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# Differential effects of fasting and pharmacological interventions on cancer and cancer stem cells

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# ABBREVIATIONS LIST

2DG - 2-Deoxy-D-Glucose

8-Br-cAMP - 8-Bromoadenosine 3',5'-cyclic mono-phosphate

ABC - ATP-binding cassette

ABCC11/MRP8 - multidrug-resistant protein-8

ABCG2/BCRP - breast cancer resistance protein

AC - adenylate cyclase

ADP - adenosine diphosphate

ALDH1 - aldehyde dehydrogenase 1

alt-NHEJ - alternative non-homologous end-joining

AR - androgen receptor

ATR - ataxia telangiectasia and Rad3-related

BCT - breast conserving surgery

BER - base excision repair

BL1 - basal-like 1

BL2 - basal-like 2

BTLA - T lymphocyte attenuator

CDK - cyclin-dependent kinase

Chk1 - Checkpoint kinase 1

CP - cyclophosphamide

CR - caloric restriction

CRP - C-reactive protein

CSC - cancer stem cell

CTLA-4 - cytotoxic T lymphocyte antigen 4

CVD - cardiovascular disease

DBS - DNA double-strand breaks

DDR - DNA-damage response

DHH - desert Hedgehog

DSR - Differential Stress Resistance

DSS - Differential Stress Sensitization

DXR - doxorubicin

EGF - epidermal growth factor

EMT - epithelial-mesenchymal transition

EMT epithelial-mesenchymal transition

ER - estrogen

$ER^+$BC - ER positive breast cancer

ET - endocrine therapy

FGF - fibroblast growth factor

FMD - Fasting Mimicking Diet

FZD - frizzled family protein

GH/IGF-1 - growth hormone/insulin growth factor-1

GHR - growth hormone receptor

GLUT - glucose transporters

GSI - γ-secretase inhibitor

HER2 - human epidermal growth factor receptor 2

$HER2^+$BC - HER2 positive breast cancer

HIF-1α - hypoxia-inducible factor-1α

HK - hexokinase

HR - homologous recombination

IF - intermittent fasting

IGFBP1 - IGF1 binding protein 1

IHH - Indian Hedgehog

IIS - insulin/insulin-like growth factor signaling pathway

IM - immunomodulatory

JAK/STAT - janus family of kinases/signal transducer and activator of transcription

LAR - luminal androgen receptor

LRP - low-density lipoprotein receptor-related protein

MAPK/ERK - mitogen-activated protein kinase/extracellular signal-regulated kinase

MCL1 - myeloid cell leukemia 1

MDR - multidrug resistance

MEK1/2 - mitogen-activated protein kinase 1/2

MHCI - major histocompatibility class I

MRP1/ABCC1 - multi-drug-resistant protein 1

MS - metabolic syndrome

MSL - mesenchymal stem-like

mtDNA - mitochondrial DNA

MUC-1 - glycosylated form of mucin 1

NAC - neoadjuvant chemotherapy

NSAID - non-steroidal anti-inflammatory drug

OCR – oxygen consumption rate

OS - overall survival

OXPHOS - oxidative phosphorylation

PARP - poly ADP ribose polymerase

pCR - pathological complete response

PD-1 - programmed cell death protein 1

PDGF - platelet derived growth factor

PF - prolonged fasting

PFS - progression free survival

PI3K - phosphatidylinositol-3 kinase

PKA - adenylate cyclase-protein kinase A

PKB - protein kinase B

PR - progesterone

ROS - reactive oxygen species

RTKs - receptor tyrosine kinases

S6K - ribosomal protein S6 kinase

SASP - senescence-associated secretory phenotype

SHH - sonic Hedgehog

SRC - sarcoma family kinase

SRSP - self-renewal signaling pathway

SSB - single strand breaks

STS - Short-Term Starvation

TGFβ - transforming growth factor β

TIC - tumor initiating cells

TIL - tumor infiltrating lymphocytes

TLR - Toll-like receptor

TNBC - triple negative breast cancers

TOR - target of rapamycin

TP53 - tumor protein 53

TSH - thyroid stimulating hormone

VEGF - vascular endothelial growth factor

WBI - whole breast irradiation

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**Introduction**



**Results**

# ABSTRACT

Approximately 10-15% of breast carcinomas are classified as triple receptor-negative breast cancer (TNBC) subtype because of the lack of expression of hormone receptors. Despite the advent of new therapeutic strategies, tumor relapses remain the major challenge in TNBC management. Several studies show that treatment failure and cancer recurrence are primarily due to drug resistance acquisition and self-renewal that are specific properties of cancer stem cells (CSCs). Here I show that a fasting mimicking diet (FMD) reduces the percentage of the staminal population in mouse models of TNBC, increasing cancer free survival, and that the mechanism through which it affects CSCs is glucose dependent and mediated, at least in part, by the down-regulation of the protein kinase A (PKA) pathway. Moreover, the use of RNA-seq analysis on TNBC tumor masses, after FMD, allowed the identification of druggable escape pathways, in particular PI3K/AKT, mTOR and CCND/CDK4-6 axis, activated selectively by differentiated cells. My results show that addition of FMD to inhibitors of these pathways promotes TNBC regression, leading to complete tumor shrinkage. Notably, FMD protects also from hyperglycemia induced by PI3K pathway inhibitors, preventing side effects associated with it. Taken together, these data indicate that FMD has wide but differential effects reaching normal as well as differentiated cancer cells and CSCs, thus representing a promising strategy for the treatment of TNBC, which can be hopefully translated into the clinic.

# INTRODUCTION

## 1. Aging and age-related diseases

Aging is a complex process which affects function at the molecular, organelle, cellular and extracellular levels. It is characterized by a progressive tissue degeneration that impairs the structure and function of vital organs and increases sensitivity to chronic diseases and death (Kirkwood TB, 2005; Longo VD et al., 2008; Fontana L et al., 2010). Aging is determined by a time-dependent accumulation of cellular and molecular damage, the consequent alterations in gene expression and epigenetic factors due to DNA damage and structural modifications of the DNA, including telomere shortening (Campisi J et al., 2001). However, these are just a few among the many alterations associated with aging (Figure 1) (López-Otín C et al., 2013).

Hayflick suggested that aging is not a disease itself, but a process that increases susceptibility to disease (Hayflick L, 1965). Age related pathologies include a wide range of diseases, among which cardiovascular diseases, type 2 diabetes, pulmonary fibrosis, neurological disorders, cognitive decline and cancer; more than 70% of people over 65 experiences at least two chronic conditions (Hung WW et al., 2011; Fabbri E et al., 2015). Although in the last 100 years the average life expectancy in humans has raised drastically, this has not been associated with an equivalent improvement in health-span (Hung WW et al., 2011). According to the World Health Organization, within 2050 the absolute number of people over the age of 60 years is expected to increase from 605 million to 2 billion, with a consequent increase in frailty due to age-related disorders but also in age-related diseases including cancer (Fontana L et al., 2014). For this purpose, the study of aging process is necessary to identify possible approaches to slow aging, to delay and prevent disease onset for many chronic conditions of adult and old age/ age-related (Longo VD et al., 2015).

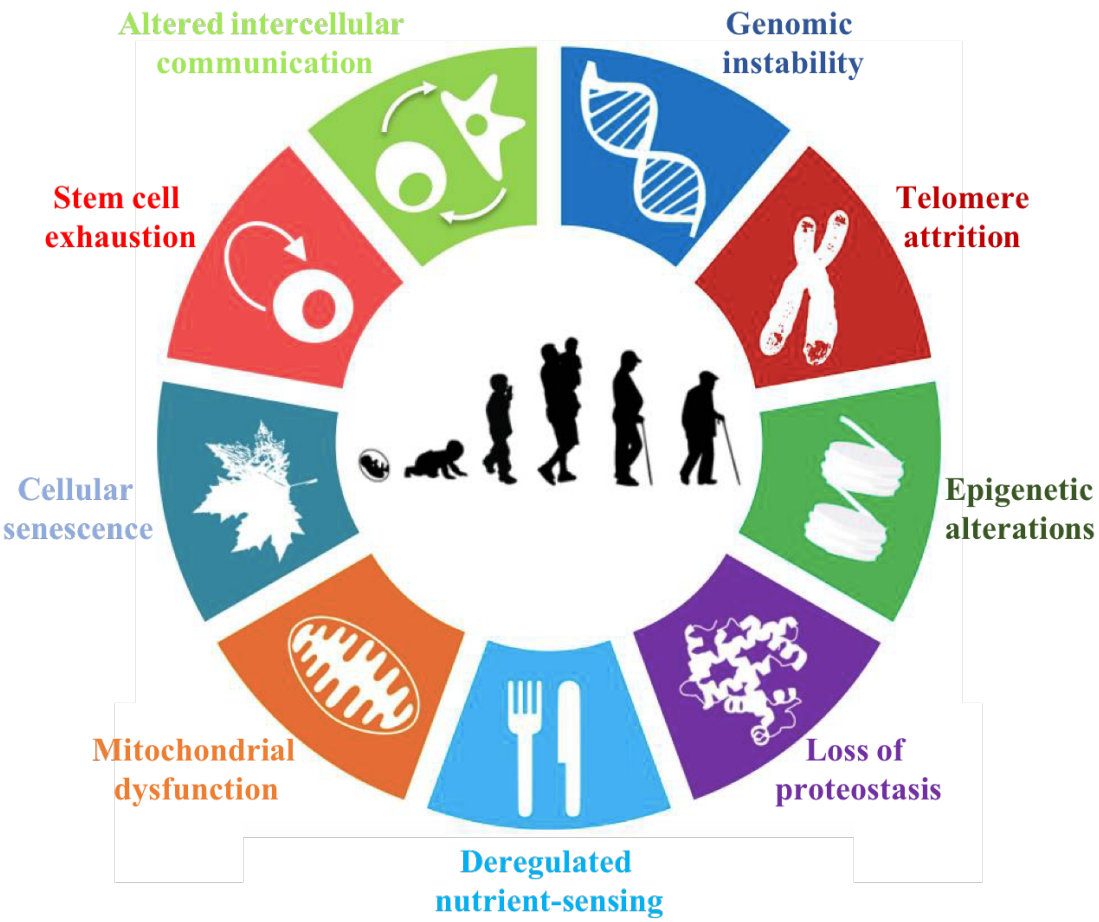



**Figure 1. The hallmarks of aging.** Nine hallmarks are involved in the aging process: altered intercellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion. (adapted from López-Otín C et al., *Cell*, 2013).

## 1.1 Molecular basis of aging

Aging is highly complex process which involves multiple mechanisms at different levels and which has been explained by different theories:

*Somatic mutation theory* sustain that the DNA repair is the major determinant of the rate of aging at cellular and molecular level; in fact, numerous studies reported that aging is associated to an increase in somatic mutations and other forms of DNA damage, suggesting a relationship between longevity and DNA repair (Promislow DE, 1994). It has been demonstrated that high activity of the enzyme poly (ADP-ribose) polymerase-1

(PARP-1), a key player of DNA repair machinery, is associated with longer life span in different species (Grube K and Burkle A, 1992).
In addition, aging is explained through the *replicative senescence theory*, since every cell division is followed by an incomplete duplication of the telomeres (Hayflick, 1965; Saretzi G and Von Zglinicki T, 2002). Telomeres are regions at the end of chromosomes that protect DNA from degradation and recombination, supporting genome stability (Chan SR and Blackburn EH, 2004). In most mammalian somatic cells, telomeres shorten with each cell cycle leading to a progressive loss of telomere protective sequences. This process is due to the lack of telomerase, an enzyme normally expressed only in germ cells and in a few adult stem cells, which protect the end of the chromosome from DNA damage by adding telomeric DNA repeats (Kim SH et al., 2002; Von Zglinicki T, 2002). Therefore, cell divisions result in telomere shortening of chromosomes until cells are no longer able to divide and enter in a cell state defined as cellular senescence (Hayflick L, 1965). Senescence is a form of long-term cell-cycle arrest, caused by DNA damage and elevated level of oxidative stress, accompanied by suppressed apoptosis and secretion of multiple factors (the senescence-associated secretory phenotype, SASP). A persistent upregulated SASP is involved in the development of age-related diseases (Franceschi C et al., 2000). The progressive accumulation of senescent cells is in fact considered a hallmark of aging (López-Otín C et al., 2013). Recent studies show that the ablation of senescent cells extends lifespan and health span (Zhu Y et al., 2015; Xu M et al., 2018).

The *mitochondrial theory* shows the connection between aging and the accumulation of mitochondrial DNA (mtDNA) mutations (Wallace DC, 1999). Cells with high level of mtDNA mutation result to suffer from impaired ATP production which leads to tissue failure (Brierley EJ et al., 1998; Cottrel DA et al., 2001; Taylor RW et al., 2003).

The *altered protein theory* finds that the accumulation of damaged proteins, due to the age-related impairment of protein turnover, contributes to different age-related disorders, such as Alzheimer's and Parkinson's diseases (Powers et al., 2009; Hartl FU et al., 2011). Proteostasis involves the functions of different mechanisms, the autophagy-lysosomal system and the ubiquitin-proteasome system, which help to restore or remove and degrade damaged and ubiquitinated proteins (Hartl FU et al., 2011). There is evidence that both systems decline with age (Calderwood SK et al., 2009; Rubinsztein DC et al., 2011).

Most of gerontologists supports theories of aging as non-adaptive, due to stochastic accumulation of damages at cellular level. Recent studies show that longevity is genetically determined and depends on evolutionary conserved pathways. These findings have contributed to the Programmed Longevity Theory, which proposes that aging is the result of the end or the weakening of a longevity program that ensures that all of the cells and systems of an organism function in a highly effective way until a specific age at which reproduction is expected to have been completed or optimized (Longo VD et al., 2005)

## 2. Dietary interventions to slow aging and age-related diseases

A large body of studies show that caloric restriction (CR), a dietary intervention that reduces calorie intake without incurring malnutrition, extends lifespan and retards age-related chronic diseases. Evidence that CR retards aging was first presented by McCay in 1930 (McCay CM et al., 1935). Thereafter, similar observations have been made in different organisms including yeast, flies, worms, rodents and monkeys (Barrows CH and Kokkonen G, 1978; Weindruch R and Walford RL, 1988; Lane MA et al., 2002; Wei M et al., 2008; Anderson RM et al., 2009; Grandison RC et al., 2009; Fontana L et al., 2010; Colman RJ et al., 2009; Colman RJ et al., 2014). A caloric restricted diet was found to extend lifespan and protect against age-related disorders and decline in functions in mice and monkeys (Anderson RM et al., 2009), while in humans it slows metabolism, decreases oxidative damages and reduces risk factors for diabetes, cardiovascular diseases and cancer (Fontana L and Klein S, 2007).

Aging, age-related diseases and the subsequent mortality can be promoted by the activation of nutrient sensing pathways that regulate metabolism and growth and that are down-regulated by CR. Mutations or inactivation of these pathways can mimic CR effects on health and longevity (Figure 2) (Fontana L et al., 2010).

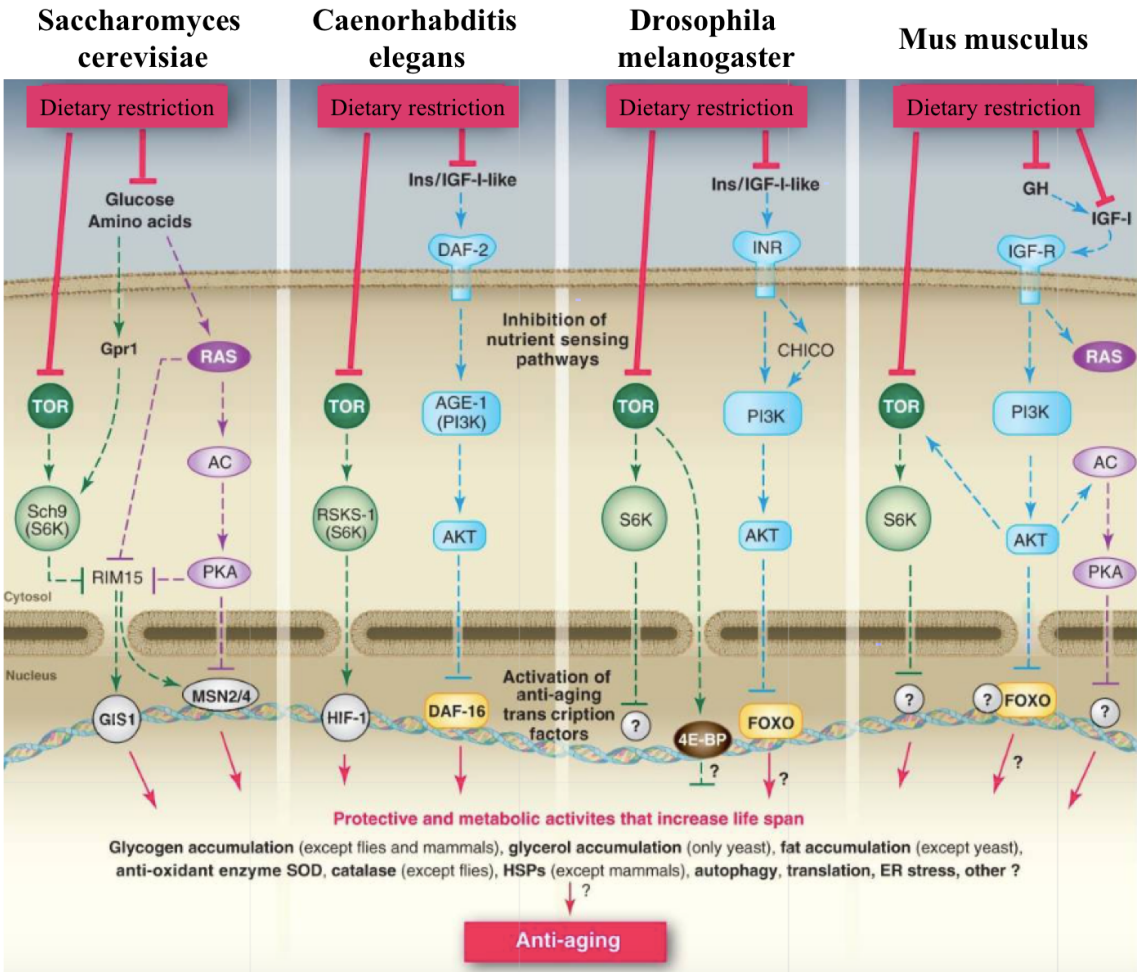



**Figure 2. Dietary restriction effects on aging.** Regulation of nutrient sensing pathways mediated by caloric restriction, in different models (adapted from Fontana et al., *Science*, 2010).

The growth hormone/insulin growth factor-1 (GH/IGF-1) pathway and its downstream effectors such as target of rapamycin (TOR), ribosomal protein S6 kinase (S6K) and the adenylate cyclase-protein kinase A (PKA) promote aging in different eukaryotic model organisms (Fontana L et al., 2010).

Yeast and worms were the first organisms used to study the role of nutrient sensing pathways in aging. Chronological lifespan studies in *Saccharomyces cerevisiae* led to the identification of two life span regulatory pathways, the axis between TOR and the serine-threonine kinase Sch9, which is the homologous version of the human protein kinase B (PKB), also known as Akt, and the axis between Ras, adenylate cyclase (AC) and PKA pathways. In presence of glucose and other nutrients, these pathways are activated and inhibit the serine/threonine kinase Rim15, an important meiotic regulator, and

consequently the activity of stress resistance transcription factors Msn2/Msn4, which play an important role in lifespan regulation (Mirzaei H et al., 2014). The deletion of the gene coding for Sch9 extends chronological lifespan, reduces genome instability and promotes stress resistance (Fabrizio P et al., 2001; Fabrizio P et al., 2004; Madia F et al., 2008). The inhibition of both TOR-Sch9 and Ras-AC-PKA axis are also implicated in dietary restriction-dependent increase of chronological lifespan (Wei M et al., 2008). The effect of the insulin-IGF-1 pathway on longevity was first demonstrated in the nematode worm *Caenorabditis elegans* (Johnson TE, 1990; Kenyon C et al., 1993). It has been found that reduced activity of the insulin/insulin-like growth factor signaling pathway (IIS) and the consequent activation of the Forkhead FoxO transcription factor daf-16, a regulator of genes involved in defensive activities such as cellular stress response, increase lifespan in worms. The TOR pathway interacts with IIS and, as in yeast, TOR and S6 kinase reduction contributes to extend lifespan in *C.elegans*. (Johnson, 2008; Hansen M, 2008). Reduced IIS activity and down-regulation of TOR pathway can increase lifespan also in the fruit fly *Drosophila melanogaster* (Kapahi P et al., 2004; Piper MD et al., 2008; Bjedov I et al., 2010).

As in yeast, worms and flies, reduced activity of nutrient sensing pathways can increase lifespan also in mice. Mutations in GH and IIS genes, mTOR pathway inhibition by rapamycin or deletion of S6K1 extend lifespan in mice, as in other model organisms, reducing incidence of age-related disorders including bone, immune, motor dysfunctions and insulin resistance (Selman C et al., 2009; Fontana L et al., 2010). As in yeast, disruption of PKA signaling also causes life span extension in mice, in which it also causes reduction in age dependent tumors (Fabrizio P et al., 2001; Enns LC et al., 2009). Mice deficient in GH and IGF-1 plasma level present a 50% increase in life span (Brown-Borg HM et al., 1996; Coschigano KT et al., 2000; Holzenberger M et al., 2003). Mice carrying homozygous mutations in the Prop-1 and Pit-1 genes are deficient in the generation of the anterior pituitary cells that produce GH, thyroid stimulating hormone (TSH) and prolactin, and are consequently one third of the size of control mice but survive more than 40% longer (Brown-Borg HM et al., 1996). Furthermore, dwarf mice with high GH plasma level but a 90% lower circulating IGF-1 show an increase in life expectancy and mice lacking one copy of IGF-1 receptor (IGF-1R) live 33% longer than their wild type controls (Coschigano KT et al., 2000; Holzenberger M et al., 2003). Similarly, dietary restricted mice present a 60% increase in lifespan in part by delaying the occurrence of many chronic diseases (Anderson RM et al., 2009). Restriction of specific

amino acids delays tumor incidence, decrease glucose, insulin and IGF-1 concentrations at serum level and the production of reactive oxygen species, inducing less oxidative damage (Orentreich N et al., 1993; Miller RA et al., 2005; Ayala V et al., 2007).
Taken together, all these studies show that these nutrient sensing pathways may play a partially conserved role in the regulation of aging and age-related disorders in organism ranging from yeast to mice (Longo VD and Finch CE, 2003).
Alterations in GH-IGF-1 axis have been studied also in humans; human Laron syndrome is caused by mutation in growth hormone receptor (GHR) which interrupts functional GH signaling, thereby lowering secretion of IGF-I by the liver. Studies on Laron syndrome patients (growth hormone receptor deficient or GHRD) show that these individuals displayed very low cancer mortality or diabetes rates, but they do not reach the 40% lifespan extension observed in GHRD mice, possibly due to overeating and obesity (Guevara-Aguirre J et al., 2011).

## 2.1 Fasting, Fasting Mimicking Diet (FMD) and aging

CR restriction is defined as a continuous reduction of the daily caloric intake on the order of 20-40%, without causing malnutrition, and is associated with an increase in lifespan and health in organisms ranging from yeast to mammals (Colman RJ et al., 2009; Kenyon CJ, 2010; Signer RA and Morrison SJ, 2013).
Differently from CR, fasting is the most extreme of the dietary interventions; in fact, it involves the complete elimination of nutrients. There are different forms of fasting normally used on animal models, rodents and lower eukaryotes in particular: the intermittent or alternate day fasting (IF) which requires 24 hours cycles during which water but not food can be consumed, on every other day for long periods of time (Trepanowsky JF et al., 2011), and the prolonged fasting (PF) which involves 2 or more days cycles of water only fasting at least one week apart (Longo VD and Mattson MP, 2014).
Fasting has been shown to extend lifespan (Figure 3) and age-related disorders in different model organisms (Longo VD and Mattson MP, 2014).

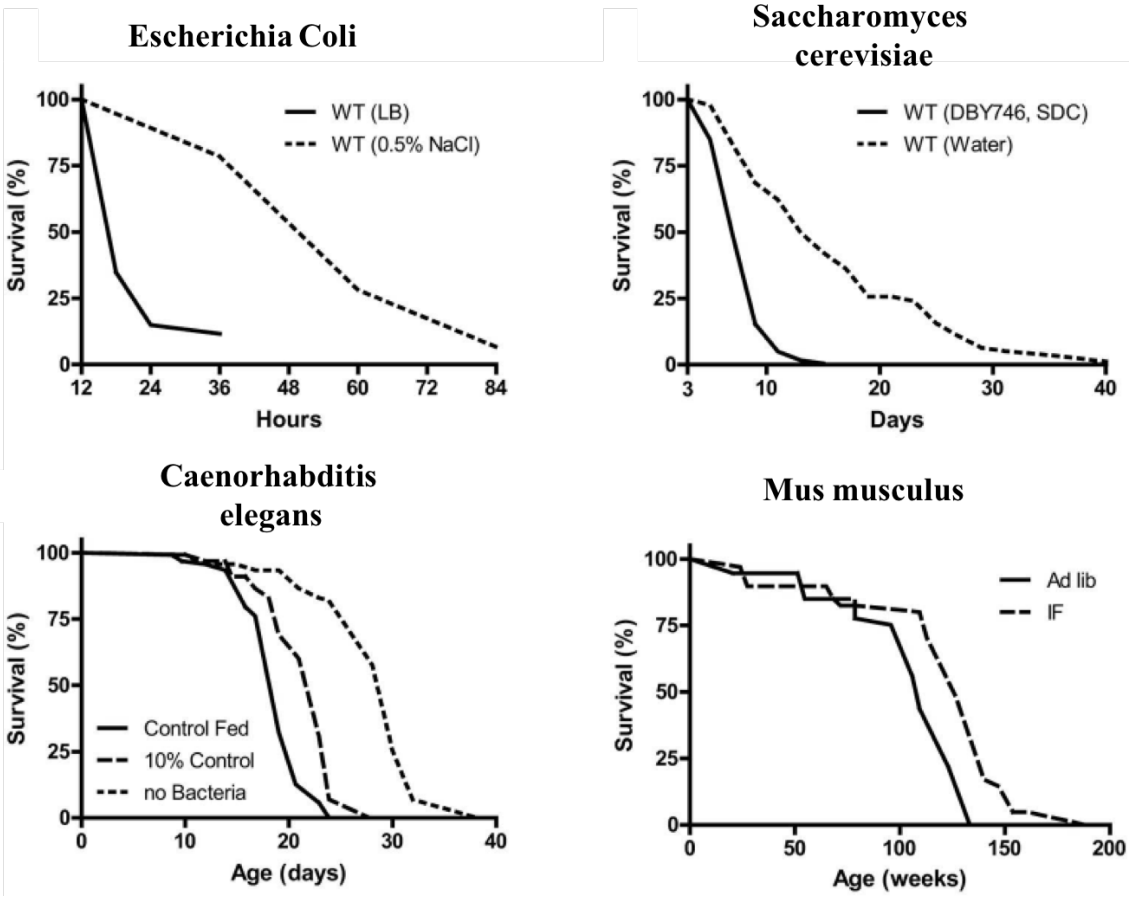



**Figure 3. Different types of fasting prolong survival in several model organisms**. Nutrient free medium in Escherichia Coli, water in Saccharomyces Cerevisiae, medium with a 90% reduction or complete removal of bacterial food in Caenorhabditis elegans and intermittent day fasting in mice, extend life span.

In yeast *Saccharomyces cerevisiae,* the switch from medium supplemented with glucose to water leads to the downregulation of the TOR-S6K and Ras-AC-PKA nutrient sensing pathways followed by the activation of the stress resistance transcription factors Msn2/4. These mechanisms promote stress resistance and longevity increase (Wei M et al., 2008). Mice subjected to IF cycles exhibit less neuronal dysfunction and degeneration, a delay in the progression of myocardial infarction, diabetes, stroke, Alzheimer's and Parkinson's diseases (Duan W and Mattson MP, 1999; Longo VD and Mattson MP, 2014; Mattson MP, 2014). IF, in rodents, prevents and reverses all aspects of metabolic syndrome (MS), reduces abdominal fat, blood pressure, inflammation, insulin resistance and protects against ischemic renal and liver injury (Wan R et al., 2003; Castello L et al., 2010). On the other hand, PF cycles protects mice against cancer progression and chemotherapy

adverse effects and to promote stem cell regeneration and immune system rejuvenation (Longo VD and Mattson MP., 2014; Cheng CW et al., 2014).

In addition, PF causes a 30% and 40% decrease in circulating insulin and glucose respectively, a decline in IGF-1 levels by 70%, causes the downregulation of TOR-SK6 and Ras-AC-PKA nutrient signaling pathways, such as in yeast, and decreases the phosphatidylinositol-3 kinase (PI3K)-AKT pathway activity, which is a key regulator of cell cycle (Lee C et al., 2010; Cheng CW et al., 2014).

In humans, 5 days of fasting can lead to a major decrease in circulating IGF-1 and a 5-fold increase in IGF binding protein 1(IGFBP1). This effect is mediated largely by protein restriction; therefore, chronic caloric restriction may not lead to IGF-1 decrease unless combined with protein restriction (Thissen JP et al., 1994, Fontana L et al., 2008; Fontana L et al., 2010).

Periodic fasting cycles provide a much more viable strategy than continuous CR to achieve beneficial effects against aging and disease. However minor side effects have been reported, such as headache, nausea, anemia and weakness, especially in frail subjects (Thomson TJ et al., 1966; Lee C and Longo VD, 2011). Thus, water-only fasting remain a challenging option for the majority of population. For this reason, our laboratories have recently identified a periodic, short-term, dietary intervention which mimic the metabolic effect generated by fasting, the Fasting Mimicking Diet (FMD). FMD is a low-calorie diet composed by low levels of protein and sugar, and high levels of unsaturated fats. The effect of FMD on health, longevity and age-related diseases, such as diabetes and cancer, was tested in multiple mouse studies. Middle-aged mice subjected bimonthly to 4 days of FMD twice a month display a 40% decrease in blood glucose levels and a ~9-fold increase in ketone bodies production; moreover, FMD in mice reduces insulin level and reduce IGF-1 by 45% while increases IGFBP-1 by 8-fold, similarly to what happens during 72h of fasting. After a single cycle of FMD, these markers return to normal levels within one week of refeeding (Brandhorst S et al., 2015). In addition, cycles of a 4 day FMD extend lifespan, reduce visceral fat deposits with a consequent reduction in body weight, lead to a decrease in kidneys, heart and liver weight, possibly promoting their regeneration upon refeeding, retard bone mineral density loss and restore insulin secretion and glucose homeostasis in type 1 and 2 diabetes mouse models (Brandhorst S et al., 2015). Studies in middle aged-mice found also that FMD can reduce tumor incidence by 45%, while protecting against inflammation and inflammation-associated skin lesions (Brandhorst S et al., 2015), promoting immune system regeneration and rejuvenation (Cheng CW et al.,

2014), improving motor learning and hippocampus-dependent short and long-term memory and promoting neurogenesis, probably through the reduction in IGF-1 levels and PKA pathway activity (Cheng CW et al., 2014; Brandhorst S et al., 2015).
In two recent clinical trial, healthy humans subjected to 5 days of FMD every month for 3 months, displayed a 3% reduction in body weight and trunk fat percentage, accompanied by an increase in relative lean body mass, indicating that only fat mass is lost. Moreover, FMD cycles reduced serum glucose by 11.3% and IGF-1 by 24%, decreased serum level of C-reactive protein (CRP) which is a marker of inflammation and a risk factor for cardiovascular disease (CVD) and increase mesenchymal stem and progenitor cells in the peripheral blood mono-nucleated cell population (Brandhorst S et al., 2015; Wei M et al., 2017). It was found that markers and risk factors for aging and age-related disorders were more beneficially affected in subjects at risk of age-related disease than in subjects who were not at risk (Wei M et al., 2017).
Other clinical trials evaluating FMD effects on patients with diagnosed diseases are in progress.

## 2.2 CR, Fasting, FMD and cancer

There is mounting evidence that high level of IGF-1 promotes mutations and neoplastic lesions in several model organisms (Kennedy MA et al., 2003; Guevara-Aguirre J et al., 2011). The possible involvement of IGF-1 in cancer was first demonstrated in *in vitro* studies which show that IGF-1 enhances the growth of different cancer cell lines, acting directly on cells through the IGF-1R which is normally overexpressed in many kinds of tumors (Macaulay WM, 1992; LeRoith D et al., 1995). Furthermore, epidemiologic analysis shows that high level of IGF-1 is associated with an increased risk of several cancers including colorectal, breast and prostate cancer in people older than 40 (Chan JM et al., 2000; Yu H and Rohan T, 2000).
Chronic moderate CR is known to reduce IGF-1 at serum level by 30-40%and to inhibit a variety of spontaneous and chemically induced neoplasia by more than 50% in mice (Weindruch R and Walford RL, 1982; Gross L and Dreyfuss Y, 1984; Gross L and Dreyfuss Y, 1990). Studies extended to primates, the *rhesus monkeys*, show that 30% CR delays disease onset and death, in part by reducing cancer incidence by 50% (Colman RJ et al., 2009). These findings suggest that the reduction of IGF-1 level induced by CR is fundamental to counteract tumor growth (Longo VD and Fontana L, 2010). However, the

decrease in blood glucose and IGF-1 levels caused by dietary restriction is 15% and 25% respectively, a very small reduction compared with the decrease induced by 2-5 days of fasting (75%) (Lee C et al., 2010).

Furthermore, in humans, a caloric-restricted regimen causes a progressive loss of weight and muscle mass and impairs immune function, resulting in a significant risk to cancer patients receiving chemotherapy or surgery (Reed MJ et al., 1996; Kristan DM et al., 2008; Fontana L et al., 2010). Thus, a 3-5 days fasting period followed by several weeks of a normal diet represents a more powerful and feasible option for oncological patients.

