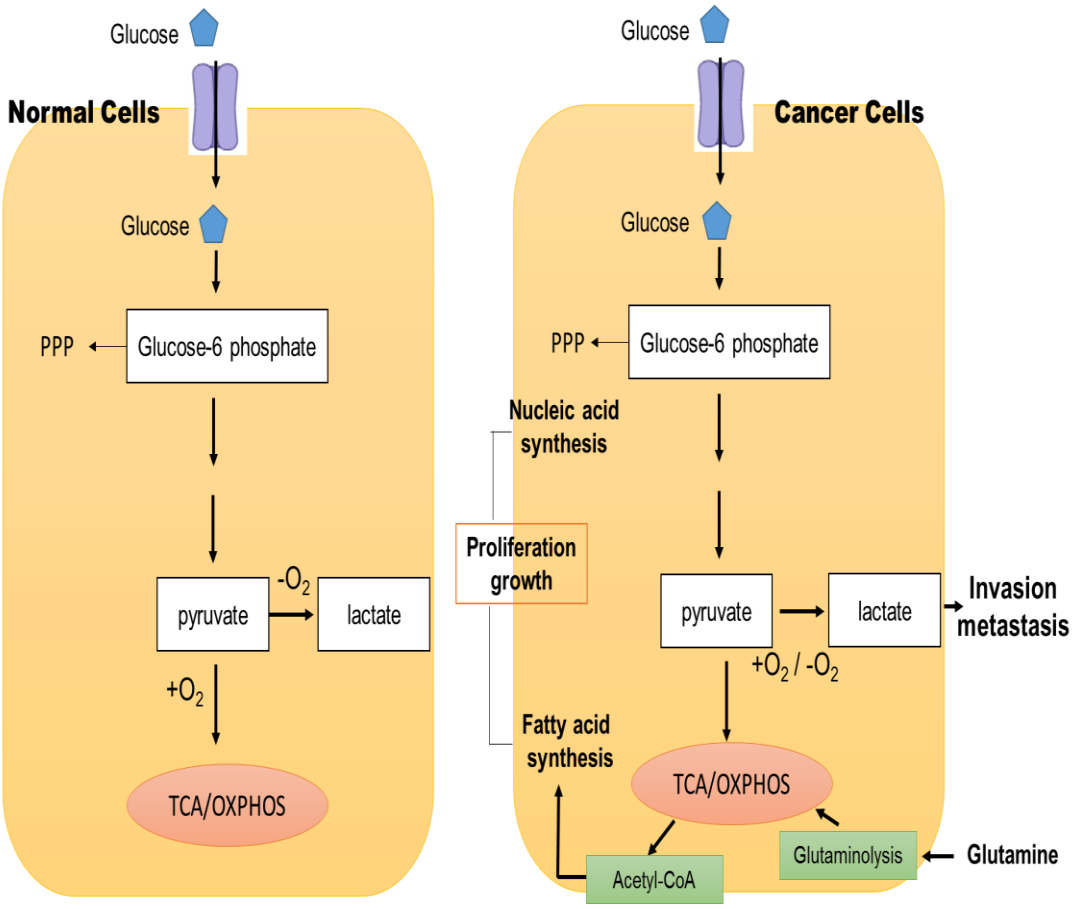

Figure 1.3: Metabolic differences between normal and cancer cells (extracted from Jóźwiak et al., 2014).

In normal cells, glucose is metabolised to pyruvate and oxidised to CO_2 through the TCA cycle and OXPHOS in the mitochondria. Lactate will be produced when the O_2 is limited. In cancer cells, glucose is converted to lactate regardless of O_2 availability. Nucleic acid and fatty acid synthesis can promote the proliferation and growth of cancer cells.

1.6.2 Warburg effect

Otto Warburg, in 1920, was the first to observe numerous uptakes of glucose into cancer cells and to use aerobic glycolysis as a method of energy production. This Warburg effect, in which glucose is converted into lactate, is widely regarded as a hallmark of many cancer cells (Liberti and Locasale, 2016). In addition, aerobic glycolysis is very beneficial to cancer cells, as its glycolytic intermediates can be used to fuel multiple biosynthetic pathways. One example is the pentose phosphate pathway (PPP), which generates ribose-5-phosphate and NADPH, both of which are required for the biosynthesis of nucleic acids and lipids. Furthermore, NADPH production can maintain an adequate level of glutathione (GSH) to protect cancer cells from chemotherapeutic agents by maintaining their redox status (Backos et al., 2012; Traverso et al., 2013).

1.7 Process of aerobic glycolysis

1.7.1 Glucose uptake through glucose transporter in cancer cells

The hydrophilic nature of glucose prevents it from penetrating the lipid bilayer of the cell membrane. As glycolysis occurs in the cytoplasm, facilitative glucose transporters are required for the transport of glucose into cells, making this the first-rate limiting step in glucose metabolism. Cancer cells multiply at a faster rate and require more energy (Arora et al., 2015). In order to compensate for the inefficient energy obtained by the Warburg effect, cancer cells were shown to express more GLUTs and consume glucose at a higher rate.

Class 1 (GLUT1-4, 14); Class 2 (GLUTs 5, 7, 9, and 11); and Class 3 (GLUTs 6, 8, 10, 12, and 13) are the three sequence-based classifications of the 14 human facilitative GLUTs (Scheepers et al., 2004). As shown in Figure 1.4, despite the fact that all three classes of GLUTs share a high degree of sequence homology and sequence features, their N-linked glycosylation positions are distinct (Joost et al., 2001). In addition, distinct types of GLUTs may have varying affinities for glucose or other sugars, depending on tissue expression (Table 1.1). Certain tissues can also express multiple types of GLUTs, such as in the muscle, where GLUT3-5, 10, and 11 have been identified (Ancey et al., 2018).

Recent studies have identified GLUT1 and GLUT3 as the distinct agents responsible for the acceleration of cancer metabolism (Pliszka and Szablewski, 2021). Overexpression of both glucose transporters has been associated with an increase in cancer cells' aggressivity and invasiveness which correlates with an acceleration of the metastasis process. In addition, the overexpression of GLUT1 and GLUT3 has been linked to poor survival and a worsening prognosis in cancer patients (Szablewski, 2019). Therefore, the inhibition of GLUTs by natural or synthetic inhibitors, glucose transporter-specific antibodies, siRNA, and shRNA can become a potential method for cancer treatments (Pliszka and Szablewski, 2021).