Our laboratory showed that an *in vitro* condition mimicking fasting, referred as Short-Term Starvation (STS) and characterized by low levels of glucose and serum, enhances the efficacy of chemotherapeutic agents on different types of cancer cells while inducing the protection of normal cells from the toxic side effects and protects normal cells but not a wide range of cancer cells from reactive oxygen species (ROS) inducing agents, such as hydrogen peroxide (Raffaghello L et al., 2008). These phenomena are known as "Differential Stress Sensitization" (DSS), due to the incapability of cancer cells to reprogram their cell cycle in accordance to nutrients availability, and "Differential Stress Resistance" (DSR), based on the ability of all cells and organisms but not cancer cells to enter a low or no division high protection mode in nutrient-low environments (Raffaghello L et al., 2008; Lee C et al., 2012; Longo VD and Mattson MP, 2014; Brandhorst S et al., 2015; Di Biase S et al., 2016). Fasting induces DSS both in *in vitro* and *in vivo* models in part by reducing PKA activity, circulating IGF-1 and glucose levels and by regulating genes involved in DNA repair (REV1) and cell death (p53) (Raffaghello L et al., 2008; Lee C et al., 2010; Lee C et al., 2012; Cheng CW et al., 2014). In particular, we found that *in vitro* STS sensitizes a wide range of cancer cells to doxorubicin (DXR) and cyclophosphamide (CP) while protecting normal cells from side effects (Raffaghello L et al., 2008; Lee C et al., 2012). In the same way, 48-60 hours of fasting induces DSS against oxidative stress and chemotherapy also in xenograft and allograft mouse models (Raffaghello L et al., 2008; Lee C et al., 2012). We found that 2 cycles of 48 hours fasting are as effective as 2 cycles of chemotherapy treatment in retarding tumor growth in syngeneic mouse models injected with murine breast (4T1), melanoma (B16), glioma (GL26) cancers and xenograft of human breast (MDA231) and ovarian (OVCAR3) cancer cell lines. The greatest therapeutic outcome is observed after combining fasting cycles with chemotherapy (Figure 4) (Lee C et al., 2012).

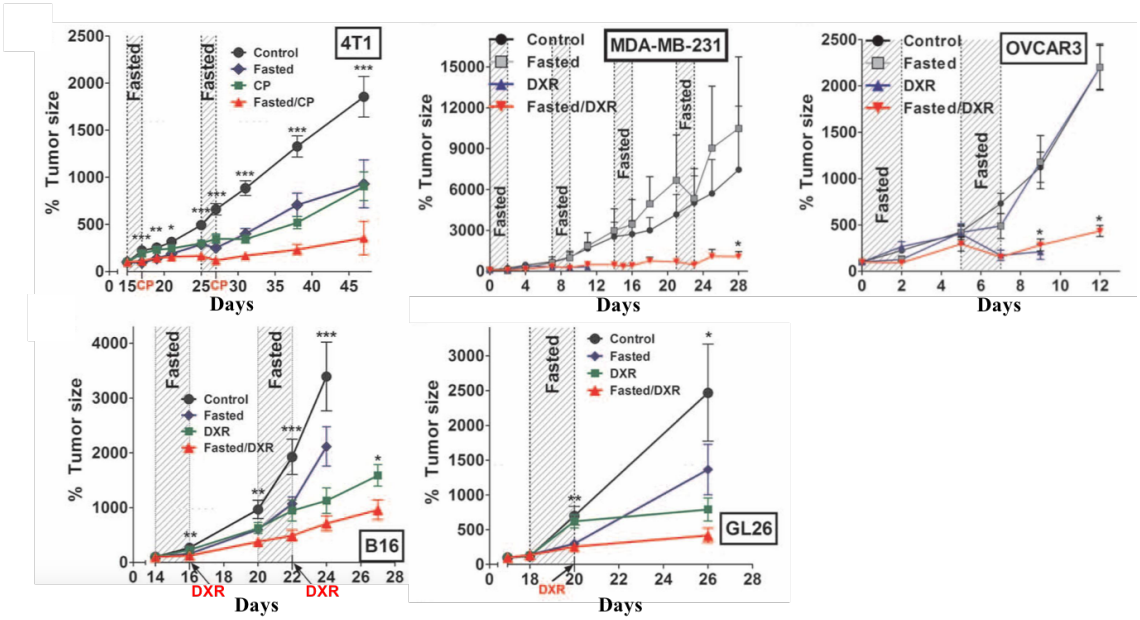



**Figure 4. Fasting sensitizes tumors to chemotherapeutic agents in mouse models.** Fasting cycles potentiate the effect of doxorubicin (DXR) and cyclophosphamide (CP) in delaying tumor progression in xenograft models of human breast (MDA231) and ovarian (OVCAR3) cancers and on allograft models of murine breast (4T1), melanoma (B16) and glioma (GL26) cancers (adapted from Lee C et al., *Sci Transl Med*, 2012).

Furthermore, we found that fasting can enhance the survival of mice with metastatic breast, melanoma and neuroblastoma cancers receiving chemotherapy (Lee C et al., 2012).

In addition, fasting promote the switch of cancer cell metabolism from aerobic glycolysis to oxidative phosphorylation (OXPHOS), generating an "anti-Warburg effect", leading to an increase of ROS production in tumor cells (Lee C et al., 2012; Bianchi G et al., 2015). The increase in ROS induced by fasting contributes to make cancer cells more sensitive to chemotherapy (Lee C et al., 2012).

Moreover, our laboratory found that FMD cycles alone or combined with chemotherapy, is as effective as water only fasting in reducing tumor progression in syngeneic mice injected with murine breast and melanoma cancer cells (Figure 5) (Di Biase S et al., 2016). FMD, as fasting, reverses chemotherapy-induced immunosuppression; in fact, we found that FMD in combination with DXR promotes the accumulation of CD8+ tumor-infiltrating lymphocytes (TIL) in the tumor bed and reduces tumor-associated Tregs, in part through the downregulation of Heme-Oxygenase 1 (HO-1) (Di Biase S et al., 2016). Moreover, selective depletion of CD8+ TIL reverses the combinatory effect of FMD and

DXR on tumor progression, demonstrating the key role of TIL in FMD-mediated DSS to chemotherapy (Di Biase S et al., 2016).

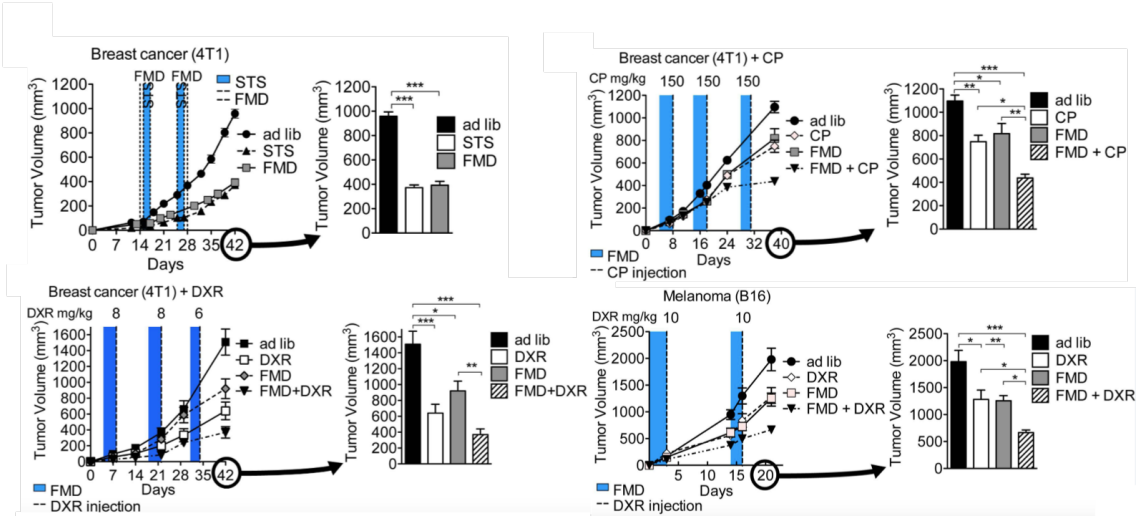



**Figure 5. FMD has the same effect of STS in delaying tumor progression, both alone or combined with chemotherapy.** FMD cycles are as effective as STS in reducing tumor growth on allograft models of murine breast (4T1) and melanoma (B16) cancers, alone or in combination with chemotherapy (adapted from Di Biase et al., *Cancer Cell*, 2016)

These findings suggest that fasting conditions have the potential to enhance the efficacy of standard cancer therapy and provide a foundation for an effect of FMD cycles in providing an alternative to chemotherapy to treat cancer at early stages or to decrease the risk for recurrence (Lee C et al.,2012). Recent studies have shown that FMD is safe and feasible when combined with standard anti-cancer therapies and one recent clinical trial reports the beneficial effects of FMD as an adjunct to neoadjuvant chemotherapy in breast cancer patients (de Groot S et al., 2020).

# 3. Breast cancer

## 3.1 Breast cancer incidence and subtypes

Breast cancer is the most frequently diagnosed and the second leading cause of tumor mortality in women worldwide after lung cancer (American Cancer Society, 2010). Breast cancer accounts for about a 25% of all cancer and 15% of cancer death in women globally. 1 in 8 women will develop breast cancer in their lifetime, and the risk of developing breast cancer increases with age (Siegel RL et al., 2018). It remains a major challenge, mainly due to its heterogeneity; in fact, breast cancer greatly differs among different patients, called inter-tumor heterogeneity, and within each tumor, called intra-tumor heterogeneity, due to the presence of heterogeneous cell populations within an individual tumor (Ellsworth RE., 2016; Turashvili G and Brogi E, 2017). Understanding the molecular and cellular mechanisms of tumor heterogeneity is relevant to make specific diagnosis and develop therapies for each kind of breast cancer (Blows FM et al., 2010).

Standard breast cancer treatment is based on several factors, including tumor morphology, clinical stage, tumor size, presence of lymph node metastases and biomarker profile, and is affected by the patient's age and menopausal status (Harris LN et al., 2016). Accurate grouping of breast tumors into different biological subtypes is fundamental to make specific therapeutic decision and evaluate the disease-specific outcome (Millar EK et al., 2009; Voduc KD et al., 2010).

The evaluation of standard biomarkers that can be assessed with immunohistochemistry analysis lead to the traditional classification of breast cancers in estrogen (ER) and progesterone (PR) positive, human epidermal growth factor receptor 2 (HER2) positive and triple negative phenotype (TNP) (Figure 6). ER, PR and HER2 are cell surface receptors that promote cell growth, differentiation and metastasis formation when overexpressed. These biomarkers are consolidated predictive and prognostic factors and their expression is fundamental to determine a specific patient therapy (EBCTCG, 2005; Harris LN et al., 2016).

HER2 is a transmembrane tyrosine kinase receptor and is overexpressed in approximately 15-20% of primary breast carcinomas (Slamon DJ et al., 1987). HER2 positive breast

cancer ($HER2^+$BC) shows high rate of response to anti-HER2 targeted therapy (Dean-Colomb W and Esteva FJ, 2008).

ER positive breast cancer ($ER^+$BC) is characterized by an overexpression of ER and usually, but not always, is matched by high levels of PR. Up to 80% of breast carcinomas are $ER^+$ and 60-70% are positive for PR expression (Harvey JM et al., 1999; Bardou VJ et al., 2003). $ER^+$BC co-express PR ($ER^+/PR^+$) in 70-80% of cases; the remaining 20-30% is $ER^+/PR^-$ or, rarely, $ER^-/PR^+$. Patients with ER+BC benefit from endocrine therapy, with the best response in $ER^+/PR^+$ tumors (approximate rate of 60%) (Bardou VJ et al., 2003). Depending on the concurrent expression or absence of HER2, ER+BC can be classified into Luminal A subtype (tumors with ER or PR positivity and HER2 negativity) and Luminal B subtype (tumors with ER or PR positivity and HER2 positivity) (Vallejos CS et al., 2010).

Breast cancers characterized by the absence of ER, PR and HER2 expression are defined triple negative breast cancers (TNBCs) or basal like tumors, and represent 15-20% of all invasive breast carcinomas. This is a histologically, genetically and clinically heterogeneous category of breast cancer, characterized by poor prognosis, mainly due to the lack of specific targeted therapies (Vuong D et al., 2014).

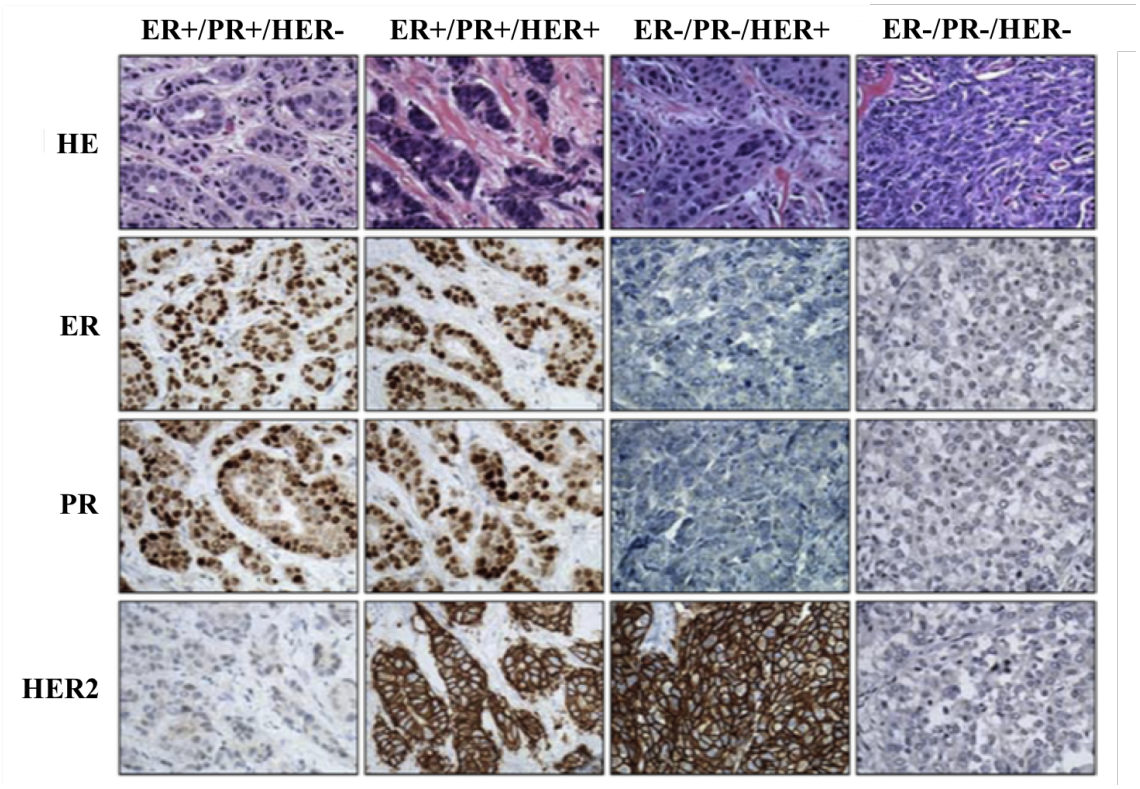


**Figure 6. Classification of breast cancers subtypes based on expression of ER, PR, HER2.** ER, PR, HER2 expression is visualized, through immunohistochemical analysis, in representative examples of invasive breast carcinomas (figure adapted from Rivenbark AG et al., *The American Journal of Pathology*, 2013**.**

Other biomarkers have been investigated for potential diagnostic, prognostic and therapeutic implications in breast cancer including Ki-67, a growth and proliferation factor, p53 or EZH2, that are invasion and metastasis biomarkers, WNT5A, a marker for epithelial-mesenchymal transition, and many others (Lee E. and Moon A., 2016).
The advent of high-throughput technologies for gene expression analysis, such as microarrays, show that cancer response to therapy is determined by molecular features that can be probed by molecular methods (Sotiriou and Pusztai, 2009; Weigelt B et al., 2010). The molecular portrait revealed by gene expression analysis proves that the variation in growth rate, in specific signaling pathway activity, in the cellular composition of the tumors are related to relevant variation in the expression of specific genes subsets (Perou CM et al., 2000). These findings allow to further sub-classify breast cancers and help clinicians in choosing the most effective treatment (Goldhirsch A et al., 2013). However, the clinical application of gene expression profiling remains difficult, mainly due to the lack of full standardization and its excessive cost.

## 3.2 Therapies for breast cancer

Breast cancer treatment is a multidisciplinary approach. To establish a diagnosis and make a decision on management of the primary tumor is relevant a diagnostic imaging work-up and biopsy analysis. Furthermore, also cancer stage and size and patient's age play a key role in selecting a specific therapy. For the majority of women with early-stage breast cancer, the best approach is a breast conserving surgery (BCS) accompanied by radiotherapy or mastectomy. These are both well-established local therapies for invasive breast cancer. Multiple clinical studies show that BCS has survival outcomes equivalent to mastectomy when patients present stage 1 and 2 breast cancers (Van Dongen JA et al., 2000; Fisher B et al., 2002; Veronesi U et al., 2002). BCS provides for excision of the tumor, called lumpectomy, followed by adjuvant whole breast irradiation (WBI). WBI is used to remove potential remaining microscopic disease in breast tissue; it's reported that WBI following lumpectomy reduces local recurrence rates by 50%, increasing patient's

survival (Poggi MM et al., 2003; Darby S et al., 2011). However, recent studies show a 10-22% increase in locoregional recurrence rates in patients subjected to BCS compared to patients undergoing mastectomy (Van Dongen JA et al., 2000; Fisher B et al., 2002; Poggi MM et al., 2003). Mastectomy is chosen when tumor is large relative to breast size or in case of serious connective tissue diseases, such as scleroderma which make patients sensitive to radiotherapy side effects. Patients with large tumor size can undergo to neoadjuvant chemotherapy (NAC), a treatment applied before surgery; NAC is used to reduce the tumor so it can be surgically removed with the purpose to facilitate breast conservation. Administration of NAC is also reported to significantly reduce axillary metastases rate in clinically node-negative women (Fisher B et al., 1998). A meta-analysis of patients treated with NAC compared to surgery followed by chemotherapy doesn't show differences in locoregional recurrence rate and survival, while a 17% decrease in the mastectomy rate is reported in patients receiving NAC (Mieog JS et al., 2007). However, adjuvant therapies are always recommended after surgical resection of the primary breast cancer to eradicate any potential micro-metastasis. Selection of adjuvant systemic treatments is based on different risk factors, including number of lymph nodes, tumor size and disease biology, determined by hormone receptor and HER2 status (EBCTCG, 2005).

Patients with hormone receptor positive breast cancers may be treated with endocrine therapy (ET), which comprises different classes of drugs targeting hormone receptors, such as ER in case of $ER^+$BC. Patients may be treated with ET for up to 5-10 years or longer, mainly as an adjuvant, but also in a neo-adjuvant setting. Tamoxifen is an example of ET used in premenopausal and postmenopausal women, while aromatase inhibitors, such as anastrozole, letrozole and exemestane, are only used in postmenopausal women (Burstein HJ et al., 2014). It is reported that tamoxifen reduces by 50% the risk of recurrence during the first years after surgery, with a continuous risk reduction of over 30% in later years (EBCTCG et al., 2011). Instead, in case of $HER2^+$BC, patients are treated with a HER2 targeted therapy, such as trastuzumab or pertuzumab, a monoclonal antibody directed against the HER2 receptor. Clinical trials show that trastuzumab plus chemotherapy reduces recurrence rate by 50% compared to chemotherapy alone (Piccart-Gebhart MJ et al., 2005; Romond EH et al., 2005; Perez EA et al., 2014; Cameron D et al., 2017).

Patients with TNBC do not benefit from ET or HER2 targeted therapy because of the lack of target receptors. Hence, surgery and chemotherapy represent the only treatment option.

# 4. Triple Negative Breast Cancer (TNBC)

TNBC is a very heterogeneous subtype of breast cancer characterized by poor prognosis. The term "triple negative" refers to the fact that cancer cells don't present ER or PR and HER2. TNBC occurs in approximately 15%-20% of all diagnosed breast cancers and affects predominantly women younger than 40 years of age, of African-American or Hispanic origin and those with a BRCA1 gene mutation (Anders CK and Carey LA, 2009; Carey LA et al., 2010). TNBC comes as an invasive, poor differentiated, highly proliferative ductal carcinoma, characterized by large overall size. It frequently metastasizes to lung and brain, unlike other breast cancer subtypes which usually disseminate to the bone and soft tissues (Dent R et al., 2007).

Clinical studies report that only 30-45% of patients with TNBC achieves a pathological complete response (pCR) after preoperative chemotherapy (Liedtke C et al., 2008; Masuda H et al., 2011). This is mainly due to TNBCs intra- and inter-tumor heterogeneity. Large-scale gene-expression studies allowed TNBCs to be subdivided in six subclasses: basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal-like (M), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR) subtype (Lehmann BD et al., 2011).

BL1 tumors present high levels of cell cycle and DNA-damage response (DDR) genes and are characterized by high expression of Ki67. Antimitotic agents such as taxanes (paclitaxel or docetaxel) and DNA-damaging agents such as cisplatin are the therapies most commonly used for this kind of cancer, and 52% of patients achieve a pCR (Henderson IC et al., 2003). Instead, patients with BL2 tumors rarely achieve a pCR; this TNBC subtype is characterized by high levels of proliferation genes, survival-mediated receptor tyrosine kinases (RTKs) and metabolic signaling genes.

IM tumors are enriched for immune response-mediated cell signaling with high expression of genes encoding immune antigens and cytokines. Gene expression analysis show that IM tumors are characterized by the presence of tumor infiltrating lymphocytes (TILs). It's reported that the high levels of TILs inside the tumor is associated with a better prognosis and increased rates of pCR (Liu F et al., 2012; Loi S et al., 2014). An immune-based therapy approach blocking immune-checkpoint receptors or targeting immunosuppressive factors is very effective for IM tumors (Stagg J and Allard B, 2013). However, only 30% of these patients achieve a pCR (Masuda H et al., 2013).

The M and MSL subclasses are characterized by increased expression of epithelial-mesenchymal transition (EMT) and growth factor pathways and low levels of genes involved in proliferation accompanied by a low mitotic index (Lehmann BD et al., 2011). In particular, MSL tumors present an up-regulation of transforming growth factor β (TGFβ) receptor type III (TGFβRIII) which is involved in cell migration and invasion (Jovanovic B et al., 2014). In vitro studies show that cell lines with this subtype result to be sensitive to sarcoma family kinase (SRC) and PI3K/mTOR inhibitors (Lehmann BD et al., 2011). pCR rates in patients with this kind of tumors is overall moderate, 23-31% (Masuda H et al., 2013).
The LAR subtype is enriched for hormone regulated signaling pathways, such as steroid synthesis and androgen receptor (AR) signaling. LAR tumors frequently present mutation in PI3K subunit α (PI3KCA) and preclinical studies report that this subtype benefit from combined treatments targeting AR and PI3K (Lehmann BD et al., 2014). LAR cancers are chemo-resistant and have a lower pCR rate (10%) with traditional neoadjuvant chemotherapy. However, pre-clinical and clinical studies are ongoing to investigate the effect of novel anti-androgenic agents, such as orteronel or bicalutamide (NCT03055312).

Treatment for TNBC remains a major clinical challenge, mainly due to its aggressiveness and heterogeneity (Lehmann BD et al., 2011; Shah SP et al., 2012). Tumor size, lymph node status, grade and overall performance status are to consider to decide the best therapeutic approach. Surgery is recommended for patients with resectable disease; however, as TNBCs grow rapidly and are locally aggressive, BCS followed by radiation therapy, even in early stage, is not equivalent to mastectomy as in other breast cancer phenotypes (Panoff JE et al., 2011). In non-metastatic settings, patients with TNBC > 0,5cm or node positivity are treated with neoadjuvant or adjuvant chemotherapy; unfortunately, these patients have a higher risk of relapses compared to other breast cancer subtypes and pCR is achieved in 30-45% of cases following chemotherapy (Liedke C et al., 2008; Von Minckwitz G et al., 2012). Neoadjuvant anthracycline-based chemotherapy increases pCR in TNBC compared to other luminal subtypes, and the addition of platinum compounds to standard chemotherapy is reported to double pCR rates (Petrelli F et al., 2014). However, patients who fail to achieve pCR exhibit worse outcomes than other breast cancer phenotypes (Liedke C et al., 2008).

Differently from other breast cancers, gene expression analysis didn't identify any specific target that could be used in therapy, although, TNBCs are characterized by many mutations, including tumor protein 53 (TP53) mutation or loss, amplification of myeloid cell leukemia 1 (MCL1), amplification of v-Myc avian myelocytomatosis viral oncogene homolog (c-MYC), mutation or loss of retinoblastoma 1 (RB1) and mutations in PI3KCA or phosphatase and tensin homolog (PTEN) (Shah SP et al., 2012). In addition, TNBCs may present also alteration of breast cancer -1 and -2 (BRCA1 and BRCA2) genes expression. Dysfunction of BRCA1 expression is reported to confer good prognosis to cisplatin treatment (Silver DP et al., 2010). Similarly, expression of CD73, a cell surface enzyme involved in tumor neovascularization and invasiveness, is associated with DXR resistance in TNBC patients (Loi S et al., 2013).

Altogether, these findings lay the basis to identify specific therapeutic strategies for TNBC by the presence of predictive biomarkers.

## 4.1 Potential targeted therapies for TNBC

PARP inhibitors

TNBC is often associated with significant genomic instability due to DNA-repair defects. Several studies show that up to 10-20% of patients under 50 years of age with TNBC carry germline or sporadic mutations in BRCA1 or BRCA2 (Hartman AR et al., 2012; Wong-Brown MW et al., 2015). These mutations affect the ability to repair DNA double-strand breaks (DBS) through the error-free homologous recombination (HR) repair mechanism. Thus, TNBC with BRCA1/2 mutations rely on the alternative non-homologous end-joining (alt-NHEJ) and base excision repair (BER) pathways for DDR, processes which require poly adenosine diphosphate (ADP)- ribose polymerase (PARP). PARP directly binds to the DNA single strand breaks (SSB) during BER; failure to repair SSBs leads to DSB during DNA replication. PARP inhibition results in HR dependency for repairing DSBs (Rouleau M et al., 2010; Gibson BA and Kraus WL, 2012; Horton JK and Wilson SH, 2013). Several studies show that PARP inhibitors veliparib and olaparib can delay tumor development, increasing levels of apoptosis (Lord and Ashworth, 2012; Gibson BA and Kraus WL, 2012; Horton JK and Wilson SH, 2013). Moreover, the combination of olaparib with paclitaxel increases significantly apoptosis rates in breast cancer cells (To et al., 2014). Clinical studies report that patients treated with olaparib

exhibit improved progression free survival (PFS) and overall survival (OS) than those treated with chemotherapy only (Robson M et al., 2017; Robson M et al., 2019).

RTKs inhibitors

RTKs are involved is several mechanisms including cell proliferation, differentiation, cell growth, cell metabolism and promote cell survival and apoptosis (Gschwind A et al., 2004). RTKs, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), TGFβ, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and IGF-1 receptors, are often elevated in cancers, therefore, they could be considered targets in TNBCs.

EGFR or HER1 is probably the most well-known protein overexpressed in several human cancers (Corkery B et al., 2009). Large-scale genomic analysis shows that 80% of TNBC display a constitutive activation of members of EGFR family and this is frequently associated with poor OS (Corkery B et al., 2009; Banerji S et al., 2012; Shah SP et al., 2012; Stephens PJ et al., 2012). Several agents targeting EGFR were approved for use in clinic, such as monoclonal antibodies cetuximab and panitumumab or small-molecule kinase inhibitors gefitinib and erlotinib, but both preclinical and clinical studies show modest activity. Cetuximab shows limited efficacy in combination with chemotherapy against advanced TNBC (Carey LA et al., 2012; Baselga J et al., 2013). Initially patients who received combination therapy had a higher response rate compared to patients receiving single treatments, but the combination minimally increased PFS and OS. Despite the modest results obtained in clinic, a preclinical study shows that cetuximab combined with radio-sensitizing chemotherapy and PARP inhibitor completely eradicates putative breast cancer stem cells (Al-Ejeh F et al., 2013).

The PDGF family is involved in the regulation of cell migration, proliferation and survival and is known to induce self-renewal capacity, while VEGF play a key role in angiogenesis process (Coltrera MD et al., 1995). PDGF and VEGF are highly expressed in tumor cells compared to normal mammary tissue and their overexpression in breast cancer is associated with advanced tumor stage at diagnosis, malignancy and poor prognosis (Linderholm BK et al., 2009). The monoclonal antibody bevacizumab, which targets specifically VEGF, and the small molecule kinase inhibitor sunitinib, which inhibits both PDGF and VEGF family members, were tested in preclinical and clinical studies. In particular, sunitinib reduces tumor progression in TNBC xenografts, while

bevacizumab added to paclitaxel chemotherapy increased PFS but not OS (Chinchar E et al., 2014, Kumler I et al., 2014; Yadav BS et al., 2014).

Proliferative and survival dependent pathways inhibitors

RTKs signaling intermediates including PI3K/AKT, mTOR, janus family of kinases/signal transducer and activator of transcription (JAK/STAT), the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, also known as RAS/RAF/MEK/ERK pathway, are potential therapeutic target for TNBC (Schlessinger J, 2014).

Aberrant MAPK activity is known to be involved in TNBC progression and its overexpression contributes to malignancy increasing cell proliferation and resistance to apoptosis (Mirzoeva OK et al., 2009; Duncan JS et al., 2012; Giltnane JM and Balko JM, 2014). Moreover, overexpression of mitogen-activated protein kinase 1/2 (MEK1/2) present a risk factor for TNBC. Pre-clinical studies showed that combining the MEK1/2 inhibitor selumetinib with docetaxel reduces tumor progression in xenografts model (Balko JM et al., 2012). However, clinical studies on MEK1/2 inhibitors reported only modest results (Kirkwood JM et al., 2012; Gogas HJ et al., 2019).

Moreover, the PI3K-AKT-mTOR axis is frequently mutated in TNBC. PI3K is part of a lipid kinases family that phosphorylates the 3-hydroxyl group of phopshoinositides involved in the regulation of many cellular processes, including proliferation, survival and motility (Fruman DA et al., 1998); AKT is an important regulator of pro-apoptotic molecules such as BCL-2 associated death promoter (BAD), supporting cell survival and growth in response to extracellular signals, while mTOR has a key role in the regulation of translation, protein turnover and cell survival. 60% of TNBC patients presents mutation in PI3K pathway: in particular PTEN loss and mutation of PI3KCA and TP53 are the most common (Kang S et al., 2005). AKT and mTOR hyper activation are associated with poor prognostic outcome. However, a clinical study reports that inhibition of PI3K-AKT-mTOR pathway, in combination with chemotherapy, improves PFS in patients with metastatic TNBCs (Ganesan P et al., 2014). Preclinical studies show that dual inhibition of AKT and mTOR pathways represents a promising strategy to treat TNBC (Gordon V and Banerji S, 2013; Montero JC et al., 2014). Moreover, PI3K suppression confers sensitivity to PARP inhibition, delaying tumor progression in TNBC xenografts models (Ibrahim YH et al., 2012; Juvekar A et al., 2012).

The deregulation of the JAK/STAT pathway is reported to play a key role in TNBC (Marotta LL et al., 2011; Britschgi A et al., 2012). Similarly to the PI3K-AKT-mTOR axis, the JAK/STAT pathway is involved in the regulation of cell proliferation, survival, migration, differentiation and apoptosis. More than 50% of breast tumors present an overexpression in STAT3, which is correlated to poor prognosis and invasive phenotype (Shields BJ et al., 2013). Preclinical studies show that STAT3 knockdown is able to delay tumor progression and sensitizes cancer cells to chemotherapy (Shields BJ et al., 2013) and that JAK2 inhibition delays tumor growth and decreases cancer stem cells (Marotta LL et al., 2011). Clinical trials are ongoing to evaluate the effect of ruxolotinib, a JAK1/2 inhibitor, in combination with paclitaxel in TNBC patients.

DNA damage checkpoint inhibitors

The DDR plays a key role in cancer. Mutations in DDR mechanisms are involved in many stages of tumor development. Several hereditary cancer predispositions are caused by mutations in DNA repair genes (Goode EL et al., 2002; Negrini S et al., 2010). Different studies show that DDR proteins are upregulated during early stages of tumorigenesis and this may limit tumor development acting as a barrier for cancer cell proliferation (Bartkova J et al., 2005; Gorgoulis VG et al., 2005). Therefore, several malignant tumors present functional loss or de-regulation of proteins involved in DDR and cell cycle regulation.

Moreover, deregulation or overexpression of DDR components may cause resistance to different type of genotoxic therapies (Bao S et al., 2006; Oliver TG et al., 2010; Bobola MS et al., 2012). DNA damage-induced cell cycle arrest becomes an attractive target for cancer therapy; in fact, interfering with cell cycle control leads to an aberrant cell cycle progression, resulting in DNA damage accumulation and cancer cell death. Cell cycle arrest can also be activated by cancer cell as a survival mechanism, giving them the possibility to repair their DNA damages. Therefore, other potential targets for cancer therapy are DNA damage checkpoints; inhibition of checkpoints before DNA damage is completely repaired could lead to the activation of the apoptotic process. Checkpoint kinase 1 (Chk1) is a serine/threonine-specific protein kinase and is involved in DDR and cell cycle checkpoint response. It is regulated by ataxia telangiectasia and Rad3-related (ATR), forming the ATR-Chk1 pathway, which is activated in the presence of single strand DNA (ssDNA) caused by UV-induced damage or replication stress. Chk1 is highly expressed in fast-diving and genomic unstable cells, such as TNBCs. Several Chk1

inhibitors are under investigation, both in preclinical and clinical studies, for the treatment of TNBCs; UNC-01, a specific Chk1 inhibitor, is reported to abrogate the DNA damage-dependent G2 checkpoint induced by chemotherapy and to enhance cisplatin sensitivity by 60-fold (Takahashi I et al., 1987; Bunch RT and Eastman A, 1996). Other Chk1 inhibitors, such as AZD7762 and LY2606368, are in phase I and II clinical trials, both alone or in combination with chemotherapies, to treat patients carrying BRCA1/2 TNBC. Furthermore, inhibition in Chk1 shows promising results in preclinical studies in sensitizing TNBC xenografts mutated in TP53 to chemotherapy (Ma CX et al., 2012).
An additional approach to block cell cycle is to directly target cell cycle promoters such as cyclin-dependent kinases (CDKs), which are activated by cyclins, and as a complex control cells progression thorough cell cycle. Cyclin-CDK complexes are usually overexpressed in cancer, resulting in uncontrolled proliferation. Selective inhibition of CDK1 and CDK2 induces apoptosis in TNBC xenografts through the activation of a pro-apoptotic molecule, BIM (Horiuchi D et al., 2012). In addition, CDK4/6 inhibitors are reported to sensitize PI3KCA mutated TNBCs to PI3K inhibitors (Vora SR et al., 2014).

Immunotherapy

Cytotoxic T lymphocytes can play a key role in controlling tumor cells growth, detecting tumor-associated antigens presented by major histocompatibility class I (MHCI) molecules. Antigens specific for TNBC, that are not present in normal cells, offer an attractive target for immunotherapy. The glycosylated form of mucin 1 (MUC-1) is a cell surface-associated antigen expressed in TNBC cells; glycosylated MUC-1 can be considered a potential target for the treatment of TNBC. A preclinical study shows that glycosylated MUC-1 derived glycopeptide covalently linked to a Toll-like receptor (TLR) agonist, can generate a potent cellular immune response, efficacious in generating a therapeutic response (Lakshminarayanan V et al., 2012).
Malignant cells can acquire the capability of evading the adaptive immune system. To this purpose, an effective immunotherapy approach in targeting TNBC includes the inhibition of immune-checkpoint receptors which prevent immune activation by T cell exhaustion. Immune checkpoint receptors family includes cytotoxic T lymphocyte antigen 4 (CTLA-4), B and T lymphocyte attenuator (BTLA), programmed cell death protein 1 (PD-1) and its ligands, PD-L1/2. Tumors can upregulate the expression of PD-1 and PD-L1, promoting peripheral T cell exhaustion and the conversion of T effector cells to Treg cells (Francisco LM et al., 2009). A clinical trial shows that the anti-PD-1

antibody pembrolizumab improves pCR in patients with advanced TNBC treated with anthracycline/taxane-based chemotherapy (Nanda R et al., 2016). In a phase III randomized trial results show that the anti-PD-L1 antibody atezolizumab improves both PFS and OS in patients treated with nab-paclitaxel (Schmid P et al., 2018).
The optimization of other biomarkers predictive of response to immunotherapy is under investigation.

Despite the advent of new therapeutic strategies, tumor relapses remain the major challenge in breast cancer management. TNBC patients have a higher risk of early metastasis compared to patients with other types of breast cancer, and is reported that patients with residual disease after chemotherapy treatment present worse OS than patients with non-TNBC (Liedtke C et al., 2008). TNBC aggressiveness leads to treatment failure and cancer recurrence primarily due to drug resistance and self-renewal that are specific properties of a small population of tumor cells, the cancer stem cells (CSCs).

# 5. Cancer stem cells (CSCs)

Many studies suggest that cancer stem cells (CSCs) arise from normal stem cells or progenitor cells that have achieved the ability to self-renew (Cozzio A et al., 2003; Huntly BJ et al., 2004); on the other hand, other studies propose the hypothesis that differentiated cancer cells acquire stem cells properties through reversal of ontogeny based on oncogene-induced plasticity (Rapp UR et al., 2008). Moreover, another hypothesis is that cells can acquire stem cell properties through the mechanism of epithelial-mesenchymal transition (EMT) which leads to the repression of epithelial markers, such as E-cadherin, and up-regulation of mesenchymal markers, such as N-cadherin and vimentin (Wu Y et al., 2011).
CSCs are involved in cancer initiation, maintenance, invasion and recurrence (Reya T et al., 2001). Moreover, they express stemness properties, resistant features and immune evasion capability. CSCs are also called cancer initiating cells, due to their capability to auto-regenerate, proliferate and induce cancer formation (Lapidot T et al., 1994). CSCs are one of the determining factors which contributes to tumor heterogeneity; their capacity for self-renewal and differentiation makes them able to recapitulate the heterogeneity of the original tumor. Self-renewal, in fact, is a cell division which affects

only stem cells and enables a stem cell to produce another stem cell with the same replication and development potential, allowing the maintenance of an undifferentiated pool of cells. Instead, differentiation allows the production of daughter cells that become tissue-specific. CSCs are also one of the major causes for multidrug resistance (MDR), thanks to specific properties, such as slow rate of division, high capacity for DNA repairing and high expression of drug-efflux pumps, the ATP-binding cassette (ABC) transporters. Moreover, CSCs hypoxic microenvironment is involved in MDR. The activation of hypoxia-inducible factor-1α (HIF-1α), the main regulator of cellular response to hypoxia, leads in fact to the overexpression of stemness activators, such as WNT, Hedgehog and NOTCH pathways and stemness markers, such as NANOG and SOX2 and decreases the production of ROS preserving stem cell properties and leading to drug resistance (Majmundar AJ et al., 2010; Schulenburg A et al., 2015; Carnero A et al., 2016).

Due to the key role of CSCs in tumor initiation, progression, invasion and drug resistance, the isolation of this subpopulation of cells is essential to study therapeutic strategies specific for CSCs, aimed to prevent tumor relapses.

## 5.1 Breast cancer stem cells isolation strategies

Breast cancer stem cells (BCSCs) are characterized by the presence of specific surface markers, useful for CSCs identification and isolation, such as CD44. CD44 is a transmembrane protein highly expressed in BCSCs, involved in cell proliferation, survival, migration, differentiation, self-renewal, EMT and resistance to apoptosis. BCSCs present also high enzymatic activity of aldehyde dehydrogenase 1 (ALDH1), an enzyme involved in tumor stem cells differentiation (Clark DW and Palle K, 2016). Additionally, BCSCs can be identified thanks to their capability to proliferate in a serum free three-dimensional culture leading to the formation of mammospheres, exclude dye due to the overexpression of ABC or multidrug resistance transporters and form new tumors when serially transplanted into mice (Morrison BJ et al., 2012; Zinzi L et al., 2014).

In 2003, a preclinical study first showed that a subpopulation of cells with CD44+/CD24-/Lin- phenotype within breast cancer patient tissues could recapitulate tumor burden in mice (Al-Hajj M et al., 2003). In 2007, a subpopulation of cells with high ALDH1 activity was found to be capable to initiate tumors *in vitro* and *in vivo* (Ginestier C et al., 2007).

Taken together, these findings have led to consider the CD44+/CD24- phenotype and high activity of ALDH1 the "gold standard" signature for BCSCs (Li W et al., 2017). Clinically, this phenotype is associated with worse chemotherapy response, lymph node metastasis, distant metastasis, relapses and worse OS (Lin Y et al., 2012; Chen Y et al., 2015).

# 6. Targeting CSCs for the treatment of TNBC

Several studies demonstrate that CSCs are particularly enriched in TNBC. Histopathological analysis of breast cancer patient tissues reveals that TNBCs show enriched ALDH1 activity and CD44+/CD24- surface markers expression compared to other types of breast cancer (Honeth G et al., 2008; Li T et al., 2013; Ma F et al., 2014). Moreover, TNBC cells are reported to form mammospheres at a higher degree than cells of other breast cancer subtypes (Honeth G et al., 2008; Ricardo S et al., 2011; Li Y et al., 2013). Gene expression analysis of TNBC patients show that genes up-regulated in mammary stem cells are also enriched in TNBC cells compared to non-TNBC (Park SY et al., 2019). Furthermore, TNBC cells express the key transcription factors involved in the induction of EMT at the same level of CSCs (Liu T et al., 2013). Collectively, these data support a significant overlap between TNBC and CSCs phenotype, providing evidence that TNBC aggressiveness can be related to a high percentage of CSCs inside the tumor.

The targeting of specific surface markers, the modulation of signaling pathways typical of CSCs, the inhibition of drug-efflux pumps, the regulation of CSCs microenvironment signals, are few of the strategies that can be used to target CSCs.

## 6.1 Specific therapeutic strategies for cancer stem cells (CSCs)

### 6.1.1 Self-renewal signaling pathways inhibitors

Therapeutic strategies aimed to attenuate the CSC phenotype are reported to delay tumor progression, decrease metastasis formation and therapy resistance in TNBC. Self-renewal

signaling pathways (SRSPs) are highly activated in TNBC cells, and their inhibition is reported to reduce stemness.

STAT3 plays a key role in BCSCs self-renewal regulation; interleukin 6 (IL6), in fact, induces the conversion of tumor cells into CSCs activating OCT4 transcriptional activity through STAT3. Moreover, VEGF, after binding to its receptor 2 (VEGFR2) leads to STAT3 phosphorylation which, in turn, activates SOX2 and MYC promoter regions. This mechanism is reported to increase the in vivo tumorigenic potential, mammospheres forming efficiency and ALDH1 phenotype in breast cancer cells (Zhao D et al., 2015). STAT3 overexpression seems to be highly related to TNBC initiation, progression, invasion and drug resistance (Tian J et al., 2018). Different strategies are reported to block STAT3 signaling, such as ligand-receptor interaction blockage and inhibition of STAT3 phosphorylation upstream kinases, such as JAK. Ruxolitinib, an ATP-competitive inhibitor of JAK1/JAK2, is currently in clinical trial alone or in combination with chemotherapy, in patients with metastatic TNBC (NCT03012230). STAT5 is also known to sustain TNBC resistance and CSCs maintenance. In particular, STAT5 signaling promotes TNBC cells resistance to PI3K/mTOR inhibitors (Britschgi A et al., 2012) and its loss is reported to sensitize them to these therapeutic approaches and to reduce tumor cells migratory potential (Bernaciak TM et al., 2009; Britschgi A et al., 2012).

Proto-Oncogene Tyrosine- Protein Kinase Src (SRC) is a member of a tyrosine kinase family and has a key role in CSCs self-renewal regulation. SRC kinase is observed to be highly phosphorylated in mammospheres compared to cancer cells, and its inhibition significantly reduces BCSCs self-renewal and migratory potential (Thakur R et al., 2015). Furthermore, SRC kinase is involved in CSCs chemo-/radio-resistance; its activation, in fact, induces EMT in residual breast cancer cells after irradiation, increases CD44+CD24- phenotype, MDR, and migration (Kim RK et al., 2015; Gilani RA et al., 2016). The SRC inhibitor dasanitib is reported to induce differentiation in TNBC cells, sensitizing cancer cells to paclitaxel therapy (Tian J et al., 2018).

Wnt/β-catenin signaling pathway is often deregulated in many cancers. The nuclear accumulation of β-catenin is evidently higher in TNBC compared to other breast cancer subtypes (Geyer FC et al., 2011). The activation of Wnt/β-catenin signaling is mediated by the binding of Wnt ligands to their receptor, the frizzled (FZD) family proteins, and co-receptors, the low-density lipoprotein receptor-related proteins (LRPs). This signaling

pathway is known to be involved in cell migration, colony formation, self-renewal regulation and chemo-resistance, and drive TNBC tumorigenesis in mouse models (Xu J et al., 2015). TNBC is reported to overexpress genes involved in Wnt pathway, including WNT1, CBP, FZDs and LRPs (Pohl SG et al., 2017). Genetic silencing of WNT1 reduces self-renewal potential of CSCs leading to a reduction in tumorigenesis and metastasis in xenograft models (Jang GB et al., 2015). Moreover, genetic silencing of FZD6 or FZD7 decreases motility, invasion, mammosphere formation and in vivo tumorigenesis in TNBC (Yang L et al., 2011; Corda G et al., 2017). A preclinical study shows that FZD8 expression is higher in residual cells after cisplatin and tumor-related apoptosis-inducing ligand (TRAIL) treatment, and that FZD8 depletion reduces β-catenin accumulation leading to a decrease in MDR in TNBC cells (Yin S et al., 2013). Similarly, genetic silencing of LRP6 reduces the invasion and migration of TNBC cells (Ma J et al., 2017). The Wnt pathway antibody OMP-18R5, isolated thanks to its ability to bind FZD2, FZD5, FZD7 and FZD8, is reported to reduce tumorigenesis in different types of human tumor xenografts (Gurney A et al., 2012).

Notch and Hedgehog are two other signaling pathways involved in the regulation of CSCs self-renewal and differentiation.

Notch signaling cascade is involved in multiple cellular processes, such as stem cell maintenance and progenitor cell proliferation and differentiation. Notch pathway includes four different receptors (Notch-1-2-3-4) and five ligands (Delta-like-1-2-3 and Jagged-1-2) that are frequently overexpressed in multiple types of tumor. Cell-to-cell contact is required for Notch activation. Binding between ligands and neighboring cells leads to cleavages by ADAM proteases and γ-secretase resulting in the release of the intracellular domain of the receptor which translocate to the nucleus and initiates the transcription of multiple target genes (Aster JC et al., 2017). Notch3 overexpression is associated with poor overall survival (Hassan KA et al., 2013) and inhibition of Notch-1 and Notch-4 is reported to decrease CSCs properties and tumor progression in xenografts (Harrison H et al., 2010). In particular, Notch-1 receptor is overexpressed in TNBC and is associated to invasiveness and tumorigenesis (Nagamatsu I et al., 2014; Diluvio G et al., 2018). It has been shown that some anti-cancer drugs interfere with Notch signaling. For instance, DXR is reported to induce Notch-1 signaling in TNBC cell lines, which lead to multi-drug-resistant protein 1 (MRP1/ABCC1) overexpression, whereas γ-secretase inhibitor (GSI) reverts the ABCC1 upregulation induced by Notch-1, sensitizing cancer cell to

DXR (Kim B et al., 2015). Moreover, Notch-1 inhibitors improve docetaxel toxicity in TNBC, showing a potent anti-tumor activity in CSCs and patient-derived xenograft models (Qiu M et al., 2013).

Hedgehog signaling is involved in CSCs maintenance and acquisition of EMT and has a key role in cancer cell invasion, metastasis, MDR and recurrence (Li Y et al., 2012). Hedgehog pathway consists of three ligands (Desert (DHH), Indian (IHH) and Sonic (SHH) Hedgehog) and transmembrane receptors Patched (PTCH) and Smoothened (SMO). Glioma associated oncogene transcription factors (GLI-1-2-3) are the main effectors and are involved in the regulation of several target genes (Harris LG et al., 2012). SHH and GLI-1 are overexpressed in TNBC and stimulate migration, invasion and proliferation of cancer cells *in vitro* and lung metastasis dissemination *in vivo* (Kwon YJ et al., 2011; Harris LG et al., 2012). The inhibition of Hedgehog pathway is reported to reduce motility and self-renewal capacity of TNBC cells (Kwon YJ et al., 2011). Aberrant activation of Hedgehog signaling is mostly due to interaction with other molecular pathways, including PI3K/Akt/mTOR. Inhibition of Hedgehog and mTOR, with vismodegib and rapamycin respectively, is reported to decrease NANOG and OCT4 expressions and ALDH1+ cells proliferation (Zuo M et al., 2015).

Moreover, PI3K/AKT/mTOR pathway is involved in the maintenance of CSCs features. AKT overexpression is associated with chemo-resistance in breast cancer, while mTOR inhibition is reported to sensitize resistant cells to cytotoxic agents (Steelman LS et al., 2008, Choi HJ et al., 2019). Furthermore, AKT induces HIF-1 which is known to be a key factor in MDR (Li L and Ross AH, 2007). In breast cancer, PI3K and AKT inhibitors reduce the formation of mammospheres and lead to mesenchymal phenotype loss and recovery of epithelial markers (Gargini R et al., 2015). A clinical trial shows that the AKT inhibitor ipatasertib, in combination with paclitaxel, increases OS of TNBC patients compared to paclitaxel alone (Kim SB et al., 2017). Moreover, PTEN, a tumor suppressor gene and an inhibitor of PI3K pathway, is frequently mutated in cancers and its loss is linked to CSCs proliferation (Dong P et al., 2014). Furthermore, loss in PTEN is associated to AKT activation, increased activity of Wnt/β-catenin pathway and activation of Notch signaling (Hill R and Wu H, 2009).

TGF-β signaling pathway is known to promote EMT, proliferation, angiogenesis, metastasis and chemo-therapy resistance (Smith AL et al., 2012). Furthermore, it is a key

regulator of BCSCs; in fact, breast cancer cell lines exposed to TGF-β undergo EMT, acquiring stem cell properties (Asiedu MK et al., 2011). TGF-β is a member of a cytokine superfamily and is activated by binding to TGF-βRII, which in turn recruits and phosphorylates TGF-βRI, forming a receptor complex. Chemotherapy treatment is reported to increase TGF-β signaling in TNBC, while TGF-βR inhibitor prevents the re-establishment of tumors following chemotherapy in TNBC xenograft models (Bhola NE et al., 2013). A phase I clinical trial is investigating the effect of galunisertib, a TGF-βR inhibitor, in combination with chemotherapy, in metastatic TNBC patients. Other strategies, such as vaccines and antisense oligonucleotides (trabedersen) are still under investigation (Bogdahn U et al., 2011; Giaccone G et al., 2015).

### 6.1.2 Drug-efflux pumps inhibitors

The ABC transporter proteins are members of the ABC superfamily and their main function is the regulation of the efflux of small molecules and compounds from the cytosol to the extracellular medium using ATP hydrolysis. They are involved in the prevention of xenobiotics and toxic compounds accumulation in normal cells; on the other hand, ABC proteins are involved in the development of MDR, due to their ability to expel toxic chemicals (Gottesman MM et al., 2002; Lobo NA et al., 2007). Mechanism involved in ABC transporter proteins modulation may be considered potential targets for chemo-resistance in CSCs. ABCC1/MRP1, breast cancer resistance protein (ABCG2/BCRP) and multidrug-resistant protein-8 (ABCC11/MRP8) are frequently overexpressed in TNBC (Yamada A et al., 2013; Xu L et al., 2017). In particular, ABCC1 protein expression is reported to increase after neoadjuvant chemotherapy in TNBC patients, further supporting ABCC1 importance in chemo-resistance (Guestini F et al., 2019). ABCG2 has a key role in chemo-resistance of CSCs in TNBC (Britton KM et al., 2012); its downregulation, in fact, sensitizes TNBC cells to chemotherapy (Arumugam A et al., 2019). ABCC1 is reported to confer cross-resistance to multiple antitumor agents, such as anthracyclines, mitoxantrone and taxanes, whereas ABCG2 can transport 5-fluorouracil, methotrexate, DXR, irinotecan, mitoxantrone. ABCC11 role in chemo-resistance is under investigation but is known to transport 5-Fluorouracil and methotrexate (Oguri T et al., 2007; Sissung TM et al., 2010). Together, these transporters have a broad overlapping substrate specificity; therefore, the discovery of target therapies specific for ABC transporter protein is essential to overcome chemo-resistance process

in TNBC. There is a dual approach to target ABC transporter proteins, the inhibition of their activity and the inhibition of their expression. The first few generations of ABC transporters activity inhibitors were too toxic and were no selective (Hamed AR et al., 2019). Multiple non-steroidal anti-inflammatory drugs (NSAIDs) are shown to be able to sensitize resistant cells overexpressing ABCC1 to cytotoxic drugs (O'Connor R et al., 2004); NSAID sulindac, for instance, in combination with epirubicin, reports anti-tumor activity in a phase I clinical trial on patients with aggressive breast cancers (O'Connor R et al., 2007). Moreover, PZ-39 inhibits ABCG2 activity and accelerates its degradation (Peng H et al., 2009). Small interfering RNA (siRNA) and microRNA are used, as a novel approach, to attenuate ABC transporter-mediated chemo-resistance. RNA interference (RNAi) based drugs are reported to block ABCG2 and ABCC1 protein expression in resistant cell cultures, restoring the therapeutic benefits of cytotoxic drugs (Wang Y et al., 2016). Currently, a wide range of low toxic natural products, able to inhibit ABC transporter proteins activity, are under investigation to be safely combined with chemotherapy (Hamed AR et al., 2019).

## 6.2 CSCs metabolism

CSCs possess the ability to survive under the hypoxic conditions present inside the tumor niche by obtaining different energy sources depending on substrate availability. Indeed, different reports show that the preference for glycolysis or OXPHOS is context dependent. In fact, several studies suggest that glucose is an essential nutrient for CSCs, since its presence in the microenvironment enhances their percentage inside the tumor, while glucose uptake inhibition induces CSCs depletion *in vitro* (Liu PP et al, 2014). Moreover, glycolysis is found to be the favorite metabolic process in radioresistant sphere-forming cells in nasopharyngeal carcinoma and in hepatocellular carcinoma staminal population (Chen CL et al., 2015; Shen YA et al., 2015). In other studies, performed in many tumor types including breast, lung, ovarian and colon cancers is reported that CSCs present higher levels of glucose uptake, glycolytic enzyme expression and ATP content compared with cancer differentiated cells, and this phenotype seems to be associated to a decrease in mitochondrial oxidative metabolism and differentiation potential (Emmink BL et al., 2013; Ciavardelli D et al., 2014, Liao J et al., 2014). Analogously, during differentiation, the mitochondrial DNA copy number increases whereas stemness associated genes expression decreases (Lee WT et al., 2015). On the

other hand, growing evidence shows that mitochondria of CSCs present an increased mass and membrane potential, suggesting a CSCs preference for mitochondrial oxidative metabolism (Lagadinou ED et al., 2013; Pasto A et al., 2014). Moreover, CSCs seem to be susceptible to mitochondria targeted drugs and OXPHOS inhibitors; the OXPHOS complex I inhibitor metformin, in fact, is reported to induce partial suppression of stemness phenotype and delay tumor progression *in vivo* (Jung JW et al., 2011; Sancho P et al., 2015).

These context-dependent discrepancies could be explained by the metabolic adaptability that CSCs show in different microenvironments under adverse energetic conditions. Moreover, different studies report that CSCs can switch to a glycolytic metabolism when OXPHOS is blocked, and vice versa (Feng W et al., 2014; Luo M et al., 2018). Therefore, the dual blockade of glycolysis and OXPHOS could represent a way to deplete CSCs, without focusing on glycolysis or mitochondrial respiration inhibition exclusively (Cheong JH et al., 2012). Although, this could also represent a toxic intervention for normal cells and patients.

TNBC differentiated cells metabolism resembles that of CSCs. Evidence suggests that TNBC cell lines displayed increased glycolysis and lactate production compared with non-TNBC cell lines (Lim SO et al., 2016). Lim et al., in fact, observed that TNBC patient tissues present higher levels of hexokinase 2 (HK2) than non-TNBC. CSCs, likewise tumor cells, take advantage of (an)aerobic glycolysis over mitochondrial respiration, to produce more rapidly energetic sources and biosynthetic molecules even in the presence of sufficient $O_2$, phenomenon known as "Warburg effect" (Warburg 1956a, Warburg 1956b, Vander Heiden MG et al., 2009). Glycolysis upregulation is reported to be correlated with increased tumor aggressiveness and with the development of multi-drug resistance, specific properties related to CSCs (Milane L et al., 2011). CSCs glycolytic metabolism is supported by HIF-1, which stimulates the expression of glucose transporters (GLUT) and hexokinase (HK) enzymatic activity (Gordan JD et al., 2007). Moreover, anaerobic glycolysis reduces the generation of ROS produced by the electron transport chain during OXPHOS, helping in the maintenance of low ROS levels inside CSCs population (Dong C et al., 2013). A large body of studies show that BCSCs present an increase in glucose uptake, lactate production and higher ATP content as compared to differentiated cancer cells, which instead seem to be more dependent on the mitochondrial activity to produce energy. Rotenone, a mitochondrial complex I inhibitor, is reported to

reduce ATP production of cells upon differentiation, without impairing BCSCs metabolism (Ciavardelli D et al., 2014; Luo M et al., 2018; O'Neill S et al., 2019). Moreover, glycolysis metabolites can alter the microenvironment to favor BCSCs; lactate, for instance, acidifying the tumor niche, can favor the polarization of tumor associated macrophages towards an M2 phenotype, promoting, consequently, proliferation and migration (Colegio OR et al., 2014).

Glycolysis pharmacological targeting is shown to overcome drug resistance of CSCs, isolated from solid tumors, inactivating ABC transporters involved in the drugs efflux mechanism in CSCs (Nakano A et al., 2011). 2-Deoxy-D-Glucose (2DG) is a synthetic glucose analogue in which the C-2 hydroxyl group is replaced by hydrogen. Due to this modification, 2-DG is transported and quickly taken up into cells by GLUT, in particular GLUT1 and GLUT4 and, once inside the cells, is phosphorylated to 2-deoxy-d-glucose-6-phosphate (2-DG-6-P) which in turn, due to the lack of the 2-OH group, is accumulated inside the cells leading to inhibition of glycolysis and glucose metabolism. 2-DG is reported to inhibit solid tumor growth and, in combination with widely used chemotherapeutics, overcomes drug-resistant BCSCs (Zhao Y et al., 2011). Recent studies show that 2-DG impairs tumor cells migration and invasion and affects the ability of CSCs to form mammospheres. Moreover, the combination of 2-DG with DXR shows a synergic effect not only in impairing CSCs, but also more differentiated cancer cells, suggesting that this combination may target different cancer cell populations (Ciavardelli D et al., 2014; O'Neill S et al., 2019).

# AIM OF THE STUDY

Fasting/FMD is reported to enhance the efficacy of several standard and low toxic therapies on different types of cancer, including triple negative breast cancer (TNBC), while inducing the protection of normal cells from the toxic side effects. However, cyclic FMD alone or in combination with chemotherapy only slows down the progression of TNBC, but doesn't result in long term control of tumor growth. In addition, the effect of fasting/FMD on cancer stem cells (CSCs) has never been investigated.

Since TNBC progression is reported to be dependent on CSCs, which are known to rely on glycolysis compared to differentiated cells, and due to the key role of CSCs in tumor initiation, progression and drug resistance, the major aim of this study is to investigate whether the FMD affects TNBC CSCs compartment.

# MATERIALS AND METHODS

## Cell lines and culture conditions

The human triple negative breast cancer (TNBC) cell line SUM159 was purchased from Asterand; the murine TNBC cell line 4T1 was purchased from ATCC. SUM159 cells were cultured in Ham's F-12 medium (Invitrogen) supplemented with 5% FBS NA, 5 μg/mL insulin, 1 μg/mL hydrocortisone (both from Sigma), and 1%penicillin/streptomycin (Biowest, Cat. # L0022). 4T1 cells were cultured in RPMI 1640 medium (Biowest, Cat #: L0500) supplemented with 10% FBS (Biowest, Cat. #: S1810) and 1%penicillin/streptomycin. All cells were tested for mycoplasma contamination routinely. Cells were maintained in a humidified, 5% CO2 atmosphere at 37°C.

*In vitro*, FMD-like conditions are referred as Short-Term starvation medium (STS), a DMEM medium without glucose (DMEM no glucose, Life Technologies, Cat. #: 11966025) supplemented with 0.5 g/l glucose (Sigma-Aldrich, Cat. #: G8769) and 1% FBS. Standard conditions are referred as control medium (CTR), a DMEM no glucose medium supplemented with 1 g/l glucose and 10% FBS. For *in vitro* experiments, cells were seeded in 12 well plates in their maintenance media for 24hours. Cells were then washed twice with PBS and grown in CTR/STS media for a total of 48h (media were refreshed every 24hours to guarantee that glucose was not completely consumed). For rescuing experiments with glucose/FBS, cells were cultured under CTR, STS, STS + 1g/l glucose-1%FBS, STS + 10%FBS-0,5g/l glucose and STS + 0,5g/l glucose-1%FBS and single FBS components at CTR concentration level (IGF1: 250ng/ml, EGF: 200ng/ml, Insulin: 200ng/ml) for a total of 48h.

## Reagents preparation

WZB117

Glucose transporter inhibitor IV, WZB117, was purchased from MERK (Cat. #: 400036) and was dissolved in DMSO. Stock solutions of 70mg/ml were prepared for *in vivo* experiment and were stored at -80°C.

Metformin

Metformin was purchased from Sigma-Aldrich (Cat. #: D150959) and was dissolved in sterile water to a final concentration of 1M (stock solution). Stock solutions were store at +4°C.

2-Deoxy-D-Glucose

2-Deoxy-D-Glucose was purchased from Sigma-Aldrich (Cat. #: D-6134) and was dissolved in sterile water to a final concentration of 2M (stock solution). Stock solutions were stored at +4°C.

8-Bromoadenosine 3',5'-cyclic mono-phosphates (8-Br-cAMP)

8-Br-cAMP was purchased from Cayman (Cat. #: 14431) and was dissolved in sterile water. Stock solutions of 25mg/ml were prepared and stored at -20°C.

Alpelisib

Alpelisib was purchased from MedchemTronica (Cat. #: HY-15244) and was dissolved in DMSO. Stock solutions of 10mM were prepared for *in vitro* experiments and stored at -80°C.

Rapamycin

Rapamycin was purchased from MedchemTronica (Cat. #: HY-10219) and was dissolved in DMSO. Stock solutions of 20mM and 20mg/ml were prepared for *in vitro* and *in vivo* experiments, respectively, and stored at -80°C.

Pictilisib

Pictilisb was purchased from MedchemTronica (Cat. #: HY-50094) and was dissolved in DMSO. Stock solutions of 10mM and 200mg/ml were prepared for *in vitro* and *in vivo* experiments, respectively, and stored at -80°C.

Ipatasertib

Ipatasertib was purchased from MedchemTronica (Cat. #: HY-15186) and was dissolved in DMSO. Stock solutions of 10mM and 200mg/ml were prepared for *in vitro* and *in vivo* experiments, respectively, and stored at -80°C.

## Erythrosin B exclusion assay

Erythrosin B is a vital dye not permeable to biological membranes; therefore, it stains only non-viable cells with disintegrated membranes.
Cells were seeded in 12-well plates in their maintenance media for 24hours. The next days, cells were washed twice with PBS and grown in CTR/STS media for a total of 48h. After 24hours under CTR/STS conditions cells were treated with specific drugs or vehicle for the next 24 hours. In particular, for the experiment with PI3K-AKT-mTOR inhibitors, cells were treated with 10μM rapamycin, 10μM pictilisib, 20μM alpelisib and 20μM ipatasertib for a total of 24hours. At the end of each experiment, cells were harvested by trypsinization and collected to obtain a final concentration of 1x106 cells/ml. To perform the erythrosin B exclusion assay, cells were suspended 1:1 with erythrosin B 0.1% in PBS (Sigma-Aldrich, Cat. #: 200964) and counted in a Burker chamber. The percentage of cell death as the ratio of erythrosine B-positive cells with the total number of cells.

## Mammosphere forming assay

For mammosphere formation assay *in vitro*, SUM159 and 4T1 cells were were seeded in 12-well plates and grown in CTR/STS media for a total of 48h. At 24 hours, cells were treated with 5mM metformin or 4mM 2-Deoxy-D-Glucose. For rescuing experiments with PKA activator, cells were treated with 0,5mM 8-Br-cAMP, for a total of 24hours. Cells were harvested by trypsinization and collected to perform the mammosphere forming assay. In particular, cells were digested into single cells using a 21G needle and then were plated in ultra-low attachment plates at a density of 500 or 1500 cells per well. Cells were cultured in a mammary epithelial basal medium (MEMB Cat. #: CC-3151) and methylcellulose (Sigma Cat. #: M0512) for 8/10 days. MEMB was previously supplemented with heparin (1U/ml), hydrocortisone (0,5 μg/ml), insulin (5μg/ml), 1% L-glutamine, 1% penicillin/streptomycin, B-27 (40μl/ml, Gibco Cat. #: 17504044), epidermal growth factor (EGF, 40ng/ml Biomol, Cat # BPS-90201-3) and fibroblast growth factor (FGF, 40ng/ml, Peprotech Cat. #: 100-18B). For *ex vivo* mammosphere formation assay, tumor masses were excised from the flank/ mammary fat pad of the mice and chopped in small parts with a scalpel. These pieces were digested enzymatically in DMEM medium supplemented with hyaluronidase (10mg/ml, Sigma Cat. #: H4272) and collagenase (2000U/ml, Sigma Cat. #: C2674) for 3 hours, 5%CO2 at 37°C. Cells

obtained were filtered on cell strainer (100-70-40 μM) to achieve single cells and re-suspended in red blood cell lysing buffer hybrid-max (Sigma Cat. #: R7757) for 30 sec/1min. Finally, cells were plated in ultra-low attachment plates at a density of 1500 cells per well. To perform the serial sphere forming assay, mammospheres obtained were mechanically dissociated in single cells and re-plated to form secondary and tertiary spheres for 3 passages. 8/10 days after being plated, the number of mammospheres with a diameter >60 μm was counted.

## CD44CD24 flow cytometer

CD44CD24 staining was performed both on SUM159 cells and on 4T1 tumor masses. 4T1 tumor tissues were enzymatically digested to obtain a single-cell suspension as previously described. Cells were harvested and washed twice in PBS 1% BSA and pellets were resuspended in blocking buffer (PBS 10% BSA) and incubated for 30 min at 4°C light protected. After a wash in PBS 1% BSA, cells were incubated with antibodies solution (200μl/1x$10^6$ cells) for 45min at 4°C. In particular, 4T1 cells were stained with FITC-conjugated anti-murine CD24 antibody (Miltenyi Cat. #: 130-102-731) and PE-conjugated anti-murine CD44 antibody (Miltenyi Cat. #: 130-102-606), while SUM159 cells were stained with FITC-conjugated anti-human CD24 antibody (Miltenyi Cat. #: 130-112-844) and vioblue-conjugated anti-human CD44 antibody (Miltenyi Cat. #: 130-113-899). SUM159 samples were analysed by flow cytometry with Attune NxT flow cytometer and data were processed by Kaluza analysis software (Beckman coulter, version 2.0). 4T1 samples were analysed by flow cytometry with FACSCalibur (BD) and data were processed by FlowJo software.

## Aldefluor assay

ALDEFLUOR kit (STEMCELL technologies, Cat. #: 01700) is used for the identification, evaluation and isolation of stem and progenitor cells expressing high levels of ALDH. SUM159 tumor tissues were dissociated enzymatically to obtain a single-cell suspension as previously described. Cells expressing high levels of ALDH become brightly fluorescent (ALDHbr) and were identified by FACSCalibur (BD) flow cytometer. Data were processed by FlowJo software.

## Immunohistological staining

To determine the expression of Caspase-3 protein, immunohistological analysis was performed in sample tissues of SUM159 tumor masses. Paraffin sections of 3 µm thickness were baked and prepared according to the procedure. Tumor masses slides were incubated overnight (4°C) with cleaved caspase-3 antibody (Asp175) (Euroclone Cat. #: BK9661S). Images of sections were taken by microscope (Upright BX 51 Full Manual).

## Protein extraction and Western blot analysis

Cells were washed twice in ice-cold PBS and lysates were prepared in RIPA lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholic acid, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (protease inhibitor cocktail set III EDTA-free, Calbiochem, Cat. #: S39134; PhosStop, Roche).
Tumor tissues were collected and snap frozen in liquid nitrogen immediately after mice were sacrificed, and stored in -80 °C until use. For protein extraction, tumors were homogenized with Tissue lyser II (Qiagen) in RIPA buffer supplemented with protease and phosphatase inhibitors and then ultra-centrifuged (45000 rpm using MLA-130 Beckman rotor) for 1 hour. Protein concentrations were determined by BCA assay (Thermo Fisher Scientific, Cat. #: 23225). A total of 30µg of proteins were separated using pre-casted or home-made Acrylamid gels and transferred to 0.45µM nitrocellulose membranes or 0.2µM nitrocellulose membranes (depending on protein molecular weight) over night. The blots were blocked in 5% non-fat dry milk, in 1x TBS containing 0.01% Tween20 (TBST), for 1h at RT. Membranes were incubated overnight at 4°C with the following antibodies: Vinculin (1:10000, Sigma-Aldrich, Cat. #: V9131), GLUT1 (1:10000, Cell Signaling, Cat. #: 12939), CREB (1:1000, Cell Signaling, Cat. #: 4820S), Phospho-CREB (1:1000, Cell Signaling, Cat. #: 9198S), KLF5 (1:1000, Abcam, Cat. #: AB137676), G9A/EHMT2 (1:1000, Euroclone, Cat. #: BK68851T) and histone H3dimethylK9 (1:1000, Abcam, Cat. #: AB1220). Next, membranes were washed with TBST (3 x 10 min) and then incubated for 1h RT with anti-Mouse (1:3000, Cat. #: 170-6516) or anti-Rabbit (1:3000, Cat. #: 170-6515) secondary antibodies. Upon washing (3 x 10min) with TBST, specific bindings were detected by a chemiluminescence system (Thermo Scientific). Bands intensity was quantified with Image Lab software (version 5.2.1).

## RNA extraction, RT-PCR and qRT-PCR

Total RNA was isolated using the miRNeasy Mini Kit (QIAGEN, #217004) according to the manufacturer's instructions. Next, 1 μg of purified RNA was retrotranscribed by using SuperScript Vilo cDNA synthesis kit (Invitrogen, #11754050). Resulting cDNA was analyzed by real-time polymerase reaction (qRT-PCR) using TaqMan MBG probes with FAM reporter dyes. Human target gene primers for NANOG (hs02387400_g1), OCT4 (hs00742896_s1), TBX3 (hs00195612_m1) and KLF2 (Hs00360439_g1) were used.

## CD44CD24 Cell sorting

SUM159 tumor masses were excised from the flank of the mice and chopped in small parts with a scalpel; then, tumor tissues were dissociated enzymatically to obtain a single-cell suspension as previously described. Cells were washed twice in PBS 1% BSA and pellets were resuspended in blocking buffer (PBS 10% BSA) and incubated for 30 min at 4°C light protected. After a wash in PBS 1% BSA, cells were incubated with antibodies solution (200μl/1x$10^6$ cells) for 45min at 4°C. In particular, SUM159 cells were stained with FITC-conjugated anti-human CD24 antibody (Miltenyi Cat. #: 130-112-844) and vioblue-conjugated anti-human CD44 antibody (Miltenyi Cat. #: 130-113-899). Finally, cells were washed twice with PBS 1% BSA and sorted with MoFlo Astrios Cell Sorter (Beckman Coulter).

## RNA sequencing

Libraries for RNA sequencing were prepared following the manufacturer protocols for transcriptome sequencing with the Illumina NextSeq 550DX sequencer (ILLUMINA).
Total RNA was isolated from cells, previously sorted, using the miRNeasy Mini Kit (QIAGEN, #217004), according to the manufacturer's instructions, its abundance was measured using Nanodrop and its integrity was assessed using Agilent Bioanalyzer 2100 with Nano Rna kit (RIN > 8). mRNA-seq indexed library preparation was performed starting from 500 ng of total RNA with the TruSeq stranded mRNA (Illumina) according to the manufacturer's instructions. Indexed libraries were quality controlled on Agilent Bioanalyzer 2100 with High Sensitivity DNA kit, quantified with Qubit HS DNA,

normalized and pooled to perform a multiplexed sequencing run. 1% PhiX control was added to the sequencing pool, to serve as a positive run control. Sequencing was performed in PE mode (2x75nt) on an Illumina NextSeq550Dx platform, generating on average 45 million PE reads per sample.

## RNA-seq Bioinformatics Analysis

Reads were aligned to the hg38 reference genome with STAR (doi: 10.1093/bioinformatics/bts635) with default settings and using the parameter quantMode GeneCounts to count the number reads per gene while mapping. Differential gene expression analysis between groups was performed with DESeq2 (doi: 10.1186/s13059-014-0550-8). Genes with $|log2FC| > 2$ and adjusted p value $< 0.05$ were considered as significantly deregulated.

Gene Set Enrichment Analysis (GSEA) for pathways of interest was performed on the fold change rank ordered gene list with the fgsea (doi: 10.1101/060012) package of Bioconductor. Volcano plots were generated with the R package ggplot2. Rendering of the fold changes on pathway graphs was achieved with the Pathview (doi: 10.1093/bioinformatics/btt285) Bioconductor package, which allows to download KEGG pathway graph data and render them with the mapped data.

## Mouse models

The animals were housed under specific pathogen-free conditions with 12 hours day/light cycles. All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and were approved by the Italian Ministry of Health. For xenograft experiments, 8-weeks old female NOD scid gamma (NSG, Charles River) were subcutaneously injected with $1,5x10^6$ SUM159 cells resuspended in 100 µl of PBS. For syngeneic model, 6-weeks old female Balbc/Ola Hsd mice (Envigo) were injected in the mammary fat pad with $2x10^4$ 4T1 cells resuspended in 20 µl of PBS. When tumors were palpable (approximately 7 days for SUM159 and 3 days for 4T1 after inoculation), mice were randomly divided in the different experimental groups. Body weights were recorded daily, and tumor volumes were measured twice a week by a digital caliper according to the following equation: tumor volume (mm3) =

length x width x thickness x 0,5. At the end of the experiments, mice were euthanized by using CO2.

## Animal diets and treatments

Mice were fed ad libitum with irradiated VRFI (P) diet (Charles River) containing 3,89 kcal/g of gross energy. Our FMD is based on a nutritional screen that identified ingredients that allow nourishment during periods of low-calorie consumption (Brandhorst et al., 2015). The FMD diet consists of two different components designated as day 1 diet and days 2–4 diet. Day 1 diet consists of a mix of low-calorie broth powders, a vegetable powder, extra virgin olive oil and essential fatty acids; it contains 7.67 kJ/g (provided 50% of normal daily intake; 0.46 kJ/g protein, 2.2 kJ/g carbohydrate, 5.00 kJ/g fat). The day 2–3 diet is identical on all feeding days, consists of low-calorie broth powders and glycerol and contains 1.48 kJ/g (provided at 10% of normal daily intake; 0.01 kJ/g protein/fat,1.47 kJ/g carbohydrates). Mouse weight was monitored daily and during FMD cycle weight loss did not exceed 20%.

For experiments on tumor growth, mice were fed with standard diet or underwent FMD cycles (4 consecutive days per week). Before FMD cycle was repeated, mice completely recovered their original bodyweight.

For GLUT1 inhibitor experiments, mice were daily treated with WZB117 (10mg/kg in PBS) via intraperitoneal injection. For metformin experiment, mice were daily treated with metformin (150 mg/kg in PBS) via intraperitoneal injection. For the experiments with 2-Deoxy-D-Glucose, mice were daily treated with the drug (500mg/kg in PBS) via intraperitoneal injection. For the experiment with PI3K-AKT-mTOR and CDK4/6 inhibitors, mice were treated with Pictilisib for 5 consecutive days per week (100mg/kg in 10%DMSO, 40% PEG300 and 50% saline) by oral gavage, Palbociclib every other day (62,5mg/kg in 5%DMSO, 40%PEG300 and 55% saline) by oral gavage, Ipatasertib for 5 consecutive days per week (75mg/kg in 5%DMSO, 40%PEG300 and 55% saline) by oral gavage and Rapamycin every other day (2mg/kg in 2%DMSO, 40%PEG300 and 58% saline) via intraperitoneal injection.

## Limiting dilution assay

For the limiting dilution assay, 8-weeks old female NOD scid gamma mice (NSG, Charles River) were subcutaneously injected with $1,5x10^6$ SUM159 cells resuspended in 100 µl of PBS. Mice were fed with standard diet or underwent FMD cycles (4 consecutive days per week) and were daily treated with 2DG (500mg/kg, intraperitoneally) or vehicle. Before FMD cycle was repeated, mice completely recovered their original bodyweight. Body weights were recorded daily, and tumor volumes were measured twice a week by a digital caliper. After 5 weeks, donor mice were sacrificed and tumor masses were excised and enzymatically digested, as previously described. Tumor cells derived from donor mice were re-injected at different dilution (100.000, 10.000, 1000 cells) in recipient female NOD scid gamma mice. Recipient mice were always fed with standard diet and weren't treated; survival curves were calculated on the bases of whether tumor masses became palpable. Tumor initiating cell frequency was calculated with ELDA software.

## Statistical analysis

GraphPad Prism 8 was used for the analysis of the data and graphic representations. Comparisons between two groups were performed with two-tailed unpaired Student's t test. Comparison among more than two groups were performed with ANOVA analysis followed by Tukey's test. Comparison of survival curves were performed with Log-rank (Mantel-Cox) test. P values $\leq 0.05$ were considered significant.

# RESULTS

## 1. STS/ FMD reduces CSCs in SUM159 human TNBC model, increasing cancer free survival in mice.

### 1.1 *In vitro* short-term starvation (STS) reduces mammosphere growth and $CD44^{high}CD24^{low}$ population in SUM159 triple negative breast cancer (TNBC) cells.

STS is known to sensitize different kind of cancer cells to chemotherapy, including triple negative breast cancer (TNBC), while protecting normal tissues from toxic side effects (Raffaghello L et al., 2008; Lee C et al., 2012; Di Biase S et al., 2016). Chemotherapy improves patient survival but doesn't prevent tumor relapses, in part due to a small population of slow-cycling cells, named cancer stem cells (CSCs), which are chemo- and radio-therapy resistant and able to drive tumorigenesis (Dawood et al., 2010).
For these reasons, my goal was to investigate whether STS could affect CSC population. To this purpose, I used, as *in vitro* model, SUM159 human TNBC cell line, since they are enriched in cancer stem cells which are capable of sphere formation (Grimshaw MJ et al., 2008). In particular, SUM159 TNBC cells were grown in control (CTR) medium, which mimics physiological level of glucose and serum (1g/L glucose, 10% serum) and in STS medium, which mimics the decrease in glucose and growth factors induced by FMD *in vivo* (0,5 g/L glucose, 1% serum) for 48h. Cells where then collected and processed to be plated as single cells on non-adherent plates in serum-free mammosphere medium with growth factors, to perform the *in vitro* colony forming assay. This assay is an *in vitro* quantitative technique used to examine the capability of a single cell to proliferate and self-renewal in serum free and 3D culture conditions which mimic native microenvironment. Following 8 days of culture, mammospheres were observed through a stereomicroscope. I found that STS decreases sphere formation compared to CTR. Moreover, images show that STS condition affects also the morphology of mammospheres, which, in fact, become smaller, confirming a slower proliferation compared to those cultured under CTR conditions (Figure 1).

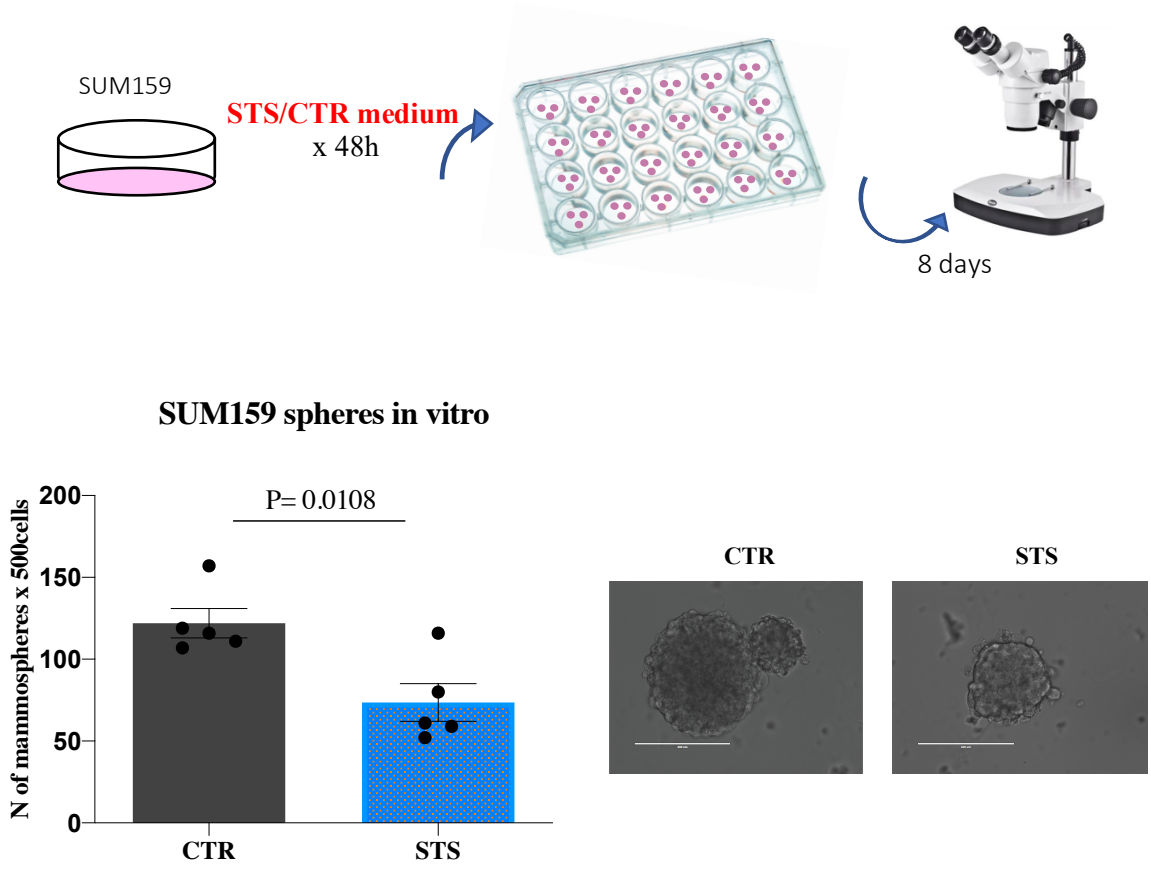



**Figure 1**. **STS condition decreases SUM159 TNBC mammospheres**.
SUM159 cells were grown under control (CTR: 1g/l Glucose, 10%FBS) and starved (STS: 0,5g/l, 1%FBS) conditions for a total of 48h. Cells were then plated as single cells on non-adherent plates in serum-free mammosphere medium with growth factors. Figure 1 shows the number and the morphology of representative SUM159 spheres (obtained from 500 cells) after 8 days of *in vitro* culture (n= 5 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

To confirm the role of STS conditions in decreasing CSCs, I measured the expression of stem cell markers by flow cytometry, in particular the high expression of CD44 and low expression of CD24 ($CD44^{high}CD24^{low}$) that are characteristic cell surface markers specific for CSCs (Honeth G et al., 2008). I found that STS condition strongly reduces the percentage of $CD44^{high}CD24^{low}$ population compared to CTR, confirming the important role of nutrient depletion in decreasing TNBC stem cells *in vitro* (Figure 2).

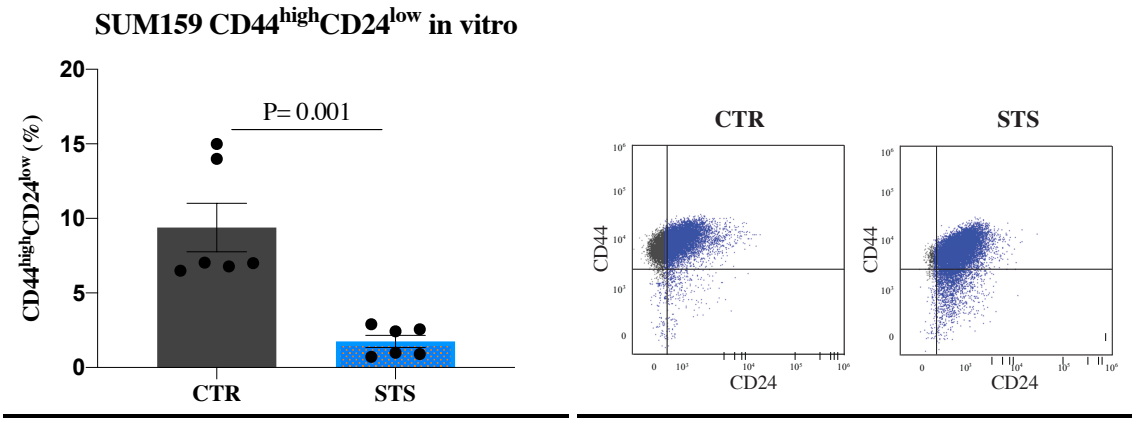



**Figure 2. STS reduces $CD44^{high}CD24^{low}$ staminal population.**

FACS analysis was performed to measure CD44 and CD24 expression in SUM159 breast cancer cell line in vitro. The percentages reflect the population of putative breast cancer stem cells defined as $CD44^{high}CD24^{low}$ (n= 6 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

## 1.2 Fasting mimicking diet (FMD) reduces tumor growth, *ex vivo* spheres formation and decreases the percentage of ALDH1+ cells in TNBC.

Based on *in vitro* results, I investigated whether FMD cycles could reduce CSCs also *in vivo*. FMD is based on severe calorie restriction, low levels of protein and sugars and relatively high fat content and is reported to delay tumor progression and sensitize cancer cells to chemotherapy, while protecting normal tissues (Di Biase et al., 2016). Immune-deficient mice bearing SUM159 xenografts were randomly assigned to two groups, one fed ad libitum (AL) with standard rodent diet and one subjected to 5 cycles of FMD (4 days of FMD every week followed by 3 days of refeeding with standard diet). FMD cycles resulted to be safe and well tolerated, as indicated by mouse bodyweight. During each FMD cycle, mice did not lose more than 20% of their initial weight, which was immediately recovered upon refeeding (Figure 3a). Moreover, blood glucose level was recorded daily and results show that during FMD cycles glycemia decreases from ~120mg/dl to ~60mg/dl (Figure 3b).

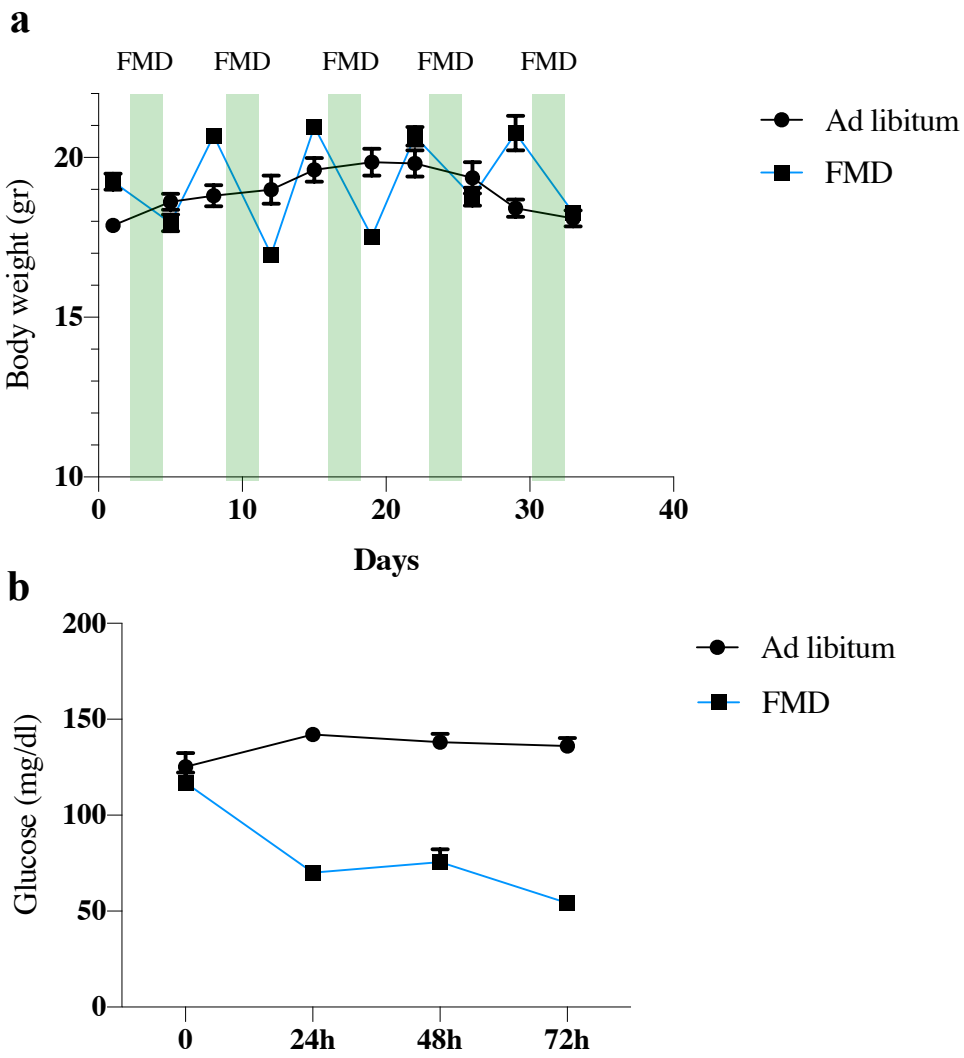



**Figure 3. FMD cycles are safe and well tolerated in immune-deficient mice.**

a) Bodyweight of nod scid mice (NSG) bearing SUM159 xenografts undergoing 4-days FMD was recorded daily (n=15 per group). b) Blood glucose level was determined through Accu chek guide instrument.

To evaluate the effect of FMD in delaying tumor progression, tumor size was measured twice a week by a digital vernier caliper. Cyclic FMD greatly delayed tumor progression and reduced cancer size compared to AL (Figure 4).

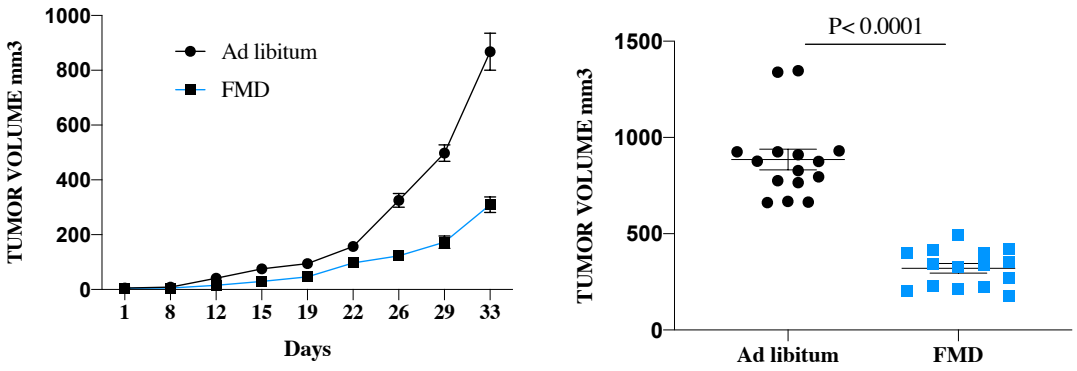



**Figure 4. FMD reduces TNBC progression and tumor size.**

8-weeks old female NSG mice were subcutaneously injected with SUM159 cells and fed with standard diet or subjected to 5 cycles of FMD. Tumor volumes before mice were sacrificed (day 33) are reported (n=15 per group). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

Starting from preliminary results obtained concerning FMD effect on SUM159 xenografts, I investigated whether the decrease in tumor size mediated by FMD was due to the activation of apoptotic processes. I performed immunohistochemical analysis to check the expression of Caspase-3 (Cas-3) protein in tumor slides, and I found that FMD causes a nearly 3-fold increase in apoptosis inside the tumor, compared to AL conditions (Figure 5).

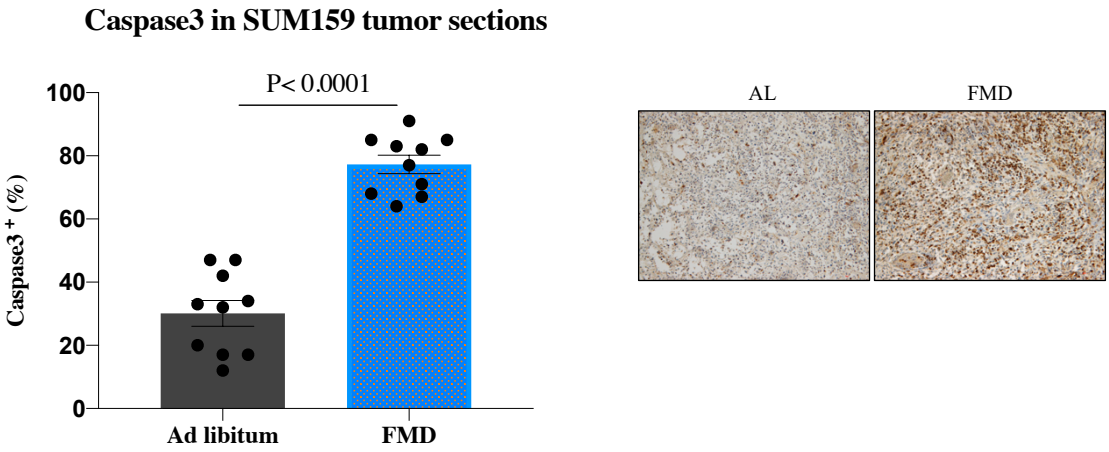
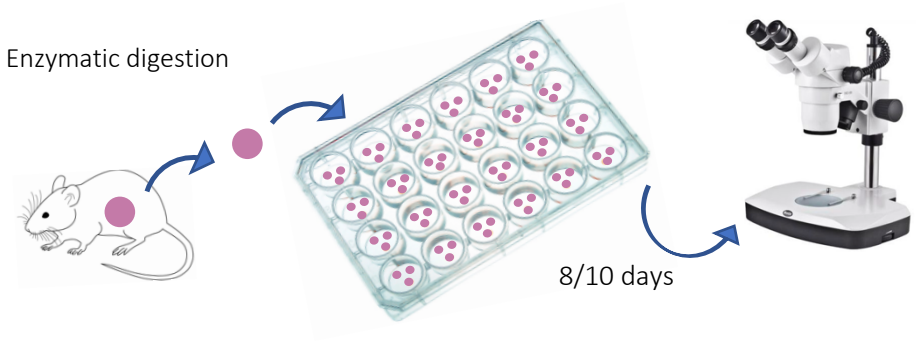


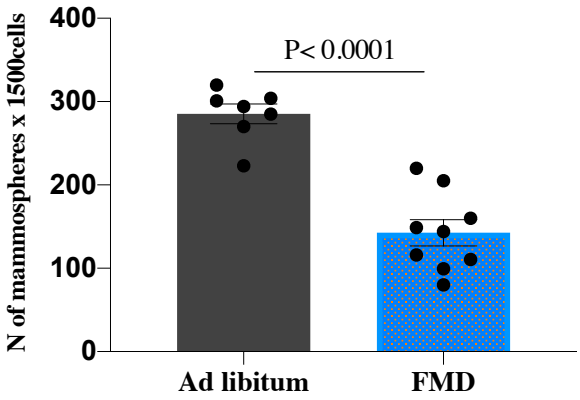

**Figure 5. FMD increases apoptotic level in SUM159 xenografts.**

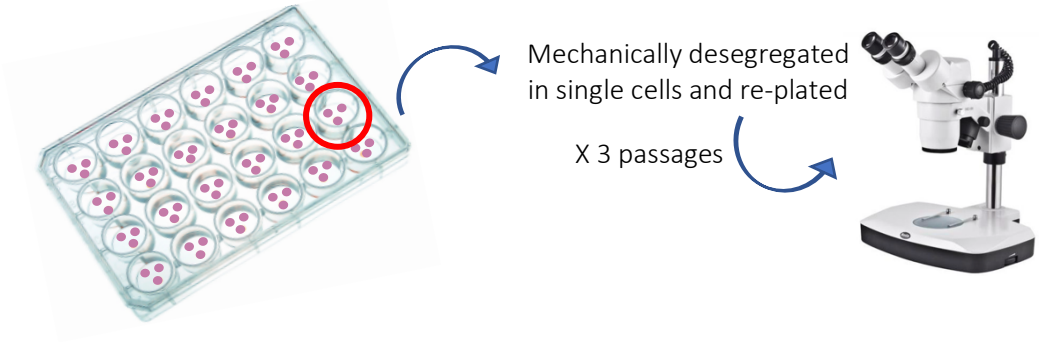
The expression of Cas-3 protein was examined by IHC staining in SUM159 tumor masses slides (n= 10 slides of different tumors, per group). Cas-3 positive cells were quantified with cell counter ImageJ plugin. Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

After 5 cycles of FMD, mice were sacrificed and tumor masses were excised and used to perform *ex vivo* primary mammosphere forming assay, in order to test the effect of fasting on CSCs survival. Firstly, tumor masses were enzymatically digested and filtered repeatedly in order to obtain single cells, which were then plated on non-adherent plates in serum-free medium, enriched in growth factors. In according with our *in vitro* results, FMD treatment *in vivo* resulted to be very efficient in reducing mammosphere formation compared to AL. I also evaluated the multiple serial propagation of the spheres in order to select cells with the highest competence to proliferate and self-renew. FMD resulted to reduce the serial spread of the spheres, even after three passages, confirming its role in decreasing CSCs within the tumor (Figure 6).




**SUM159 spheres in vivo**




**SUM159 serial spheres in vivo**

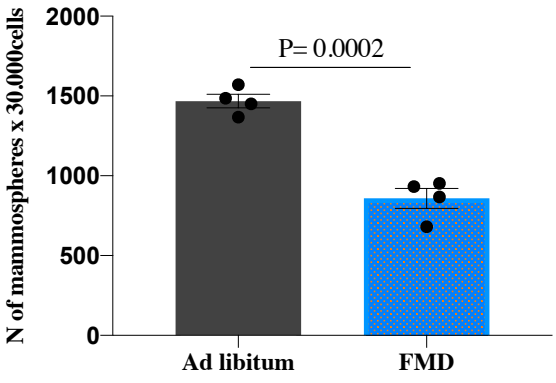


**Figure 6. FMD decreases ex vivo sphere formation and self-renewal.**

After 5 weeks of AL diet or FMD cycles, tumor masses were excised and processed for *ex vivo* primary mammospheres (obtained from 1500 cells, n=7-9 biological replicates) and for *ex vivo* serial spheres forming assay (obtained from 30.000 cells generated from dissociated secondary spheres, n= 4 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

Furthermore, stem-like cells show elevated aldehyde dehydrogenase 1 (ALDH1) activity. ALDH1 is an enzyme responsible for the oxidation of intracellular aldehydes and contributes to normal and tumor stem cell differentiation. It has been demonstrated that ALDH1-positive (ALDH1+) cells, isolated from human breast tumors, are able to self-renew and generate tumors which recapitulate parental tumor heterogeneity; therefore, ALDH1 is used as CSCs marker (Ginestier C et al., 2007; Sarkar P et al., 2018). For this purpose, I measured the percentage of ALDH1+ cells in SUM159 tumor masses and I found that the % of ALDH1+ population is much lower in FMD-treated mouse masses compared with that from AL group, suggesting that FMD decreases pluripotent cancer stem cells, which support previous results (Figure 7).

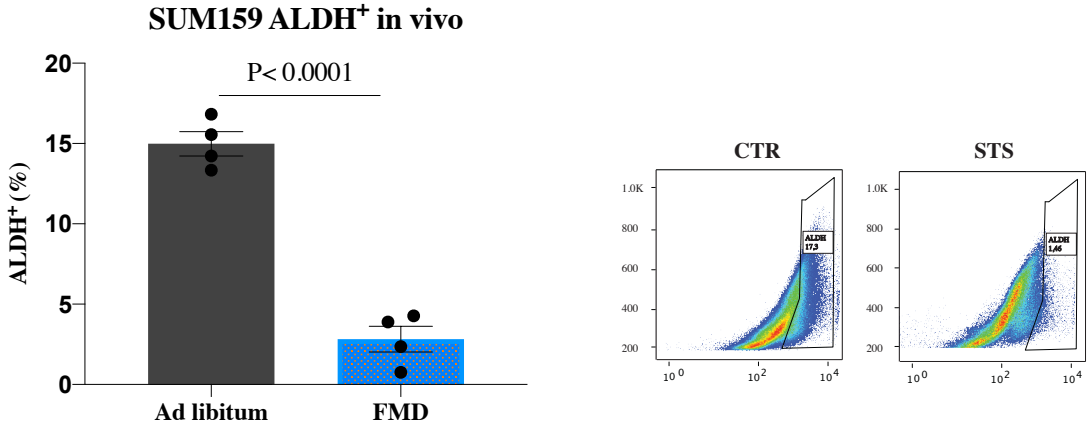



**Figure 7. FMD decreases ALDH1+ cells in SUM159 xenografts.**

Aldefluor analysis were performed by flow cytometry using the ALDEFLUOR kit, to measure ALDH1 expression in SUM159 xenografts (n=4 biological replicates). Data are represented as mean ± SEM. Unpaired t test was performed.

To further confirm the effect of FMD on CSCs, I performed the limiting dilution assay, the gold standard test to investigate CSCs, used to calculate tumor initiating cells (TICs) frequency. In particular, I used donor mice bearing SUM159 xenografts, fed with AL diet or subjected to FMD cycles. After 5 weeks, tumor masses were excised, digested and cells were injected in recipient mice, always fed with AL diet, at different cells dilution. FMD decreased TNBC initiating cell frequency, increasing the cancer free survival, compared to AL (Figure 8).

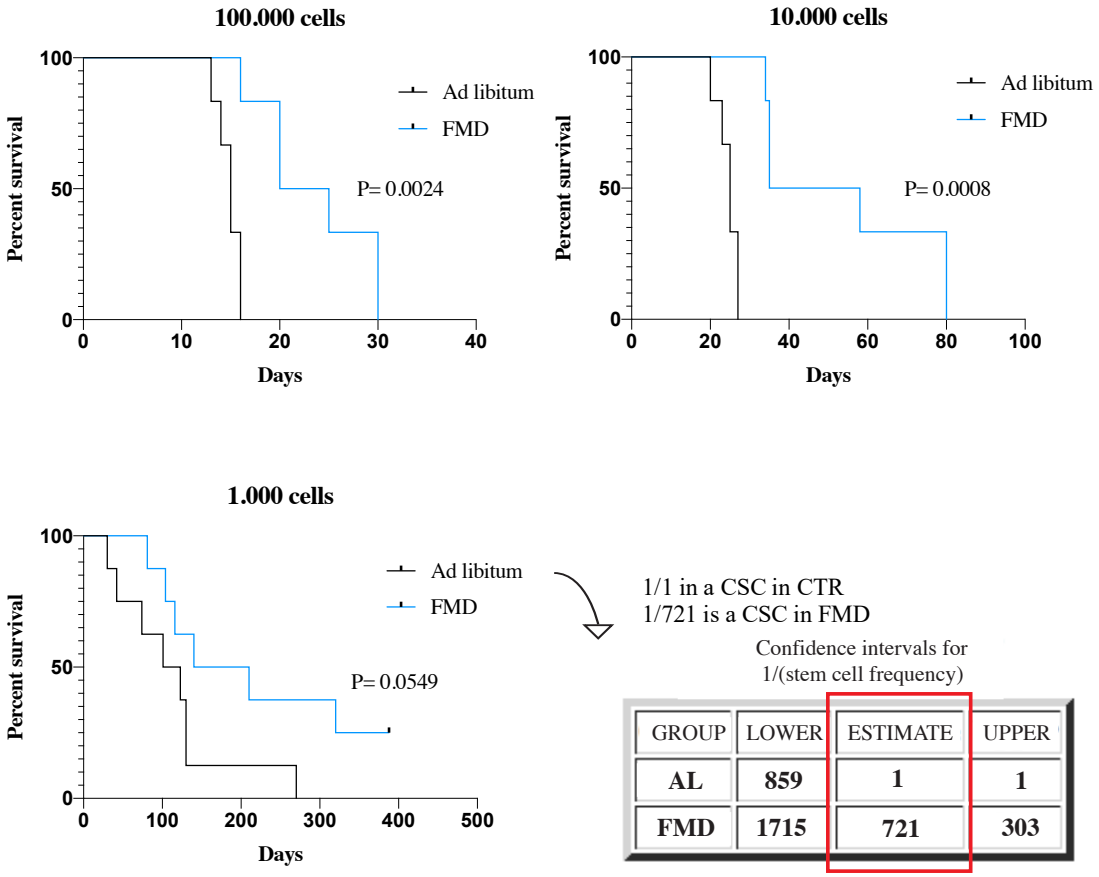
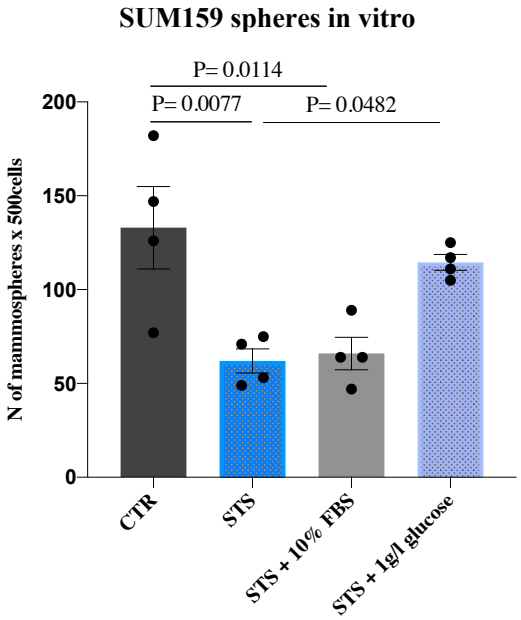



**Figure 8. FMD reduces the frequency of tumor initiating cells in SUM159 xenografts.** SUM159 tumor cells, derived from mice fed with AL or FMD diets, were injected in recipient mice at different cell dilutions, to perform the limiting dilution assay (100.000-10.000-1.000 cells), n= 6-8. The stem cell frequency was calculated using ELDA software. Survival curve are represented. Log-rank (Mantel-Cox) test was performed.

# 2. FMD induced CSCs reduction is glucose-dependent

## 2.1 STS/FMD effect in lowering mammospheres can be reversed by glucose supplementation.

Supported by results obtained on the effect of STS/FMD on staminal population within the tumor, I decided to investigate which is the nutrient affected by fasting/FMD that could affect cancer stem cell survival/number. To this purpose, I tried to reverse the effect of STS on mammosphere growth, adding separately fetal bovine serum (FBS), glucose and single serum components, at CTR concentration level, to STS medium. In particular SUM159 cells were grown in media containing low level of glucose (0,5g/L) and normal concentration of serum (10% FBS), normal glucose (1g/L) and low level of serum (1% FBS) or low glucose and serum levels (1g/L glucose, 1% FBS) with the addition of IGF-1, EGF and insulin, separately or combined. Interestingly, I found that the STS mediated sensitization is largely rescued by the addition of physiological level of glucose (1g/L), while FBS and supplementation with factors including IGF-1, EGF, and insulin didn't affect mammosphere growth. Collectively, these results indicate that the STS dependent reduction of spheres number is serum and growth factors independent and glucose-dependent (Figure 9).



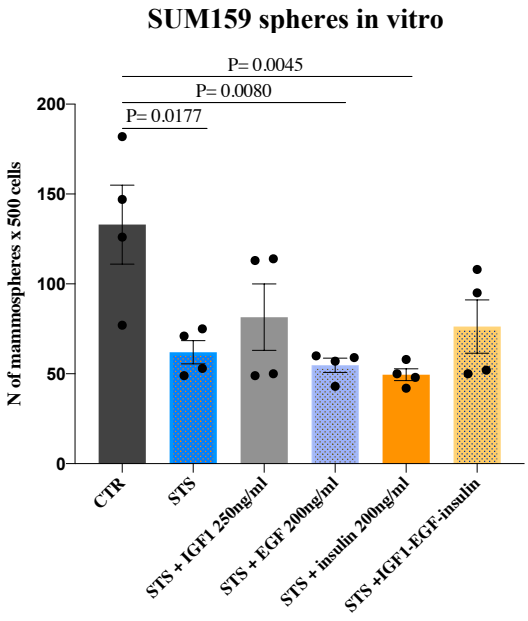
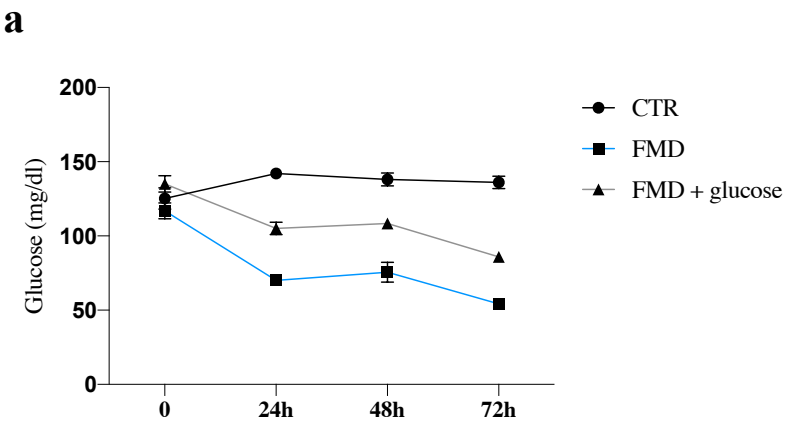



**Figure 9. STS effect on mammosphere growth is reversed by glucose supplementation.** SUM159 cells were grown under CTR (1g/l Glucose, 10%FBS), STS (0,5g/l, 1%FBS), STS + 1g/l glucose-1%FBS, STS + 10%FBS-0,5g/l glucose (graph reported on the left) and STS + 0,5g/l glucose-1%FBS and single FBS components at CTR concentration level (IGF-1: 250ng/ml, EGF: 200ng/ml, Insulin: 200ng/ml) (graph reported on the right) for a total of 48h. Cells were then plated to perform the in vitro spheres forming assay (n=4 biological replicates). Data are represented as mean ± SEM. One-way Anova was performed.

Supported by *in vitro* results, I tried to rescue FMD effect both in delaying tumor progression and in reducing CSCs, adding the 3% of glucose in mice drinking water, based on the normal concentration of sugar assumed daily by mice through the standard diet. Immune-deficient mice bearing SUM159 xenografts were fed with AL diet or subjected to FMD cycles alone or with oral supplementation of 3% of glucose in drinking water. I measured blood glucose level during the 4 days of FMD, to make sure that glucose supplementation in drinking water would lead to a blood glucose level increase, compared to FMD alone (Figure 10a). The 3% of glucose resulted to reverse only partially FMD phenotype in term of tumor progression. These data further support results previously obtained in our laboratory about the involvement of multiple pathways on tumor progression, particularly IGF-1 (Figure 10b).



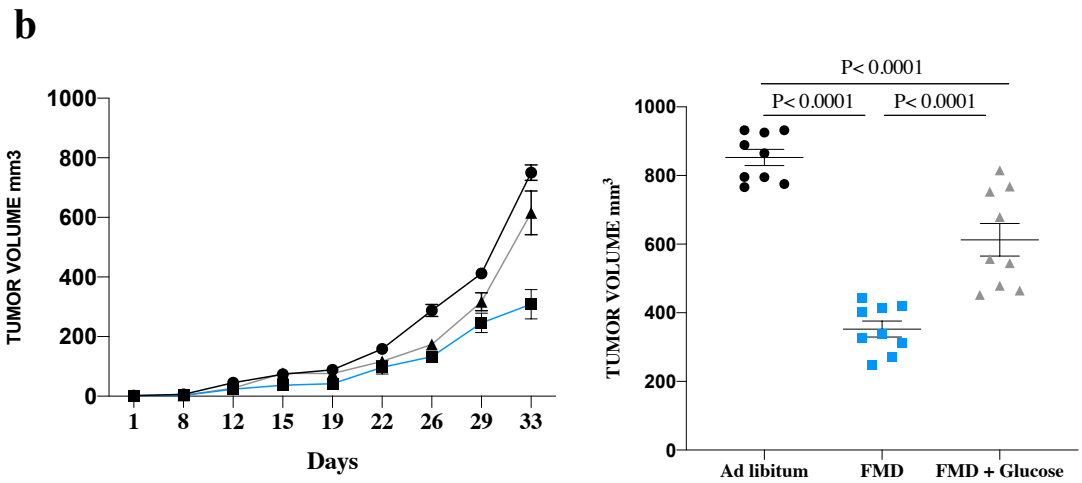



**Figure 10. Glucose in drinking water partially reverses FMD effect in delaying tumor progression.**

8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to 5 cycles of FMD alone or plus 3% glucose supplementation in drinking water. a) Blood glucose level was determined through Accu chek guide instrument. b) Tumor volumes before mice were sacrificed are reported (n=9). Data are represented as mean ± SEM. One-way Anova was performed.

At the end of the experiment, I sacrificed mice and late-stage tumor masses were excised and processed to perform *ex vivo* primary mammospheres forming assay. Accordingly, with my *in vitro* results, I found that glucose supplementation in drinking water completely rescues the FMD dependent reduction of spheres (Figure 11).

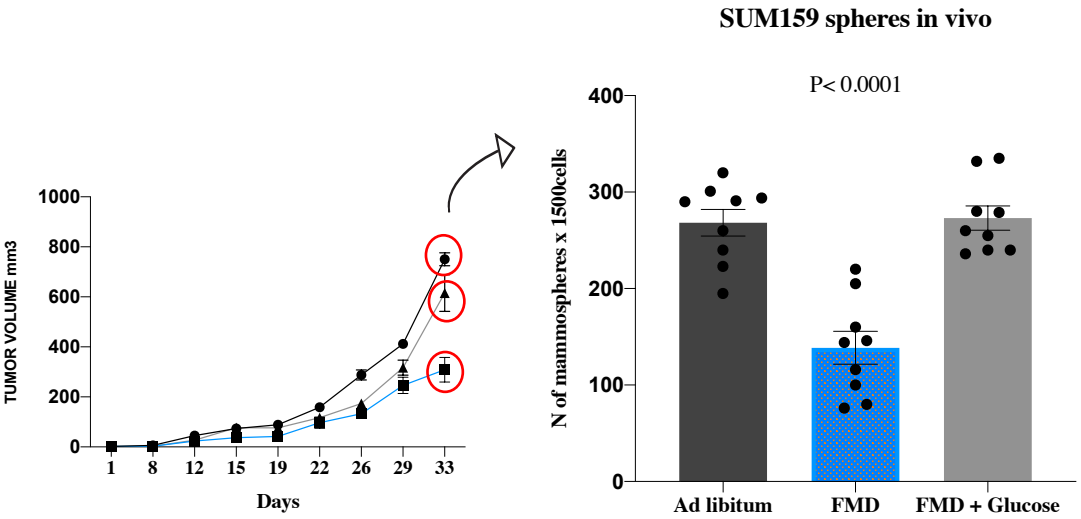


**Figure 11. Glucose in drinking water completely reverses FMD effect in reducing spheres growth.**

After 5 weeks of AL diet or FMD cycles alone or plus the addition of 3% of glucose in drinking water, tumor masses were excised and processed for ex vivo primary mammospheres (obtained from 1500 cells, n=9 biological replicates). Data are represented as mean ± SEM. One-way Anova was performed.

## 2.2 WZB117, a specific GLUT1 inhibitor, mimics the effect of FMD on CSCs.

To further investigate the toxicity of glucose restriction to CSCs, I evaluated the effect of a specific GLUT1 inhibitor, the WZB117. Liu et al, reported that this inhibitor down-regulates the expression of GLUT1 at the same level of a glucose deprived medium, which could mimic our STS *in vitro* conditions (Liu Y et al., 2012). I decided to investigate the effect of WZB117, instead of other GLUT inhibitors, because this is the glucose transporter mostly expressed in TNBC and because of its low toxicity; in fact, it is not involved in the transport of glucose to other important organs, such as brain.

Immune-deficient mice bearing SUM159 xenografts were fed AL with standard diet or with cyclic FMD and daily treated with WZB117 (10mg/kg) or PBS for 5 weeks. WZB117 reduced tumor size when compared to not treated mice fed AL, but FMD alone was more effective than GLUT1 inhibitor in delaying tumor progression. Furthermore, the drug did not potentiate the effect of fasting/FMD, probably because both FMD and the GLUT1 inhibitor lead to a similar decrease of glucose availability and their combination does not lead to a further decrease beyond this threshold. (Figure 12).

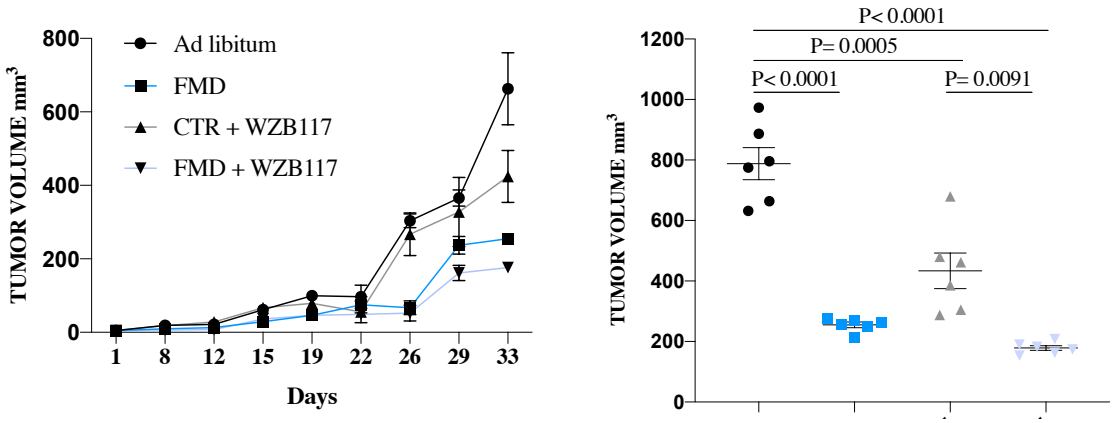


**Figure 12. WZB117 does not potentiate FMD effect in delaying tumor progression.**
8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to 5 cycles of FMD alone or in combination with WZB117 (10mg/kg) once a day, i.p. Tumor volumes before mice were sacrificed are reported (n=6 per group). Data are represented as mean ± SEM. One-way Anova was performed.

Based on my hypothesis, I measured the protein level of GLUT1 in SUM159 xenografts and I found that FMD alone greatly reduces GLUT1 expression, compared to standard conditions. This finding could explain the lack of synergistic effect between FMD and WZB117 (Figure 13).

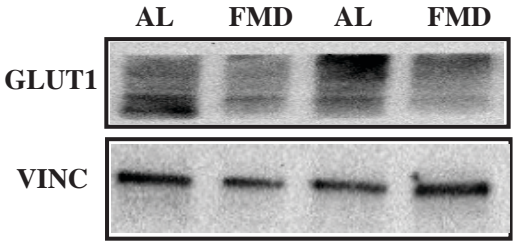



**Figure 13. FMD decreases GLUT1 expression in SUM159 xenografts.**
Detection of GLUT1 levels and VINCULIN, as loading control, in SUM159 xenografts.

Then I tested the impact of FMD, WZB117 and their combination on mammosphere growth. After 5 cycles of FMD, alone or plus GLUT1 inhibitor, mice were sacrificed and tumor masses were processed to perform the *ex vivo* primary mammosphere forming assay. I found that WZB117 mimics the effect of FMD in term of number of spheres formation, confirming my hypothesis that glucose depletion is toxic to CSCs population (Figure 14).

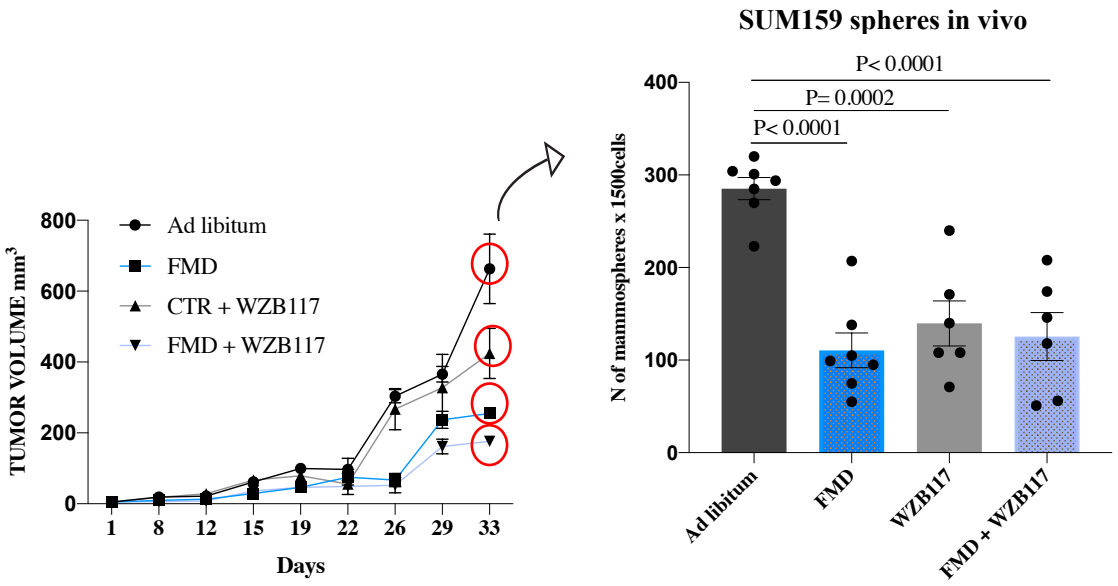



**Figure 14. WZB117 mimics FMD effect in reducing mammospheres, in SUM159 xenografts.** After 5 weeks of AL diet or FMD cycles alone or in combination with WZB117 (10mg/kg) once a day, i.p, tumor masses were excised and processed for ex vivo primary mammosphere forming assay (obtained from 1500 cells, n=6 biological replicates). Data are represented as mean ± SEM. One-way Anova was performed.

## 2.3 Metformin does not enhance FMD effect both in terms of tumor progression and mammospheres growth.

Metformin, a widely used drug for the treatment of type 2 diabetes thanks to its capability to lower blood glucose mainly through the suppression of hepatic glucose production, exhibits anti-tumor effects partly mediated by its involvement in the activation of the AMPK (Zhou et al., 2001) but also in the inhibition of mTORC1 signaling (Kalender et al., 2010).

Moreover, metformin is known to diminish CSCs, *in vitro* and *in vivo,* in combination with chemotherapy (Hirsch HA et al., 2009; Shi P et al., 2017). I investigated whether metformin could enhance FMD effect on CSCs, by further altering their metabolism. CSCs metabolism is poorly understood and, in fact, several studies suggest that CSCs are more glycolytic than other differentiated cancer cells (Liao J et al., 2014; Palorini R et al. 2014), while other studies propose that CSCs prefer mitochondrial oxidative phosphorylation (OXPHOS) (Janiszewska M et al., 2012; LeBleu VS et al., 2014; Pastò A et al., 2014; De Luca A et a., 2015). These opposing viewpoints could be explained by

the metabolic adaptability of CSCs in response to micro-environmental changes (Vlashi E et al., 2011; Flavahan WA et al., 2013).

First, I tested whether metformin, in combination with STS, could further reduce mammosphere growth, *in vitro*, and I found that metformin only slightly potentiates STS effect in decreasing spheres growth, while it is poorly effective under CTR conditions (Figure 15).

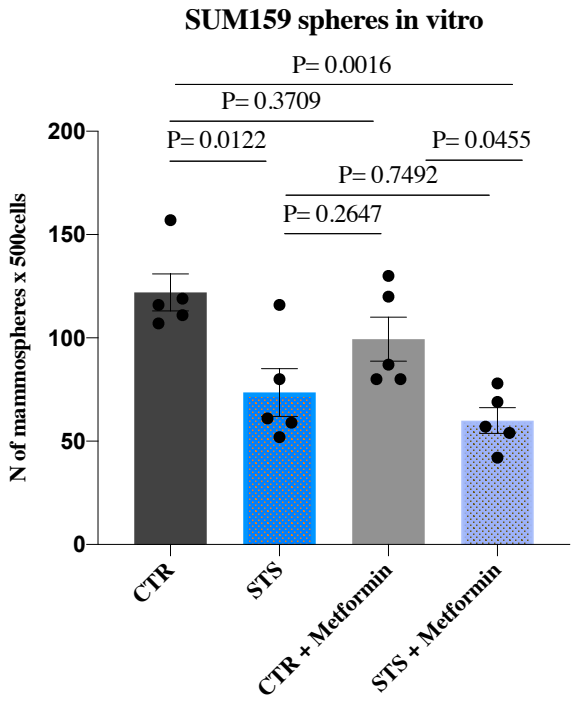



**Figure 15. Metformin does not potentiate STS dependent mammosphere reduction**. SUM159 cells were grown under control (CTR: 1g/l Glucose, 10%FBS) and starved (STS: 0,5g/l, 1%FBS) conditions for 48h, and then treated with placebo or metformin (5mM). Cells were then plated as single cells on non-adherent plates in serum-free mammosphere medium with growth factors. Figure 14 shows representative SUM159 spheres (obtained from 500 cells) after 8 days of in vitro culture. Data are represented as mean ± SEM. One-way Anova was performed.

Subsequently, to confirm *in vitro* results, I tested metformin effect in combination with FMD, in mice bearing SUM159 xenografts. Metformin is reported to dramatically reduce blood glucose levels in patients with type 2 diabetes or in obese preclinical models, but has a moderate effect in patients with normal glycemia levels (Bonanni B et al., 2012; Duca FA et al., 2015). I found that daily treatment with metformin causes a 23% decrease in blood glucose levels in those mice fed with standard diet compared to not treated mice, but doesn't potentiate the effect of FMD (Figure 16a). Consequently, I evaluated

metformin potential in reducing tumor growth. Immune-deficient mice bearing SUM159 xenografts were fed AL with standard diet or with cyclic FMD and daily treated with metformin (150mg/kg) or PBS for 5 weeks. Surprisingly, metformin resulted to be efficient alone, compared to AL conditions, but its effect was not potentiated by fasting (Figure 16b).

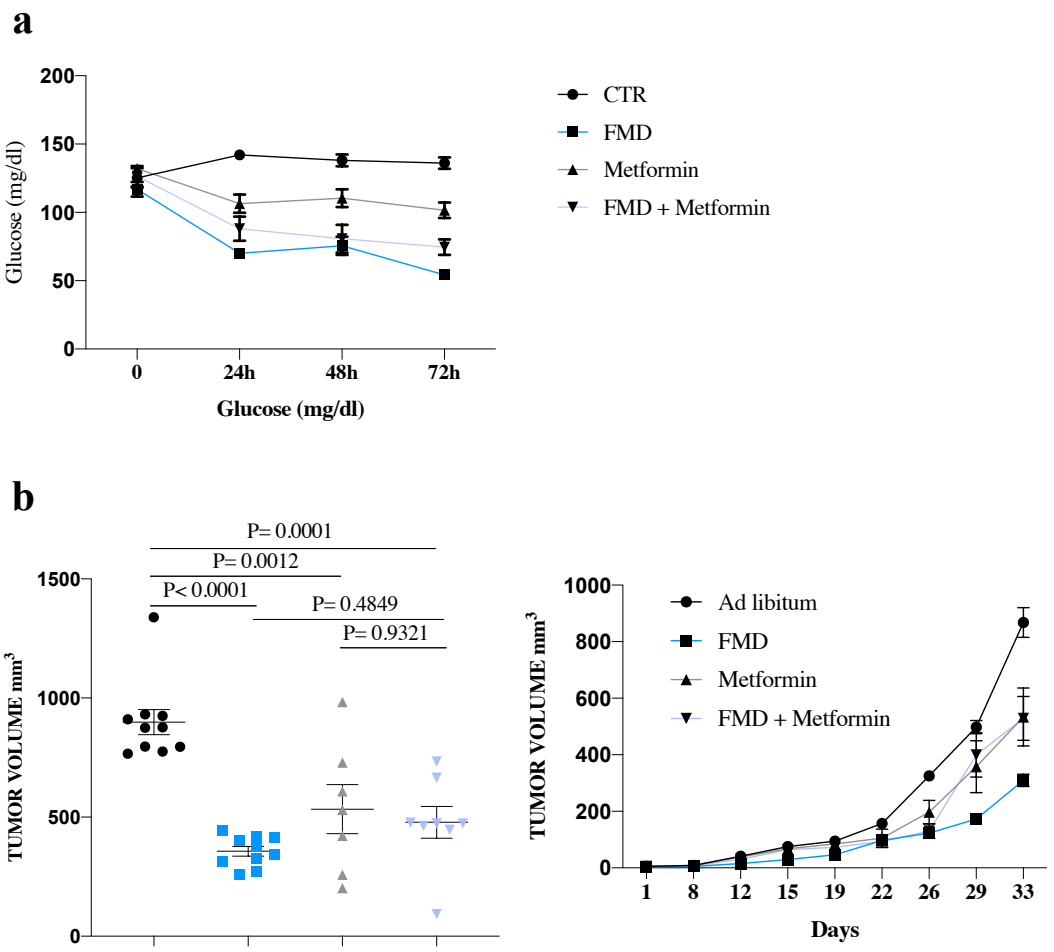



**Figure 16. Metformin does not enhance FMD effect in delaying tumor progression.**
8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to 5 cycles of FMD alone or in combination with metformin (150mg/kg) once a day, i.p. a) Blood glucose level was determined through Accu chek guide instrument. b) Tumor volumes before mice were sacrificed are reported (n=10-7). Data are represented as mean ± SEM. One-way Anova was performed.

At the end of the experiment, tumor masses were excised and used to perform ex vivo primary mammospheres assay, to test the combinatory effect of FMD and metformin on CSCs; differently from *in vitro* results, metformin decreased mammosphere number similarly to FMD. However, similarly to *in vitro* experiments, metformin combined to cyclic FMD did not result in additive or synergistic inhibition of mammosphere formation, suggesting that these approaches may be acting by shared pathways. Moreover, these effects could be partly due to the capability of metformin alone to lead to a 23% decrease in blood glucose levels, without potentiating the effect induced by cyclic FMD (Figure 17).

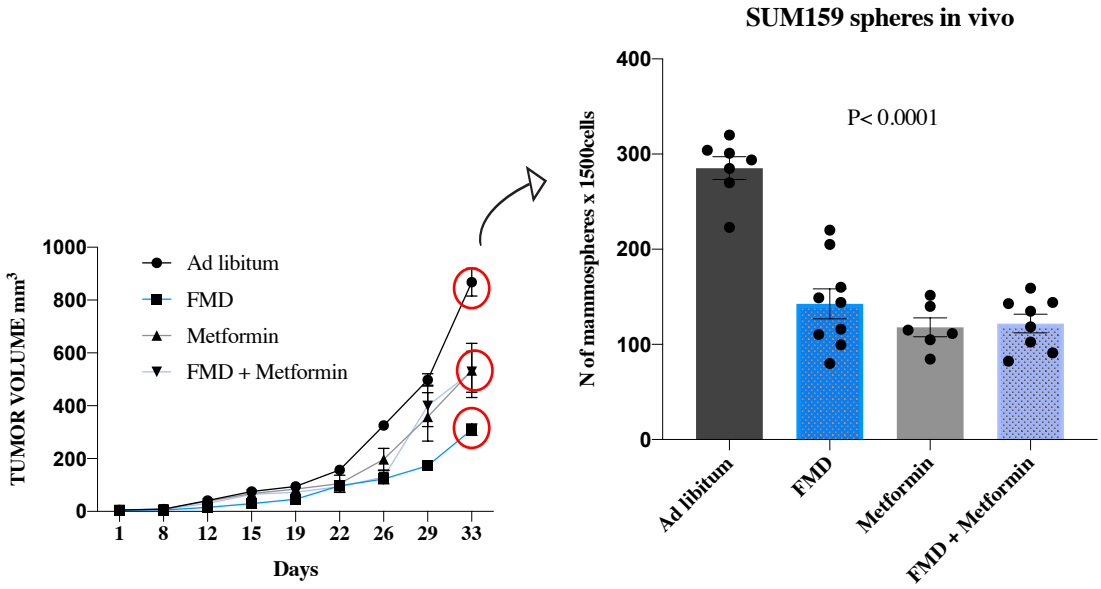



**Figure 17. Metformin reduces mammospheres growth but does not potentiate the effect of FMD.**

After 5 weeks of AL diet or FMD cycles alone or in combination with metformin (150mg/kg) once a day, i.p, tumor masses were excised and processed for ex vivo primary mammospheres forming assay (obtained from 1500 cells, n=9-7 biological replicates). Data are represented as mean ± SEM. One-way Anova was performed.

## 2.4 STS/FMD effect is potentiated by the hexokinase inhibitor 2Deoxy-D-Glucose.

I previous showed that CSCs are susceptible to glucose restriction; in fact, FMD dependent CSCs reduction results to be mediated by glucose lowering. Since a mitochondrial metabolism inhibitor, such as metformin, does not potentiate the effect of FMD on CSCs, even if it is known to reduce glucose in blood, I tried to inhibit glycolysis blocking the hexokinase enzyme, using 2Deoxy-D-Glucose (2DG). 2 DG is a glucose structure analogue and appears to selectively accumulate in cancer cells by metabolic trapping, due to high intracellular levels of hexokinase, leading to inhibition of glycolysis and glucose metabolism. Moreover, 2DG is reported to inhibit breast cancer cell growth and clonogenicity, in combination with chemotherapy (Zhao Y et al., 2011). Therefore, I tested whether 2DG could improve STS effect on mammosphere growth, *in vitro,* leading to a further decrease in glucose uptake. I found that STS-mediated inhibition of mammosphere formation is potentiated by 2DG; in fact, the dual treatment greatly reduced the formation of spheres compared to STS, while the drug alone resulted to not alter spheres proliferation, compared to CTR conditions (Figure 18).

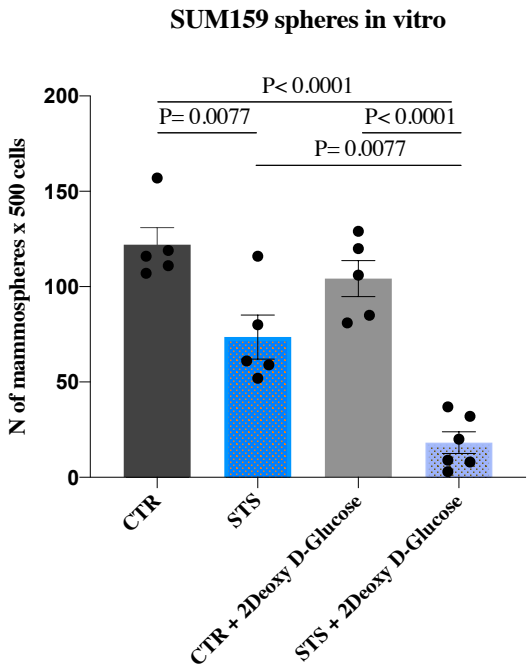


**Figure 18. 2Deoxy-D-Glucose potentiates STS effect in reducing mammosphere growth**. SUM159 cells were grown under control (CTR: 1g/l Glucose, 10%FBS) and starved (STS: 0,5g/l, 1%FBS) conditions for 48h, and then treated with placebo or 2DG (4mM). Cells were then plated as single cells on non-adherent plates in serum-free mammosphere medium with growth factors. Figure 17 shows representative SUM159 spheres (obtained from 500 cells, n=6-5 biological replicates) after 8 days of in vitro culture. Data are represented as mean ± SEM. One-way Anova was performed.

Subsequently, I tested 2DG effect in combination with FMD, both in delaying tumor progression and in reducing CSCs.

Immune-deficient mice bearing SUM159 xenografts were fed AL with standard diet or with cyclic FMD and daily treated with 2DG (500mg/kg) or PBS for 5 weeks. I obtained that FMD is much more effective than 2DG inhibitor on tumor progression, in those mice fed with standard diet. Interestingly, 2DG potentiated the anti-tumor effect of fasting, further reducing tumor volume compared to FMD alone (Figure 19).

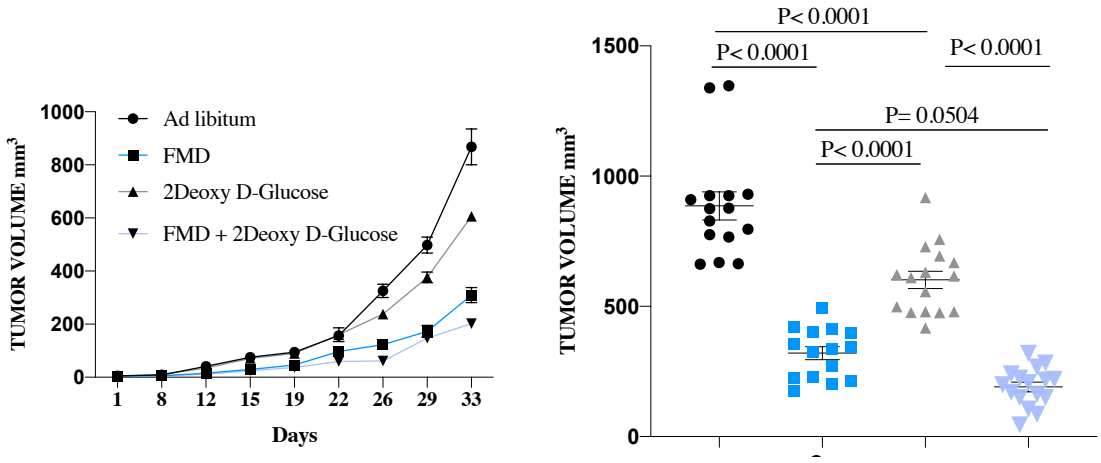



**Figure 19. FMD effect in delaying tumor progression is potentiated by 2DG.**
8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to 5 cycles of FMD alone or in combination with 2DG (500mg/kg) once a day, i.p. Tumor volumes before mice were sacrificed are reported (n=16-15). Data are represented as mean ± SEM. One-way Anova was performed.

After 5 cycles of FMD, mice were sacrificed and tumor masses were excised and processed to perform the *ex vivo* primary and *ex vivo* serial spheres forming assays, to test the combinatory effect of FMD and 2DG on CSCs with the highest self-renewal potential. 2DG resulted to potentiate the effect of FMD in decreasing the number of

mammospheres derived by SUM159 xenografts, even after a multiple serial propagation, while it had no effects in AL conditions. In fact, there are no differences between 2DG and AL conditions, in term of mammosphere number (Figure 20).

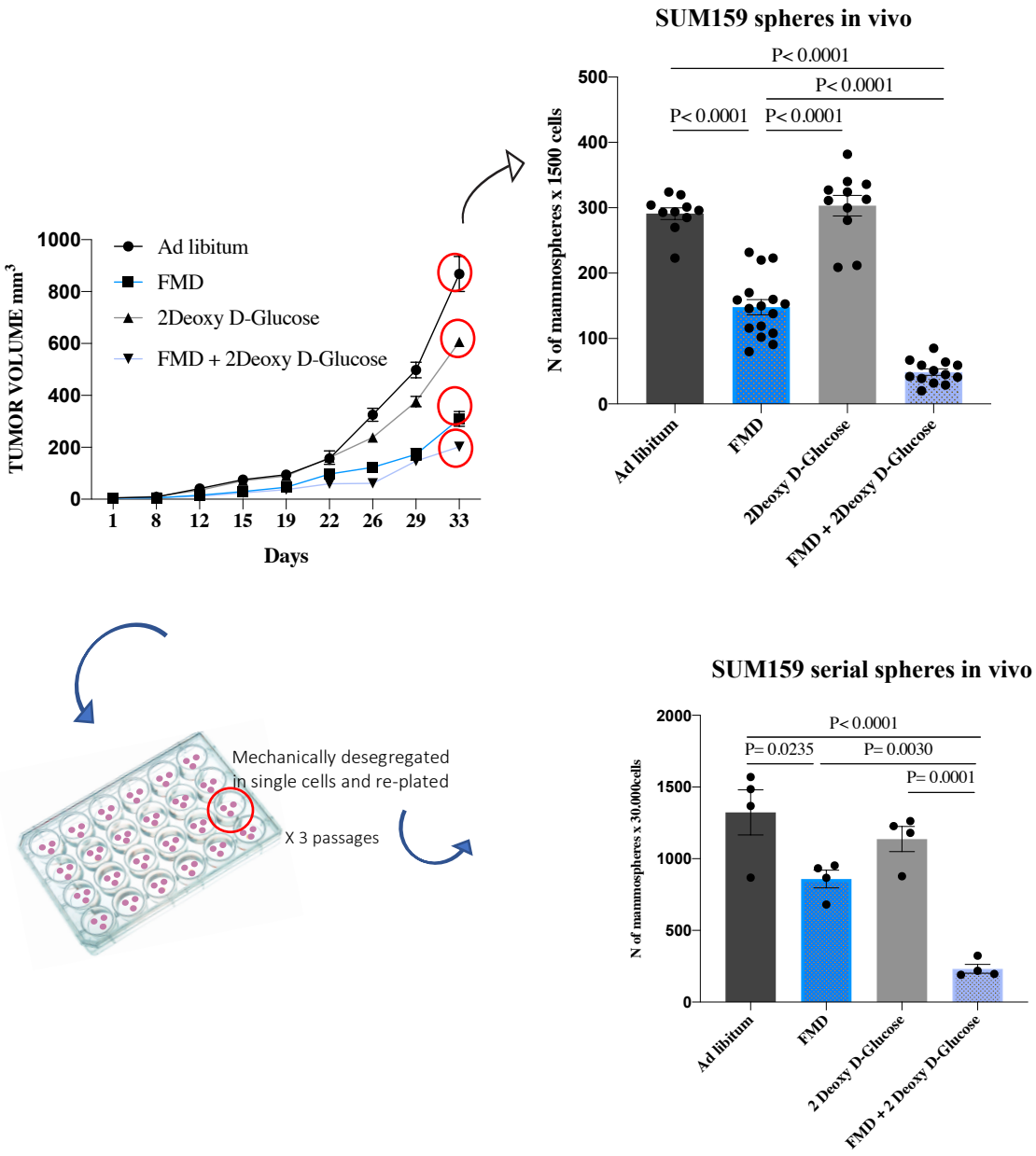



**Figure 20. 2DG potentiates FMD effect in decreasing ex vivo spheres formation and self-renewal.**

After 5 weeks of AL diet or FMD cycles, alone or plus 2DG (500mg/kg, daily, i.p), tumor masses were excised and processed for ex vivo primary mammospheres (obtained from 1500 cells, n=15-10 biological replicates) and for ex vivo serial spheres forming assay (obtained from 30.000 cells generated from dissociated secondary spheres, n= 4 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

Furthermore, I performed the limiting dilution assay to further confirm 2DG potential in enhancing FMD effect in reducing staminal population. Mice bearing SUM159 xenografts, fed with AL diet or subjected to FMD cycles, alone or treated with 2DG, were used as cancer cells donors. After 5 weeks, donor mice were sacrificed and cells derived from tumor masses were injected in recipient mice, always fed with AL diet, at different cells dilution. Notably, 2DG potentiated the effect of FMD in increasing mice survival, both compared to 2DG and FMD alone; in particular, at 1000 cells dilution, I detected a complete absence of tumor initiating cells, in FMD + 2DG group, even after 150 days post cancer cells injection. Collectively, these data confirm that CSCs are sensitive to glucose deprivation mediated by FMD and that 2DG causes a strong potentiation of FMD toxicity against CSCs (Figure 21).

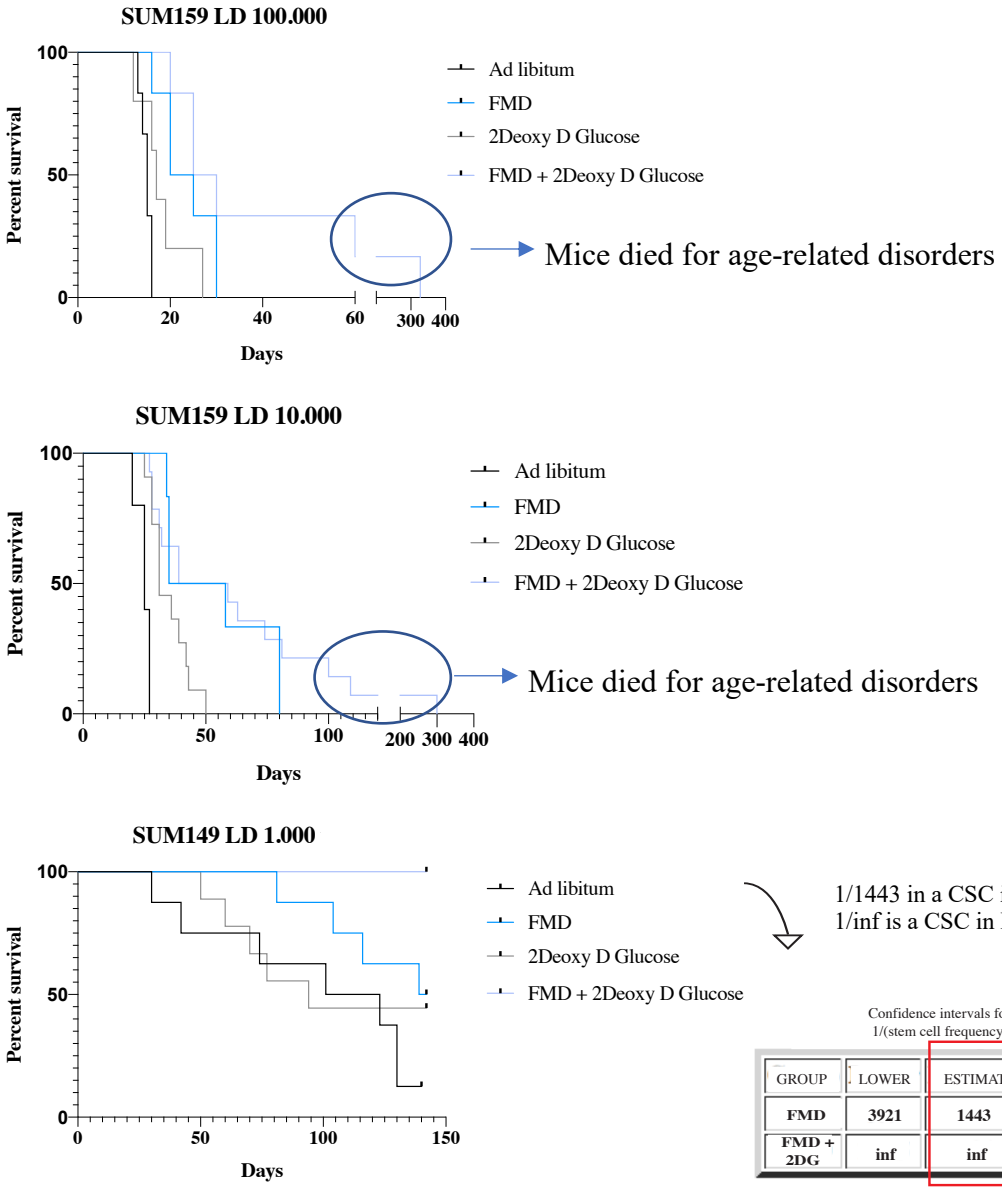


**Figure 21. FMD in combination with 2DG leads to a further decrease in stem cell frequency.** SUM159 tumor cells derived from in vivo xenografts were injected in recipient mice at different dilution to perform the limiting dilution assay. n=14-10. P values were determined by Log-rank (Mantel-Cox) test (100.000cells: Ad libitum vs FMD, p= 0.0024; Ad libitum vs 2Deoxy D-Glucose, p= 0.0660; Ad libitum vs FMD + 2 Deoxy D-Glucose, p= 0.0008; FMD vs 2 Deoxy D-Glucose, p= 0.1007; FMD vs FMD + 2 Deoxy D-Glucose, p= 0.1657; 2 Deoxy D-Glucose vs FMD + 2 Deoxy D-Glucose, p= 0.0177; 10.000 cells: Ad libitum vs FMD, p= 0.0011; Ad libitum vs 2 Deoxy D-Glucose, p= 0.0003; Ad libitum vs FMD + 2 Deoxy D-Glucose, p<0.0001; FMD vs 2 Deoxy D-Glucose, p= 0.0428; FMD vs FMD + 2 Deoxy D-Glucose, p= 0.3120; 2 Deoxy D-Glucose vs FMD + 2 Deoxy D-Glucose, p= 0.0192; 1.000 cells: Ad libitum vs FMD, p= 0.0891; Ad libitum vs 2 Deoxy D-Glucose, p= 0.3981; Ad libitum vs FMD + 2 Deoxy D-Glucose, p= 0.0001; FMD vs 2 Deoxy D-Glucose, p= 0.4714; FMD vs FMD + 2 Deoxy D-Glucose, p= 0.0123; 2 Deoxy D-Glucose vs FMD + 2 Deoxy D-Glucose, p= 0.0067). The stem cell frequency was calculated using ELDA software.

## 3. FMD reduces tumor growth and spheres number in 4T1 TNBC syngeneic model, and its effect is glucose dependent.

Supported by results described above, obtained on SUM159 human TNBC model, I also tested the effect of FMD in a syngeneic model of TNBC (4T1 allograft), in immune-competent mice. Firstly, I evaluated the effect of STS conditions on mammosphere formation, *in vitro*. In particular, 4T1 TNBC cells were grown in CTR and in STS media for 48h and then were collected and processed to be plated as single cells on non-adherent plates, in serum-free mammosphere medium with growth factors. STS greatly decreased the number of spheres compared to CTR conditions, as also in SUM159 cells (Figure 22).

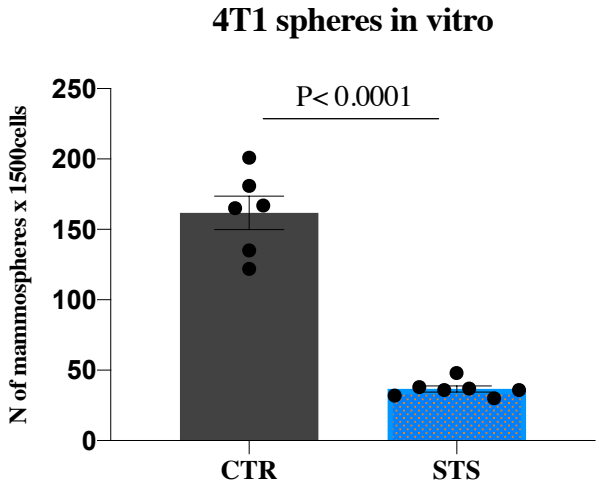



**Figure 22**. **STS conditions decrease 4T1 TNBC mammospheres**.
4T1 cells were grown under control (CTR: 1g/l Glucose, 10%FBS) and starved (STS: 0,5g/l, 1%FBS) conditions for a total of 48h. Cells were then plated as single cells on non-adherent plates in serum-free mammosphere medium with growth factors. Figure 21 shows the number of 4T1 spheres (obtained from 1500 cells) after 8 days of in vitro culture (n= 6-7 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

Therefore, I evaluated the effect of FMD in delaying tumor progression and reducing CSCs in 4T1 murine model. Immune-competent mice bearing 4T1 xenografts were divided in two groups, one fed AL with standard rodent diet and one subjected to FMD cycles. Tumor progression was also monitored with bioluminescent imaging, 1 week and

4 weeks after 4T1-luc cells injection in the mammary fat pad. I found that 4 cycles of FMD greatly reduce cancer size compared to AL, results similar to those obtained in immune-deficient mice with SUM159 cell line (Figure 23).

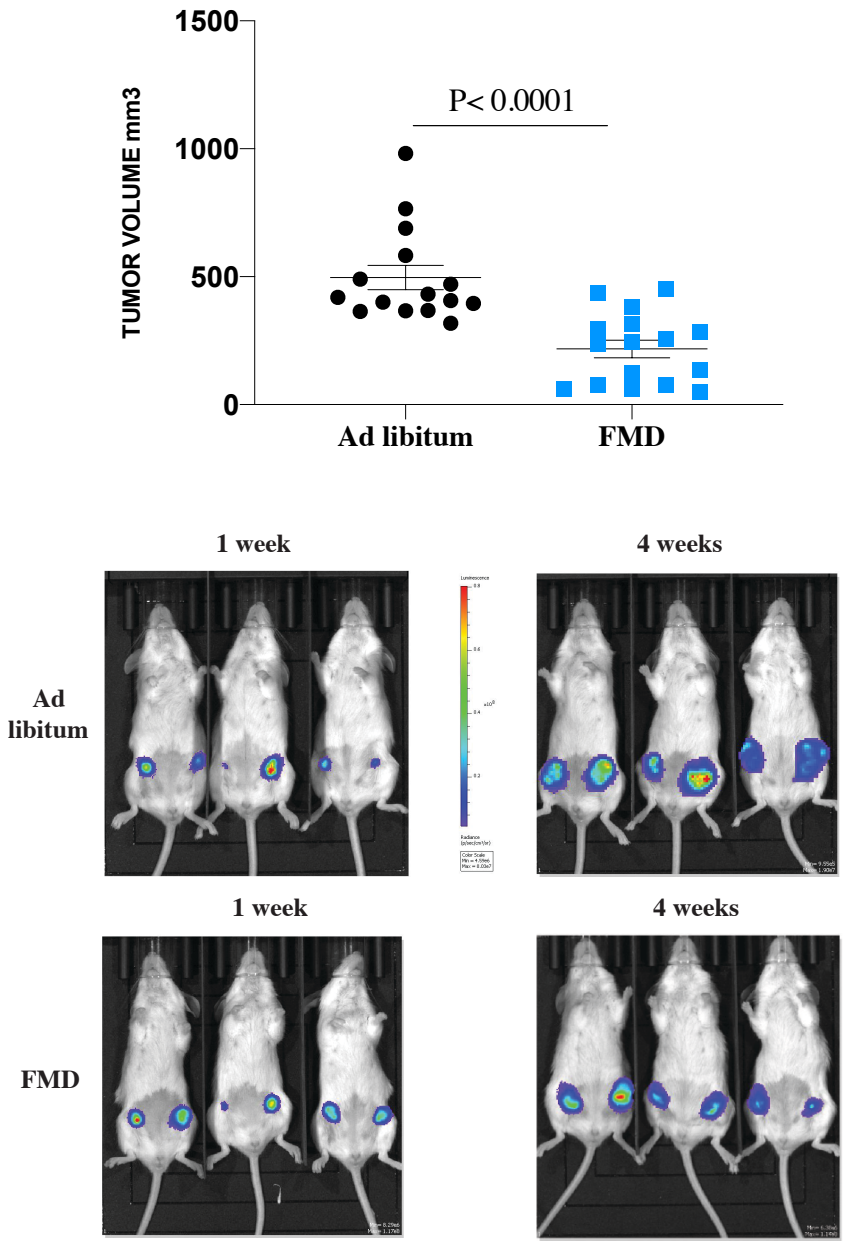



**Figure 23. FMD reduces tumor progression in 4T1 TNBC model.**

6-weeks old female Balb-c mice were injected bilaterally in the mammary fat pad with 4T1-luc cells and fed with standard diet or subjected to 4 cycles of FMD. Tumor progression was monitored with bioluminescent imaging 1 week and 4 weeks after 4T1-luc cells injection in the mammary fat pad. Tumor volumes before mice were sacrificed are reported (n=15-16 per group). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

Four weeks after cells injection, mice were sacrificed and tumor masses were excised and processed to perform the *ex vivo* primary spheres forming assay, to evaluate the effect on CSCs. In according with my previous results, FMD reduced mammosphere number compared to AL, confirming the effect of FMD on TNBC stem cells, independently of immune system (Figure 24).

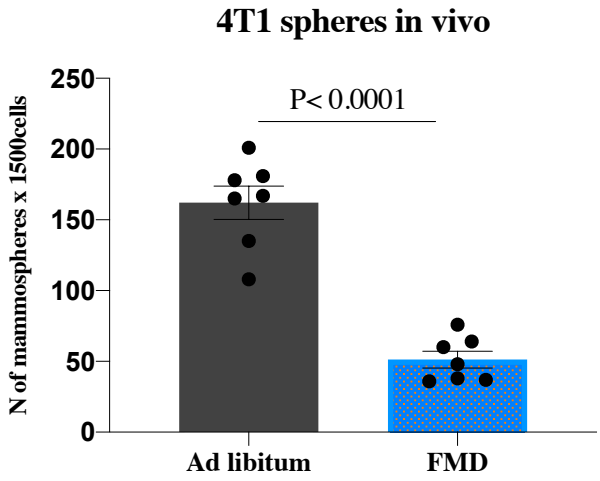



**Figure 24. FMD reduces ex vivo spheres formation in 4T1 TNBC model.**
After 5 weeks of AL diet or FMD cycles, tumor masses were excised and processed for ex vivo primary mammospheres forming assay. Figure 23 shows the number of 4T1 spheres (obtained from 1500 cells) after 8 days of in vitro culture (n=7 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

Furthermore, I measured, by flow cytometry analysis, the expression of CD44 CD24 markers, in 4T1 tumor masses, and determined that FMD strongly reduces the percentage of $CD44^{high}CD24^{low}$ population, compared to AL conditions (Figure 25).

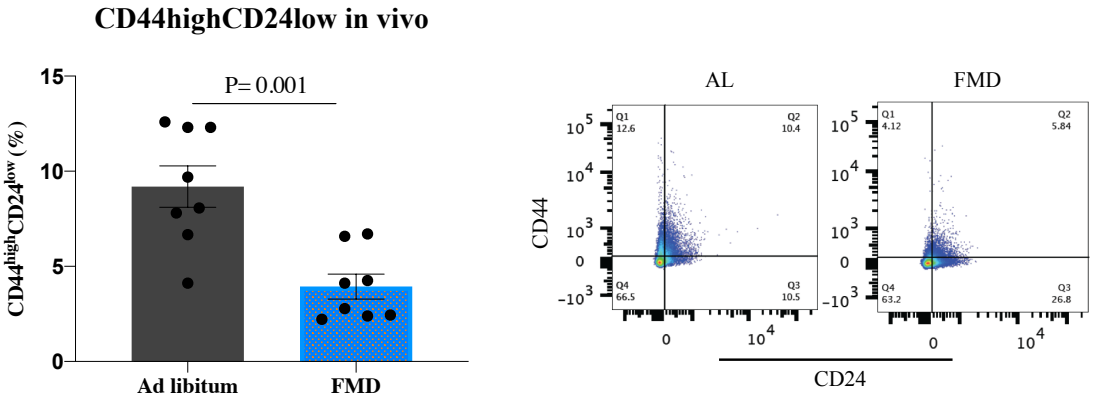


**Figure 25. FMD reduces $CD44^{high}CD24^{low}$ staminal population in 4T1 xenografts.**

FACS analysis were performed to measure CD44 and CD24 expression in 4T1 xenografts. The percentages reflect the population of putative breast cancer stem cells defined as $CD44^{high}CD24^{low}$ (n= 8 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

To confirm that glucose depletion plays a crucial role in FMD-induced mammosphere reduction also in the 4T1 TNBC model, I performed *in vitro* experiment to test whether 1 g/l of glucose is able to revert the effect of STS on spheres formation. Cells were grown in STS + glucose medium for 48h and then processed to perform the mammosphere forming assay. I found that glucose rescues almost completely STS dependent mammosphere reduction, according with data from human TNBC cells (Figure 26).

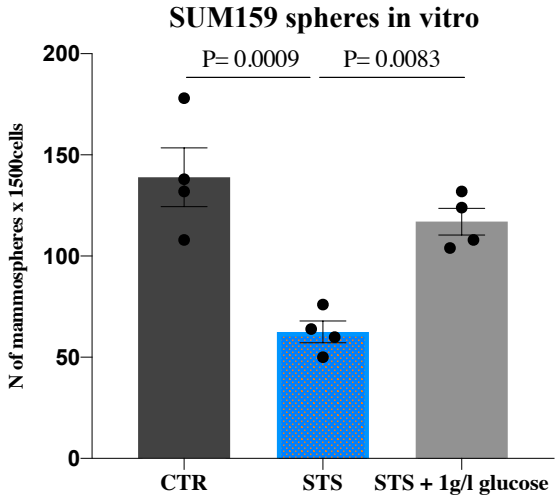
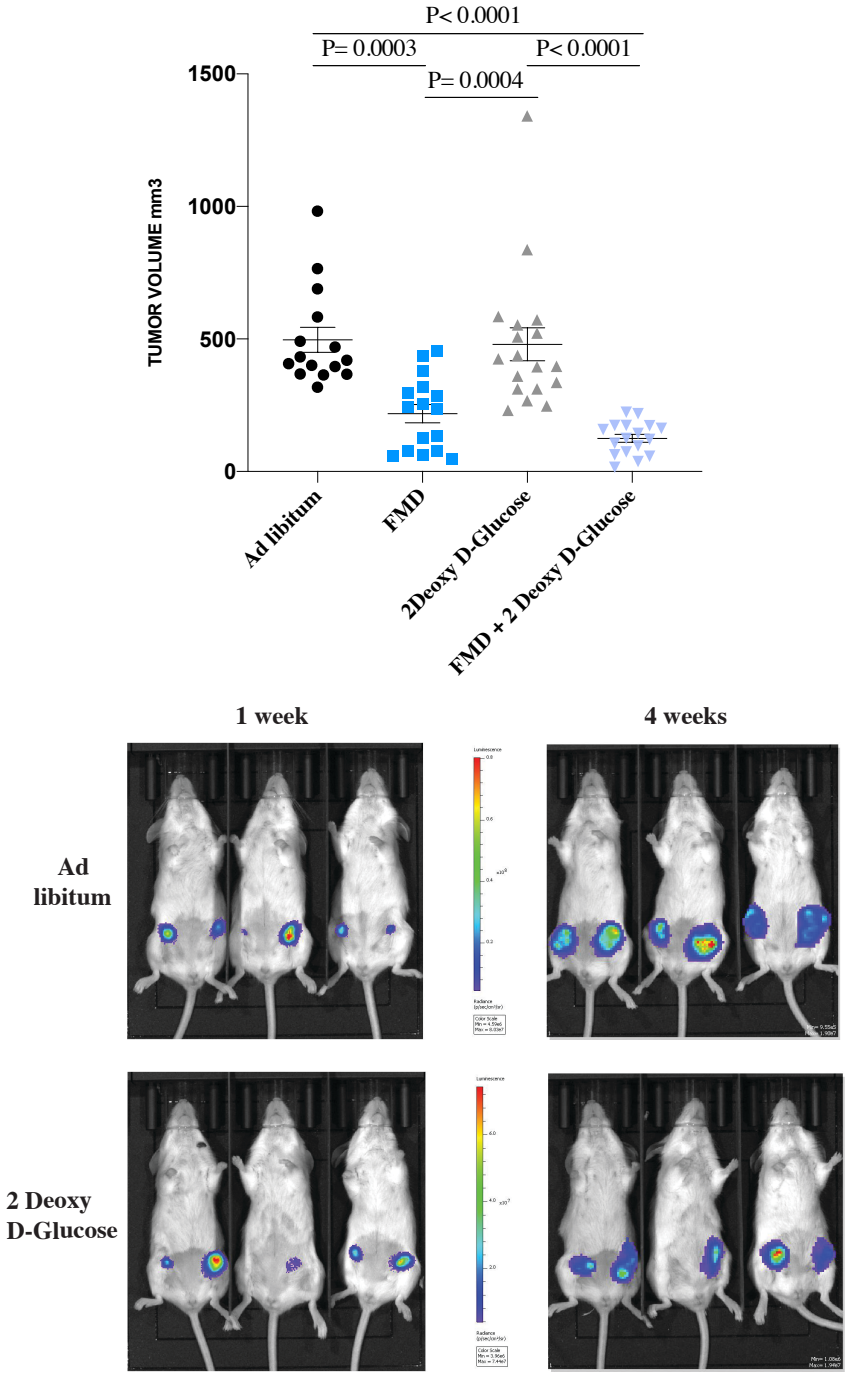



**Figure 26. Glucose supplementation reverses STS dependent mammosphere reduction in 4T1 TNBC cells.**

4T1 cells were grown under CTR (1g/l Glucose, 10%FBS), STS (0,5g/l, 1%FBS) and STS + 1g/l of glucose conditions for a total of 48h. Cells were then plated to perform the in vitro spheres forming assay. Figure 25 shows the number of 4T1 spheres (obtained from 1500 cells) after 8 days of in vitro culture (n=4 biological replicates). Data are represented as mean ± SEM. One-way Anova was performed.

Starting from promising results obtained with 2DG in SUM159 human TNBC model and after confirming that also 4T1 TNBC stem cells are sensitive to glucose deprivation, I evaluated the effect of 2DG in combination with FMD, in immuno-competent mice bearing 4T1 xenografts. Balb/c mice were daily treated with 2DG (500mg/kg) for 4 weeks and tumor progression was monitored with bioluminescent imaging, 1 week and 4 weeks

after 4T1-luc cells injection in the mammary fat pad. 2DG resulted to slightly potentiate the effect of FMD in delaying tumor progression, while 2DG, in mice fed with standard diet, did not have any effect, confirming results obtained with SUM159 xenografts (Figure 27).



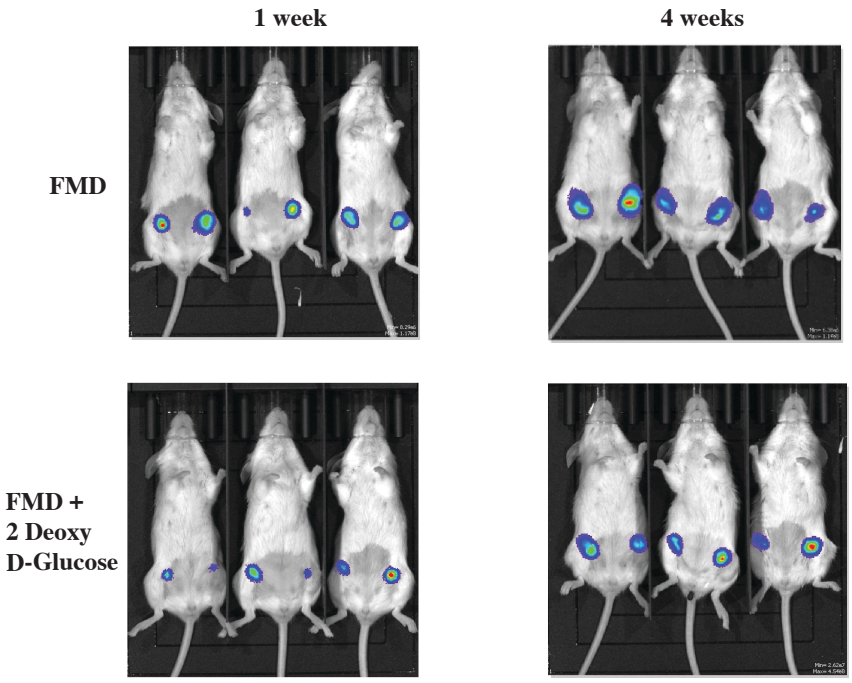



**Figure 27. 2DG potentiates FMD effect in reducing tumor volume in 4T1 xenografts.**
6-weeks old female Balb-c mice were injected bilaterally in the mammary fat pad with 4T1-luc cells and fed with standard diet or subjected to 4 cycles of FMD, alone or in combination with 2DG (500mg/kg) once a day, i.p. Tumor progression was monitored with bioluminescent imaging 1 week and 4 weeks after 4T1-luc cells injection in the mammary fat pad. Tumor volumes before mice were sacrificed are reported (n=18-15). Data are represented as mean ± SEM. One-way Anova was performed.

Four weeks after cells injection, mice were sacrificed and tumor masses were excised and processed to perform cytometry analysis, in order to evaluate the expression of CD44CD24 markers. I obtained that 2DG slightly potentiates the effect of FMD in reducing the percentage of $CD44^{high}CD24^{low}$ population compared to FMD alone. Differently from SUM159 model, 2DG alone reduced $CD44^{high}CD24^{low}$ population in 4T1 xenografts, compared to AL, but FMD alone was more effective than 2DG, in those mice fed with standard diet. (Figure 28).

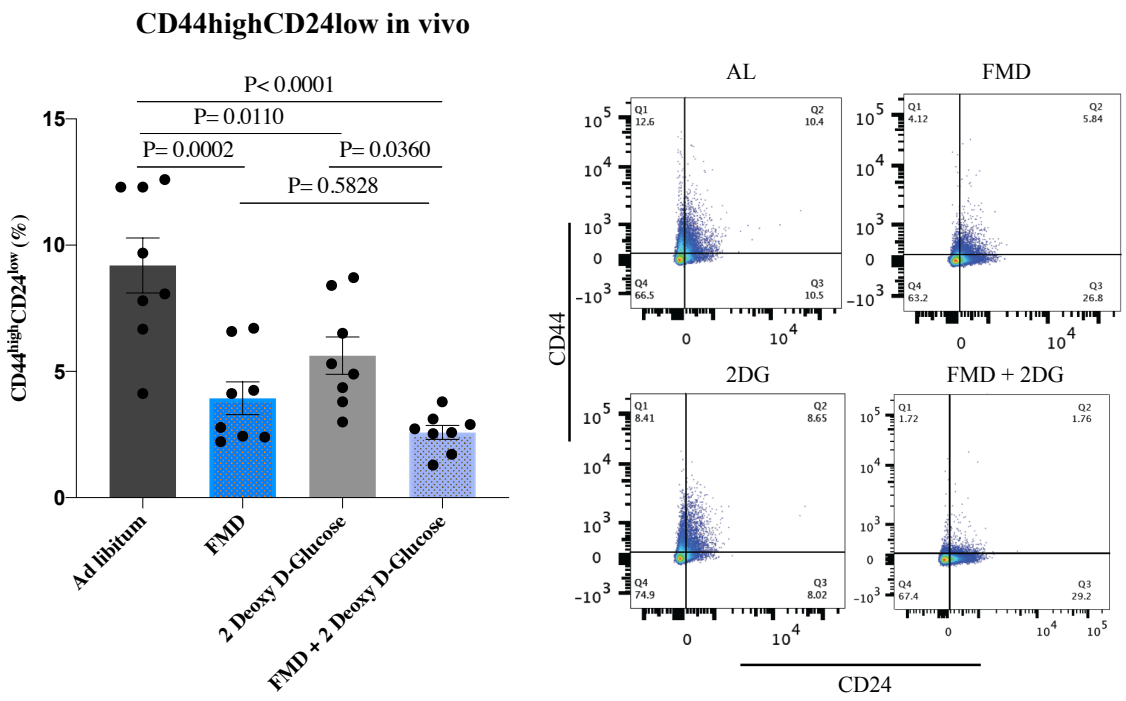



**Figure 28. 2DG slightly potentiates FMD effect in reducing $CD44^{high}CD24^{low}$ population in 4T1 xenografts.**

FACS analysis were performed to measure CD44 and CD24 expression in 4T1 xenografts. The percentages reflect the population of putative breast cancer stem cells defined as $CD44^{high}CD24^{low}$ (n= 8 biological replicates). Data are represented as mean ± SEM. One-way Anova was performed.

# 4. PKA is down-regulated by FMD and its activation reverses STS dependent mammosphere reduction.

Based on my previous results, I started to investigate the mechanism through which glucose depletion sensitizes CSCs. In normal cells and stem cells, prolonged fasting is reported to reduce the protein kinase A (PKA) (Cheng CW et al., 2014; Brandhorst S et al., 2015), a signaling pathway dependent on cellular levels of cyclic adenosine monophosphate (cAMP), the production of which is strictly related to glucose levels. In particular, prolonged fasting reduces PKA signaling in bone marrow cells, partly through the reduction of IGF-1 levels, promoting hematopoietic stem cells self-renewal (Cheng CW et al., 2014). In addition, fasting is reported to reduce PKA activity even in dentate gyrus-enriched samples derived from mice treated with cyclic FMD, inducing pro-regenerative changes, while an increase in PKA signaling is observed during the refeeding time (Brandhorst S et al., 2015). Moreover, STS/fasting down-regulates glycolysis reducing ATP synthesis in colon cancer models, leading to an increase in OXPHOS and consequently in ROS production (Bianchi G et al., 2015). Decrease in ATP molecules leads to the accumulation of AMP and consequently to cAMP depletion.

Accumulated evidence reports that cAMP regulates several cellular processes, such as survival, proliferation, differentiation and angiogenesis, through the activation of its downstream effector, PKA. Once activated, PKA phosphorylates and modulates the activity of different cytosolic and nuclear substrates, including the transcription factor cAMP response element-binding protein (CREB) and the glycogen synthase kinase-3β (GSK3β).

To investigate the FMD effect of PKA axis in SUM159 and 4T1 xenografts, I examined the phosphorylation level of CREB (pCREB). Interestingly, FMD reduced the expression level of pCREB in both models, suggesting a PKA pathway reduction (Figure 29).

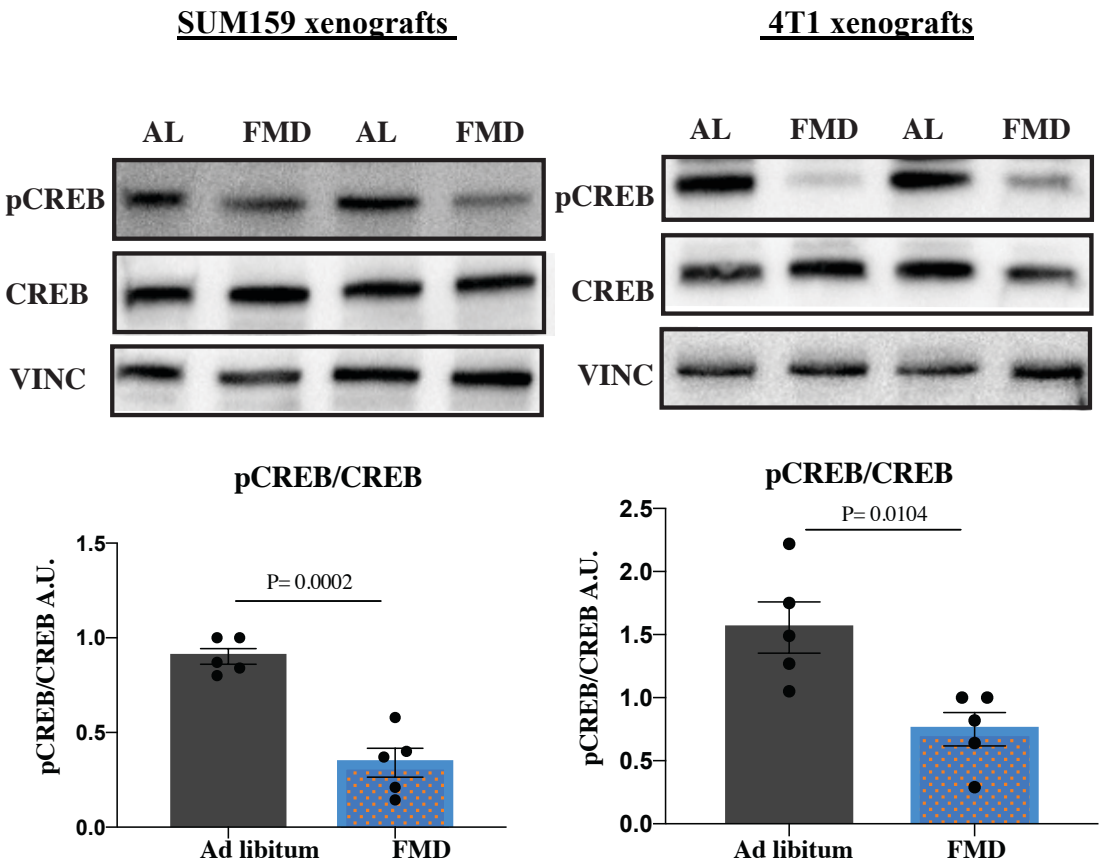



**Figure 29. FMD decreases the expression of pCREB in SUM159 and 4T1 xenografts.** Detection of phosphorylated CREB levels, total CREB and VINCULIN, as loading control, in SUM159 and 4T1 tumor masses (n=5 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

PKA regulates GSK3β, which in turn modulates the activity of human kruppel-like factor 5 (KLF5). KLF5 promotes TNBC cell proliferation, survival, migration and invasion; moreover, it directly regulates the expression of some stemness associated genes, including Nanog and Oct4 (Shi P et al, 2017). Therefore, KLF5 can be considered a potential target for TNBC stem cells.

PKA inhibition is reported to down-regulate KLF5; in fact, when PKA is downregulated, GSK3b is activated, thus leading to KLF5 ubiquitination and degradation. Based on my previous results suggesting a PKA pathway reduction mediated by FMD, I investigated the involvement of PKA pathway in FMD dependent CSCs reduction. To this aim, I measured KLF5 expression in SUM159 and 4T1 tumor masses. Consistent with FMD-induced reduction of pCREB, I found lower KLF5 levels in mice undergoing the FMD when compared with mice fed with standard diet (Figure 30).

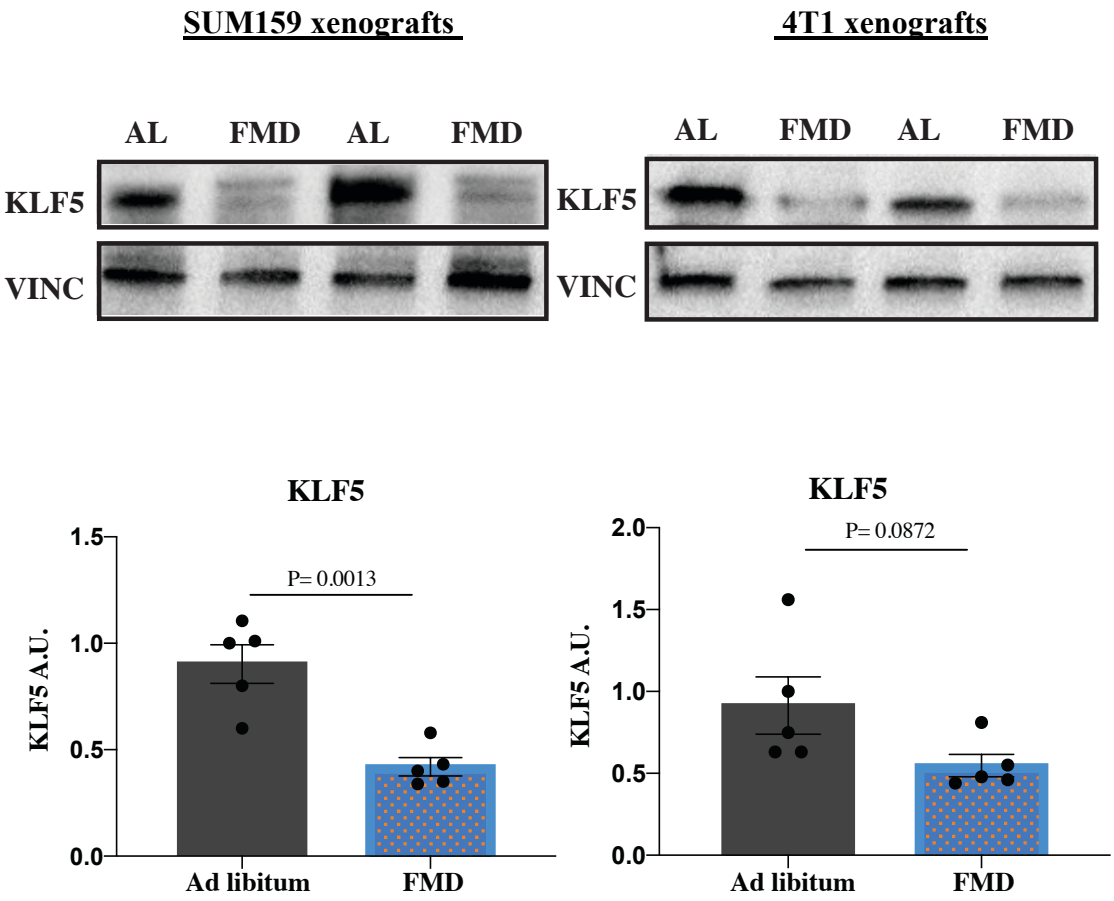



**Figure 30. FMD decreases the expression of KLF5 in SUM159 and 4T1 xenografts.**
Detection of KLF5 and VINCULIN, as loading control, in SUM159 and 4T1 tumor masses (n=5 biological replicates). Data are represented as mean ± SEM. Two-tailed unpaired t-test was performed.

To investigate whether FMD-induced inhibition of PKA plays a role in sensitizing CSCs, I tested the role of PKA in STS dependent mammosphere reduction by reactivating PKA under STS conditions. In particular, I treated cells with a PKA activator, the 8-Bromoadenosine 3',5'-cyclic mono-phosphate (8-Br-cAMP), a membrane-permeable cAMP derivate. Firstly, I verified the effectiveness of the drug, examining pCREB level after treatment, and I obtained that the phosphorylation level of CREB increases when cells are treated with 8-Br-cAMP, under STS conditions, compared to STS alone (Figure 31).

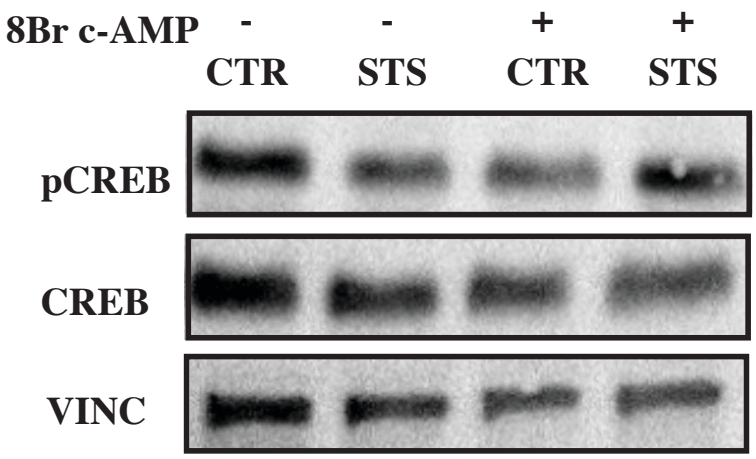



**Figure 31. 8-Br-cAMP increases pCREB expression in SUM159 cells under STS conditions.** Detection of phosphorylated CREB levels, total CREB and VINCULIN, as loading control, in SUM159 cells after 48h in STS medium (0,5 g/L glucose and 1% FBS), alone or in combination with 8-Br-cAMP.

Thereafter, SUM159 TNBC cells were grown in CTR and in STS media for a total of 48h and at 24h were treated with 8-Br-cAMP. After 24h of treatment, cells were processed to perform the *in vitro* spheres forming assay. Notably, the PKA activator completely reversed STS-dependent mammosphere reduction in SUM159 cell line. Moreover, 8-Br-cAMP also partially reversed STS effect in lowering sphere growth in 4T1 model. Collectively, my data are consistent with a central role of PKA inhibition, mediated by FMD, in stemness regulation (Figure 32).

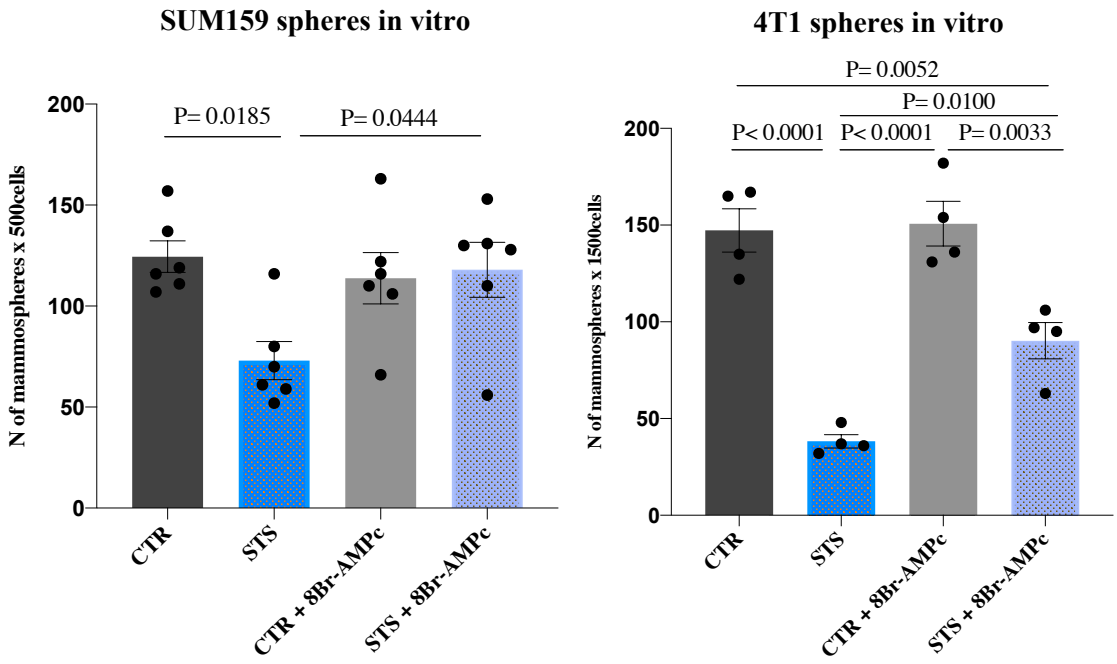


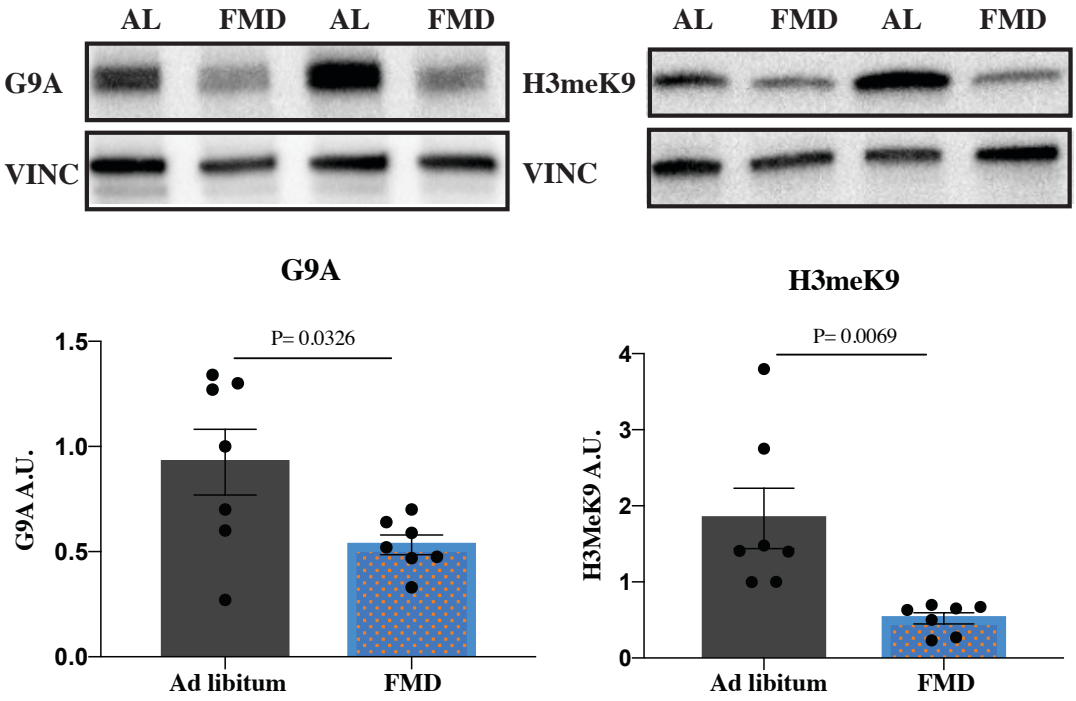
**Figure 32. 8Br-cAMP reverses STS dependent mammosphere reduction in SUM159 and 4T1 TNBC cell models.**

SUM159 and 4T1 cells were grown under CTR (1g/l Glucose, 10%FBS) and STS (0,5g/l, 1%FBS) conditions for a total of 48h. At 24h cells were treated with 8-Br-cAMP. Cells were then plated to perform the in vitro spheres forming assay. Figure 31 shows the number of SUM159 (obtained from 500 cells, n=6 biological replicates) and 4T1 spheres (obtained from 1500 cells, n=4 biological replicates) after 8 days of in vitro culture. Data are represented as mean ± SEM. One-way Anova was performed.

Moreover, PKA activates the H3K9 methyltranferase G9A, through CREB phosphorylation (Li SF et al., 2013). G9A was originally identified as a key histone methyltransferase for early embryogenesis, involved in the regulation of developmental gene expression (Tachibana M et al., 2002). Subsequently, its activity has been found to be linked to several types of cancer, including ovarian, lung, liver, breast and bladder cancers (Kondo Y et al., 2008; Hua KT et al., 2014; Bai K et al., 2016). In fact, H3K9 dimethylation mediated by G9A is known to mediate the epigenetic silencing of several tumor suppressor genes, including DSC3, MAPSIN, CDH1, and adhesion molecules (Chen MW et al., 2010). Moreover, G9A and H3K9 methylation are reported to promote cancer cell proliferation, autophagy, invasion, metastasis and cancer stemness (Kondo Y et al., 2007; Wozniak RJ et al., 2007; Chen MW et al., 2010).

G9A inhibition is shown to reduce metastasis formation in *in vivo* mouse models, override resistance to different chemotherapeutic drugs, inhibit CSCs self-renewal activity and epithelial-mesenchymal transition process (Tao H. et al., 2014; Pan MR. et al., 2016; Luo CW. et al., 2017). Since the H3K9 methyltranferase G9A is regulated by PKA and FMD reduces the expression of PKA pathway, I examined G9A and H3K9me2 levels in SUM159 and 4T1 xenografts. Of note, the FMD resulted in a remarkable reduction of G9A and H3K9me2 in both SUM159 and 4T1 xenografts (Figure 33).

## SUM159 xenografts




## 4T1 xenografts

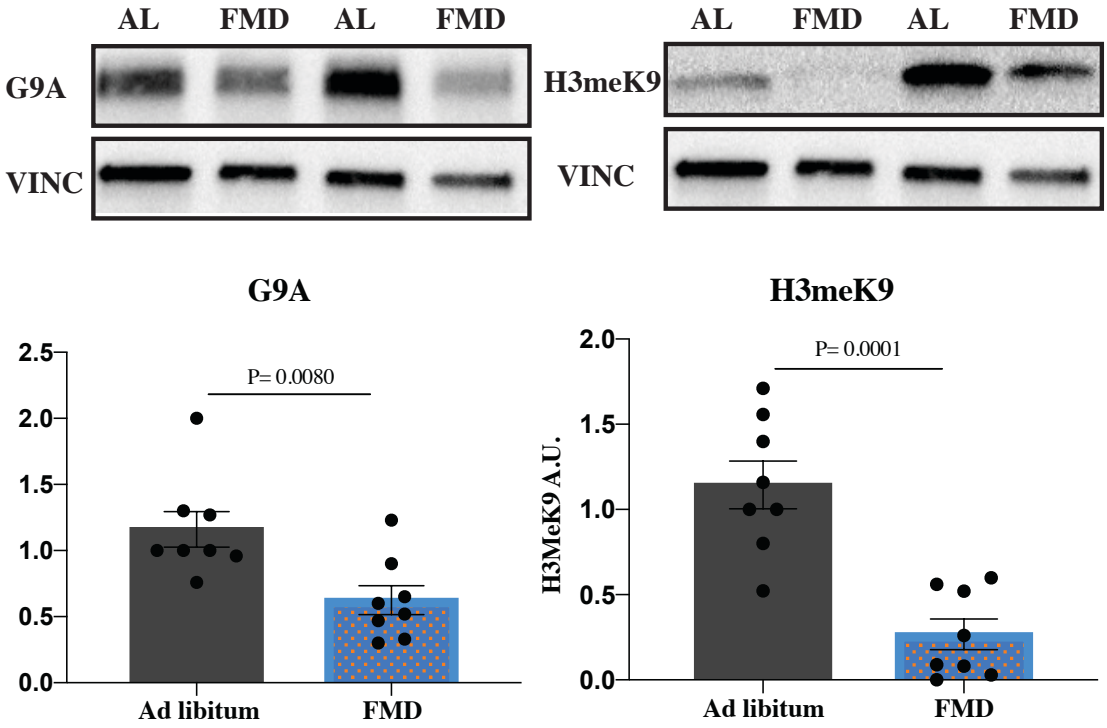


**Figure 33. FMD decreases G9A and H3meK9 expression in SUM159 and 4T1 xenografts.** Detection of G9A, H3meK9 levels and VINCULIN, as loading control, in SUM159 (n=7 biological replicates) and 4T1 (n=8 biological replicates) tumor masses. Data are represented as mean ± SEM. Unpaired t test was performed.

Both KLF5 and G9A are reported to regulate the activity of different stemness associated genes. In line with previous results, in SUM159 xenografts, the FMD reduced mRNA levels of KLF5- and G9A- downstream target stemness-associated genes OCT4 as well as NANOG, KLF2 and TBX3 that are genes regulated by the transcriptional activity of OCT4 (Parisi S et al, 2008; Luo CW et al., 2017) (Figure 34).

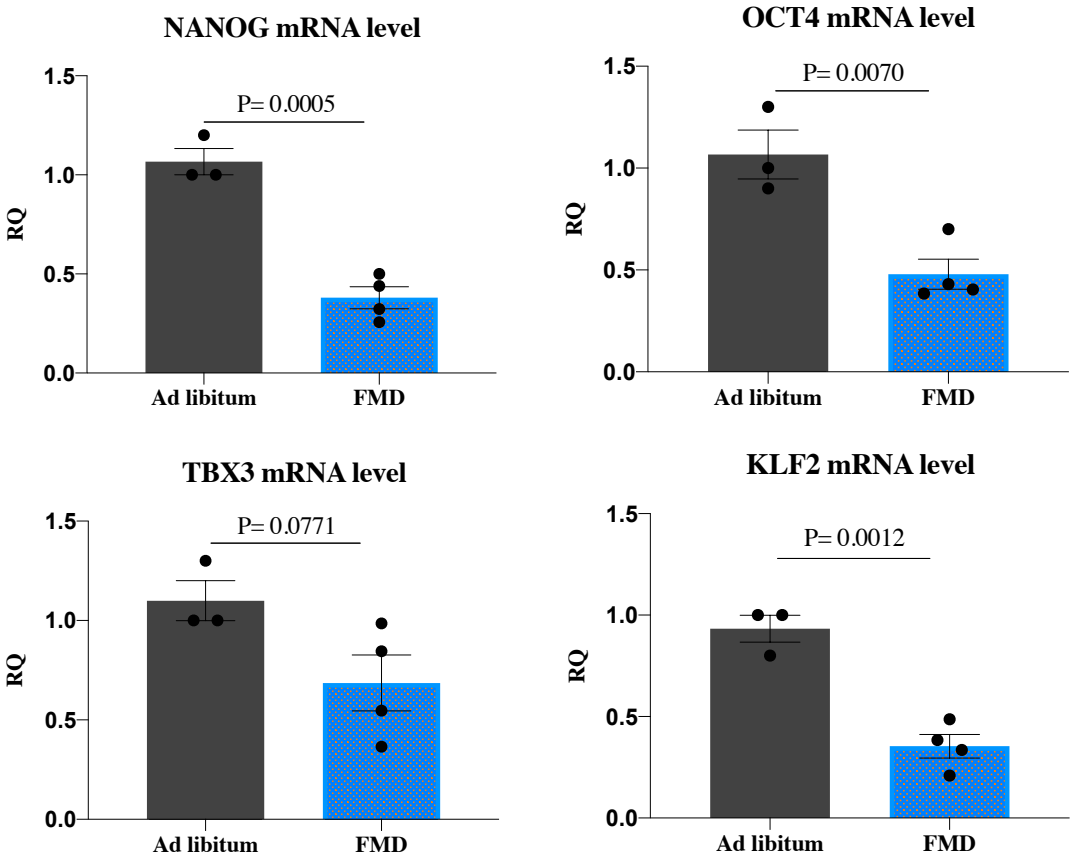



**Figure 34. FMD reduces the expression of stemness associated genes in SUM159 xenografts.** qPCR analysis was performed on SUM159 xenografts bared by mice fed with FMD or standard diet (CTR) (n=5, for each group). Data are represented as mean ± SEM. Unpaired t test was performed.

## 5. Escape pathways discovery through RNA Sequencing Analysis

I previously showed that STS/FMD greatly reduces CSCs, in two TNBC xenograft models, by reducing blood glucose levels. On the other hand, the bulk of differentiated TNBC cells could not be affected by glucose restriction as well.

To verify this hypothesis, I performed RNA sequencing analysis in order to identify a potential target therapy for TNBC. First, I sorted cancer cells, derived from SUM159 *in vivo* xenografts, with CD44CD24 human antibodies, in order to examine CSCs and differentiated cancer cells separately, to investigate more precisely the different mechanisms mediated by FMD in these two different populations inside the tumor.

Interestingly, RNA sequencing analysis revealed that the down-regulation of PKA pathway mediated by FMD occurs specifically in the $CD44^{high}CD24^{low}$ staminal population, compared to $CD44^{high}CD24^{high}$ differentiated cells, thus suggesting the selective involvement of PKA in affecting CSCs and confirming my previous results (Figure 35).

**FMD: $CD44^{high}CD24^{low}$ vs $CD44^{high}CD24^{high}$**

**PKA pathway**

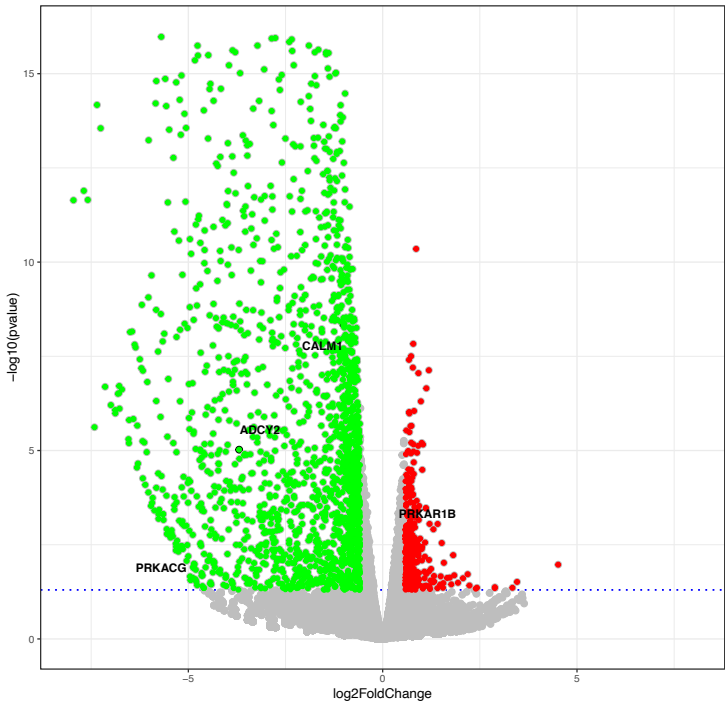


**Figure 35. PKA down-regulation mediated by FMD occurs specifically in CSCs.**
Volcano plot showing the significance versus the log2 fold-change between CD44hiCD24lo and CD44loCD24hi. Up and downregulated genes (|log2FC| > 0.58 and adj. p value < 0.05) are displayed in red and green respectively. Deregulated genes involved in the PKA pathway are highlighted.

Moreover, the FMD decreased the expression of stem cell targets in the $CD44^{high}CD24^{low}$ cell subset, compared to AL, confirming that FMD not only reduces the number of CSCs, but also their stemness potential, as illustrated by results obtained with the serial spheres forming assay (Figure 36).

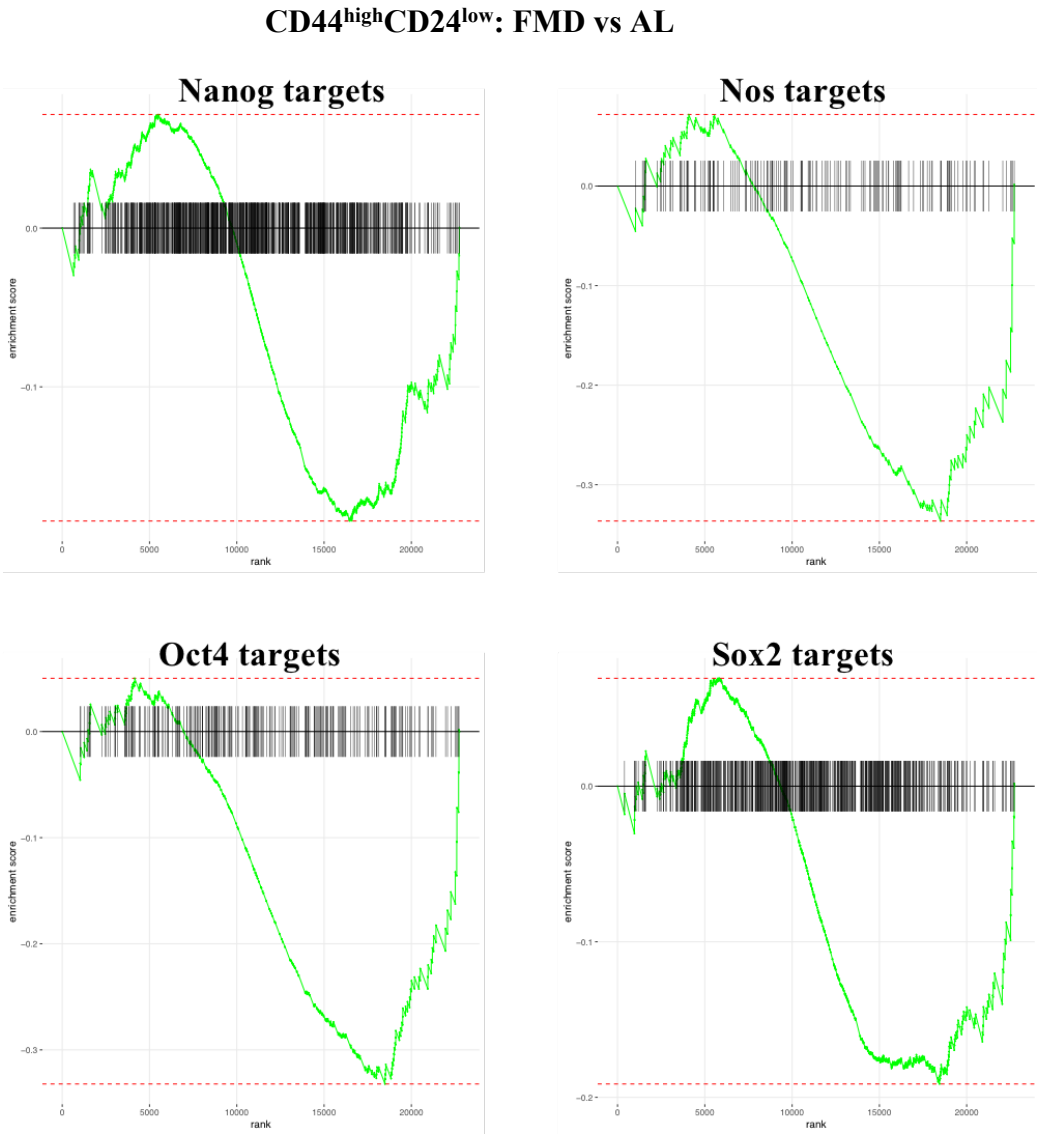


**Figure 36. FMD decreases the expression of stem cell targets in CSCs compared to AL.** Enrichment plots for Nanog, Nos, Oct4 and Sox2 target genes. The black vertical bars of each panel indicate the position of each gene in the sorted list. The green curve denotes the ES (enrichment score), the running-sum statistic calculated along the ranked list by the GSEA software.

In differentiated cancer cell subset, the FMD reduced the expression of Mps1, which is essential for chromosomes alignment at the centromere during mitosis and for centrosome duplication, and the expression of CycB and CDK1, which are essential for cell cycle transition from G2 phase to mitosis. Of note, Cyclin B overexpression is known to drive tumorigenesis and is associated with poor overall survival in breast cancer patients (Sun X, et al., 2017). At the same time, FMD upregulated the expression of CycD-CDK4/6, essential to drive cell cycle progression from G1 to S phase (Figure 37).

**$CD44^{high}CD24^{high}$: FMD vs AL**

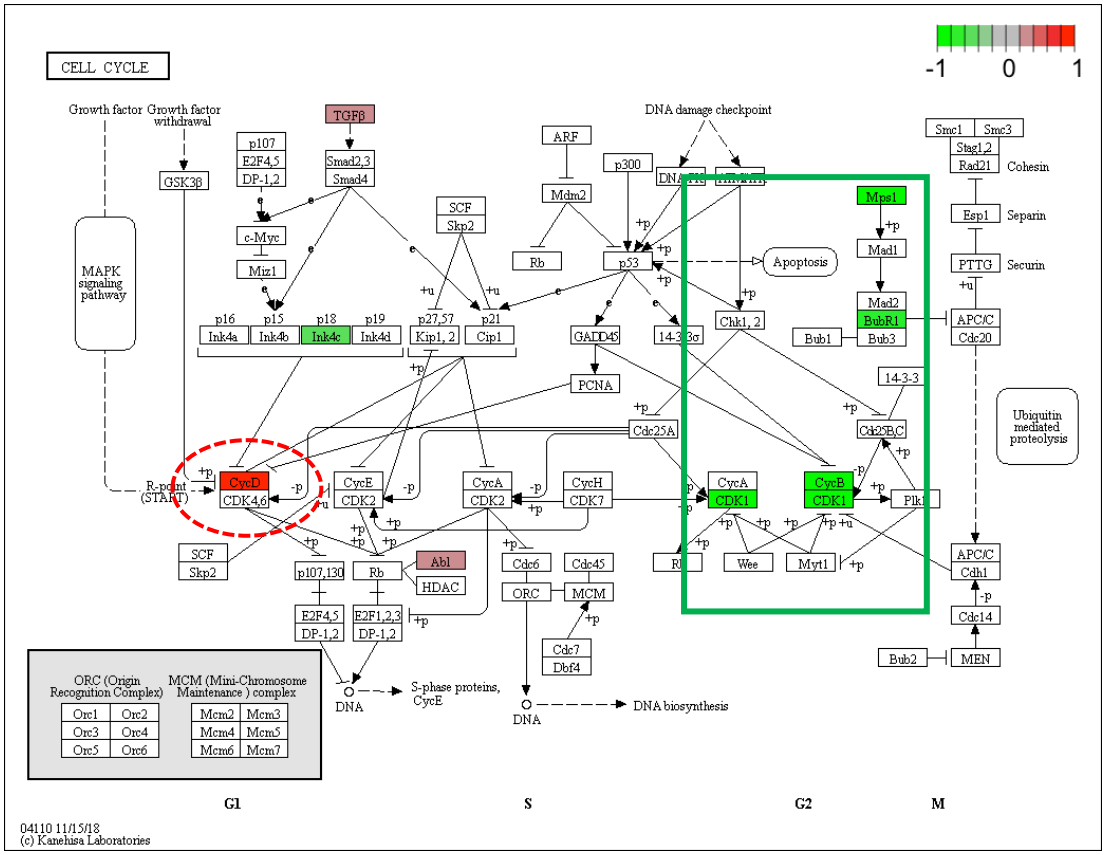


**Figure 37. FMD downregulates CycB-CDK1 while overexpresses CycD-CDK4/6, compared to AL, in differentiated cancer cells of SUM159 xenografts.**

Graph representation of KEGG cell cycle (hsa04110). Significantly up and downregulated genes (in $CD44^{high}CD24^{high}$ cells, by comparing FMD versus AL) are depicted in red and green respectively.

In differentiated cells, the FMD also resulted in the overexpression of pro-apoptotic molecules, including ASK1, a critical cellular stress sensor frequently activated by ROS, whose production is known to be increased by FMD, and Bim, a member of Bcl-2 family. These data confirm previous results showing that FMD induces apoptosis. Surprisingly, I also found that FMD results in a remarkable overexpression of genes belonging to the PI3K-AKT axis (Figure 38).

**$CD44^{high}CD24^{high}$: FMD vs AL**

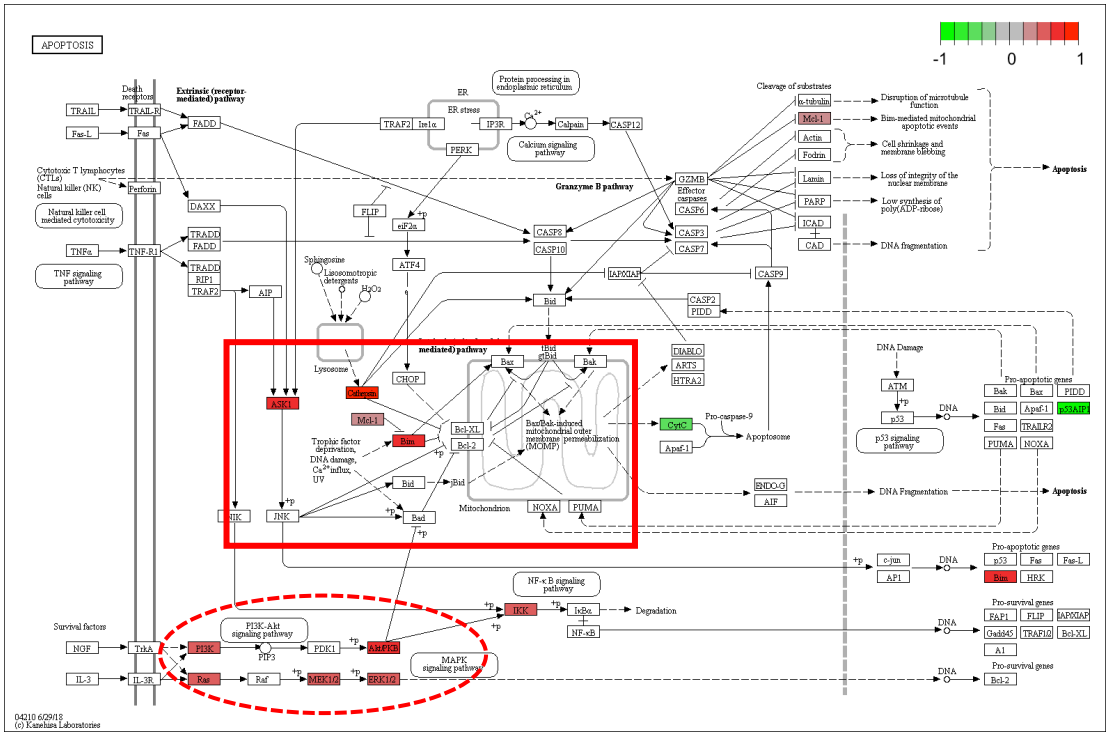



**Figure 38. FMD overexpresses pro-apoptotic molecules in differentiated cells while activates PI3K-AKT survival pathway, compared to AL, in SUM159 xenografts.**

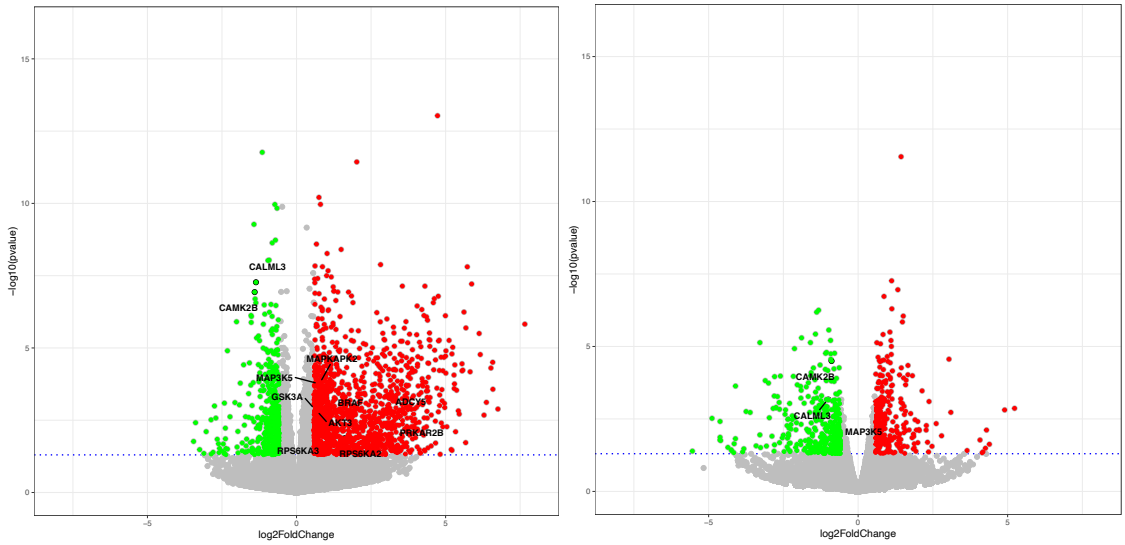
Graph representation of KEGG apoptosis (hsa04210). Significantly up and downregulated genes (in $CD44^{high}CD24^{high}$ cells, by comparing FMD versus AL) are depicted in red and green respectively.

Interestingly, more detailed analysis shows that both CycD and PI3K-AKT, mTOR signaling pathways were overexpressed by FMD only in $CD44^{high}CD24^{high}$ differentiated cells, but not in $CD44^{high}CD24^{low}$ staminal population. In the CSCs population, we did not observe the activation of survival pathways, thus indicating that the FMD might be sufficiently toxic to deplete the majority of CSCs, which are unable to upregulate pro-survival factors.

These data indicate that differentiated cells activate CycD and PI3K-AKT, mTOR survival factors as escape pathways to survive under low nutrients conditions, without undergoing apoptosis (Figure 39).

**PI3K-AKT, mTOR pathways**

**$CD44^{high}CD24^{high}$: FMD vs AL** **$CD44^{high}CD24^{low}$: FMD vs AL**



## CycD-CDK4/6

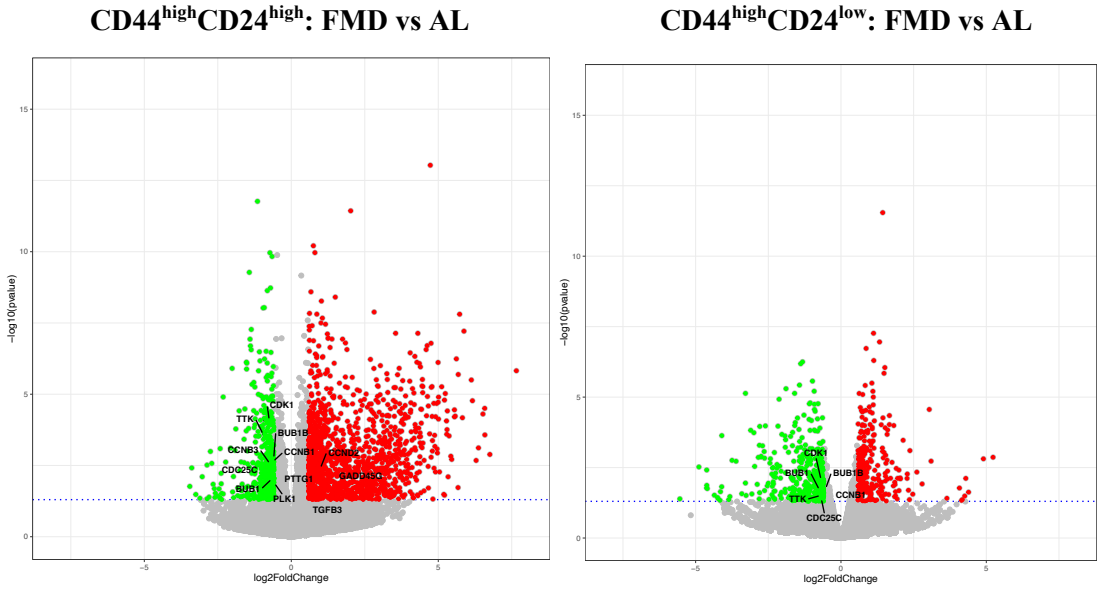



**Figure 39. FMD activates PI3K-AKT, mTOR and CycD-CDK4/6 in differentiated cells but not in CSCs.**

Volcano plot showing the significance versus the log2 fold-change in $CD44^{high}CD24^{low}$ and $CD44^{high}CD24^{high}$ populations, by comparing FMD versus AL. Up and downregulated genes (|log2FC| > 0.58 and adj. p value < 0.05) are displayed in red and green respectively. Deregulated genes involved in PI3K-AKT, mTOR pathways and CycD-CDK4/6 are highlighted.

Taken together, RNA sequencing analysis, performed on SUM159 xenografts, allow me to identify several potential escape targets to treat this kind of TNBC.

# 6. Effect of FMD in combination with pro-growth pathway inhibitors.

Starting from RNA sequencing results, I investigated the PI3K-AKT and mTOR pathway inhibitors mostly used to treat TNBC in pre-clinical models and under investigation in different clinical trials, in order to evaluate their potential in inducing cancer cell death, alone or in combination with STS/FMD conditions, first *in vitro* and subsequently *in vivo*. In particular, I evaluated the effect of pictilisib (CDC-0941), a pan-PI3K inhibitor selective for all four isoforms of class I PI3Ks, which prevents the formation of phosphatidylinositol-triphosphate ($PIP_3$), key component of PI3K pathway, alpelisib (BYL-719), a PI3Kα inhibitor, ipatasertib (GDC-0068), a highly selective pan-AKT inhibitor which binds to all three isoforms of AKT, and rapamycin (AY 22989), an allosteric mTOR inhibitor which binds to FK-binding protein 12 (FKBP12). All these inhibitors have anti-tumor activity in breast cancer models and also increase the toxicity of several drugs, reducing cancer cells resistance to treatments (O'Brien C et al., 2010; Tao JJ et al., 2014; Teo ZL et a., 2017).

In particular, SUM159 TNBC cells were grown in CTR (1 g/L glucose; 10% FBS) and in STS (0,5 g/L glucose; 1% FBS) media for a total of 48h, and at 24h were treated with vehicle or pictilisib, alpelisib, ipatasertib or rapamycin. This screening identified pictilisib as the most efficacious in inducing cancer cell death under STS conditions, while did not have any effect under CTR conditions (Figure 40).

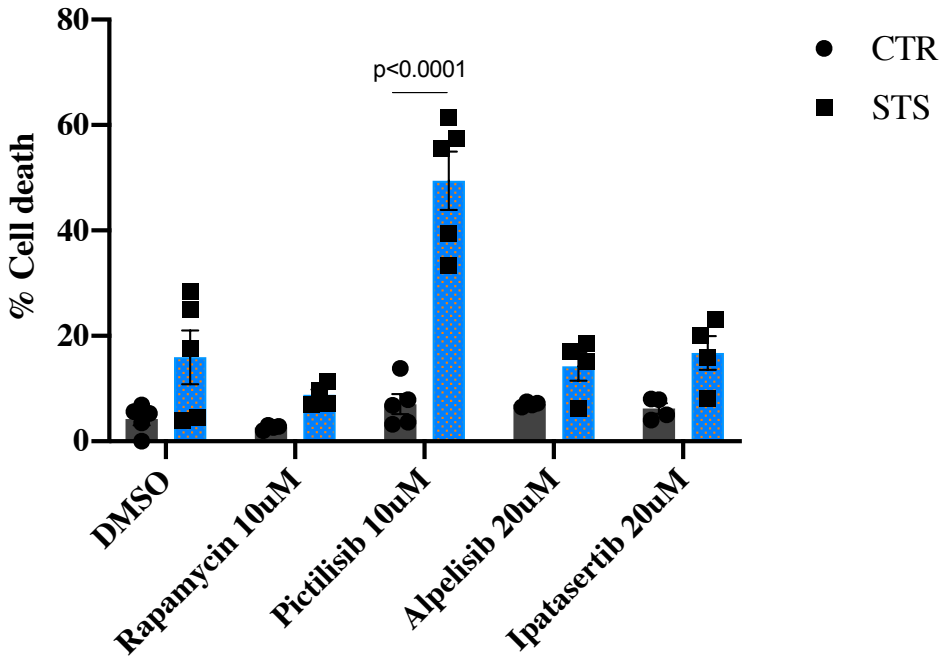



**Figure 40. Pictilisib induces cancer cells death under STS conditions, in SUM159 cells, *in vitro*.**

SUM159 cells were grown under CTR (1g/l Glucose, 10%FBS) and STS (0,5g/l, 1%FBS) conditions for a total of 48 hours. At 24h cells were treated with rapamycin (10μM), pictilisib (10μM), alpelisib (20μM) and ipatasertib (20μM), for 24hours. Viability was assessed with erythrosine stain (n= 4-5 biological replicates). Data are represented as mean ± SEM. Multiple t test was performed.

Starting from this promising result obtained *in vitro*, I decided to evaluate the effect of pictilisib alone and in combination with FMD, in mice bearing SUM159 xenografts. For this purpose, immune-deficient mice bearing SUM159 xenografts, fed with AL diet or subjected to FMD cycles, were treated with pictilisib in order to assess its effect on tumor progression and mice survival. Pictilisib alone resulted to have the same effect of FMD in delaying tumor progression, while the inhibitor, in combination with FMD, not only reduced tumor volume, but increased significantly mice survival, compared to other groups (Figure 41).

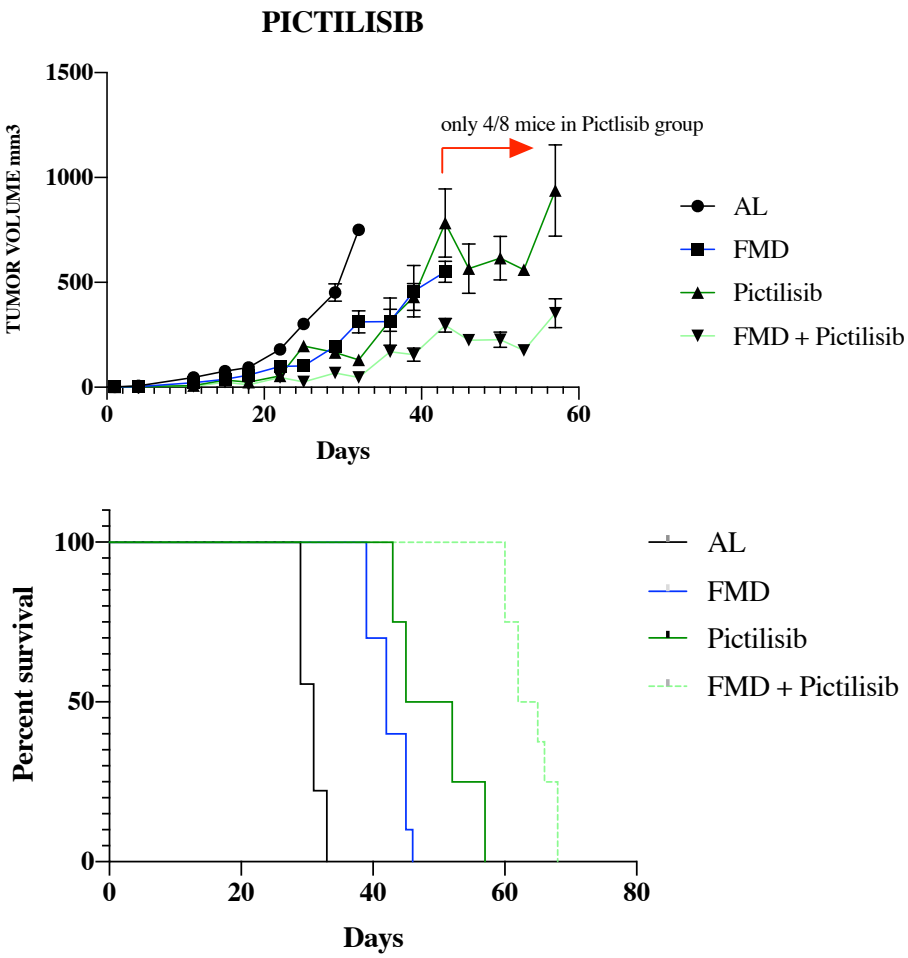



**Figure 41. Pictilisib in combination with FMD delays tumor progression and increases survival in mice bearing SUM159 xenografts.**

8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with pictilisib (100mg/kg) 5 consecutive days a week, by oral gavage. P values were determined by Log-rank (Mantel-Cox) test; AL vs FMD: $p < 0.0001$; AL vs Pictilisib: $p < 0.0001$; AL vs FMD + Pictilisib: $p < 0.0001$; Pictilisib vs FMD: $p= 0.0105$; Pictilisib vs FMD + Pictilisib: $p < 0.0001$; FMD vs FMD + Pictilisib: $p < 0.0001$.

Based on results obtained with RNA sequencing analysis, I also examined the effect of pictilisib in combination with an inhibitor of CDK4/6, in mice bearing SUM159 xenografts. In particular I used palbociclib, a selective CKD4/6 inhibitor, able to block the phosphorylation of retinoblastoma tumor suppressor gene (Rb), leading to the arrest of cell cycle in G1 phase. Palbociclib is reported to have anti-tumor activity in estrogen receptor positive breast cancers and is already in use in different clinical trials combined to hormone therapies.

First, I evaluated the effect of palbociclib alone and combined with FMD, and results revealed that the drug, both in mice fed with AL diet or subjected to FMD, delays tumor progression at the same level of FMD alone, suggesting that the inhibition of CDK4/6 is not enough to enhance FMD effect, which itself induces cell cycle arrest in G2 phase through the downregulation of CDK1 (Figure 42).

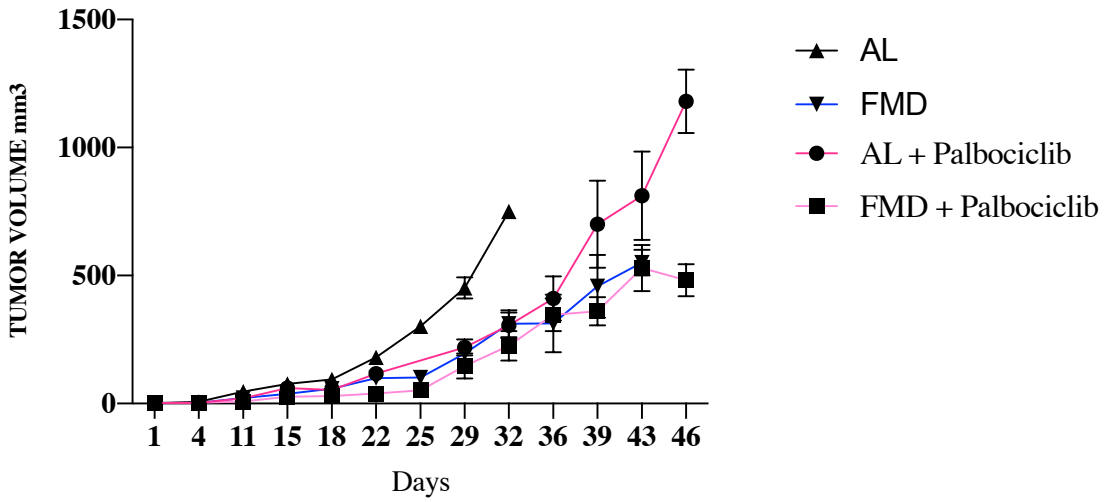



**Figure 42. Palbociclib does not enhance FMD effect in delaying tumor progression.**
8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with palbociclib (62,5 mg/kg) every other day, by oral gavage.

Thereafter, I evaluated the combined effect of palbociclib and pictilisib in mice bearing SUM159 xenografts; I found that the dual treatment is very effective in delaying tumor progression in those mice fed with standard diet, compared to AL or FMD alone, but the addition of FMD to palbociclib and pictilisib turns out to further reduce tumor volume and greatly retard cancer cell resistance to drugs. Unfortunately, mice were sacrificed because of ulcerations, even though the dimension of tumor masses was very small.
These data confirm results obtained with RNA sequencing, suggesting that differentiated cells activate survival pathways to not undergo apoptosis; indeed, the inhibition of PI3K-AKT pathway and CDK4/6, leads to tumor progression delay and the addition of FMD cycles retards drugs resistance, partly because of its effectiveness in reducing CSCs (Figure 43).

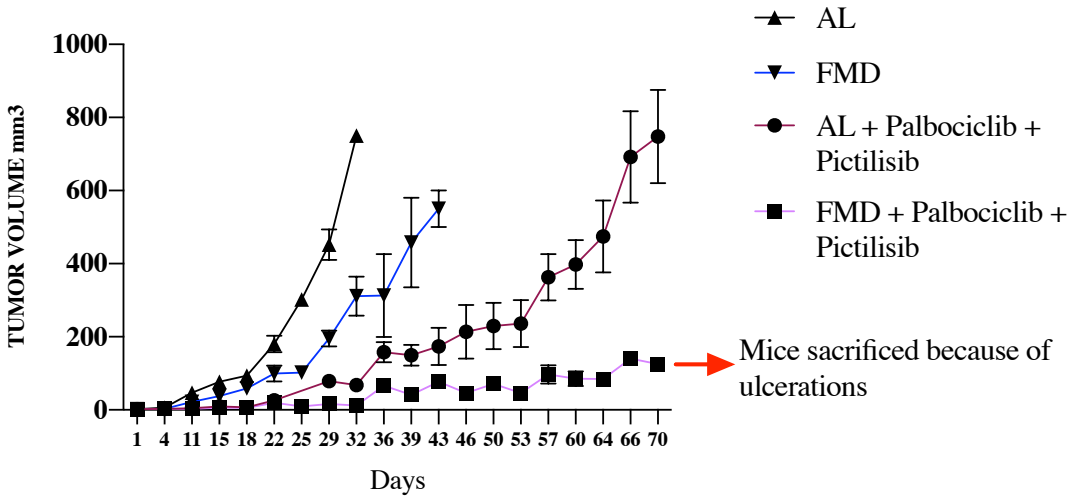
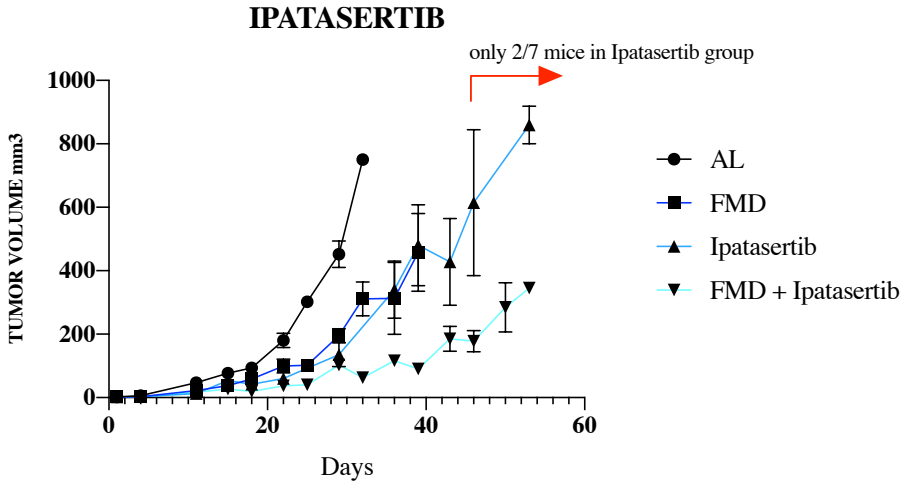



**Figure 43. Palbociclib plus pictilisib delay tumor progression and FMD addition retards drug resistance.**

8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with palbociclib (62,5 mg/kg, by oral gavage, every other day) and pictilisib (100 mg/kg, by oral gavage, 5 consecutive days a week).

Starting from promising results obtained with pictilisib alone, I decided to target more strongly the PI3K-AKT and mTOR pathways, combining pictilisib with ipatasertib or rapamycin, in a double or triple combination, +/- FMD, in mice bearing SUM159 xenografts. First, I evaluated the effect of ipatasertib alone and I found that the AKT inhibitor, in mice fed with standard diet, slows tumor progression at the same level of FMD alone, while combined with fasting/FMD it reduces tumor volume and increases significantly mice survival, similarly to pictilisib, as previously shown in figure 40 (Figure 44).



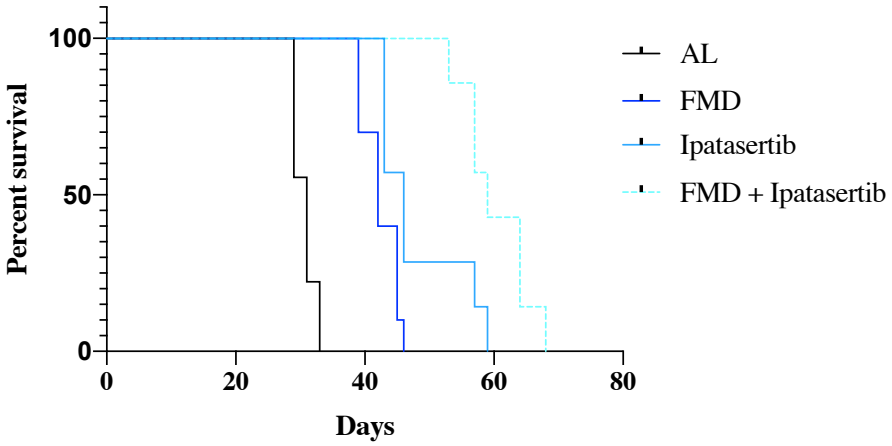



**Figure 44. Ipatasertib in combination with FMD delays tumor progression and increases survival in mice bearing SUM159 xenografts, similarly to pictilisib.**

8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with ipatasertib (75mg/kg) 5 consecutive days a week, by oral gavage. P values were determined by Log-rank (Mantel-Cox) test; AL vs FMD: $p < 0.0001$; AL vs Ipatasertib: $p < 0.0001$; AL vs FMD + Ipatasertib: $p < 0.0001$; Ipatasertib vs FMD: $p= 0.0280$; Ipatasertib vs FMD + Ipatasertib: $p = 0.0128$; FMD vs FMD + Ipatasertib: $p < 0.0001$.

Thereafter, I combined pictilisib with ipatasertib and I obtained that the double treatment is much more effective than single treatments in increasing survival, in those mice fed with standard diet, whereas it has the same effect of single inhibitors plus FMD cycles, suggesting that FMD may replace one of the two drugs. Surprisingly, the addition of FMD to the combined treatment resulted to only increase mice survival slightly, compared to other groups, suggesting that the dual treatment and FMD could act by sharing similar pathways (Figure 45).

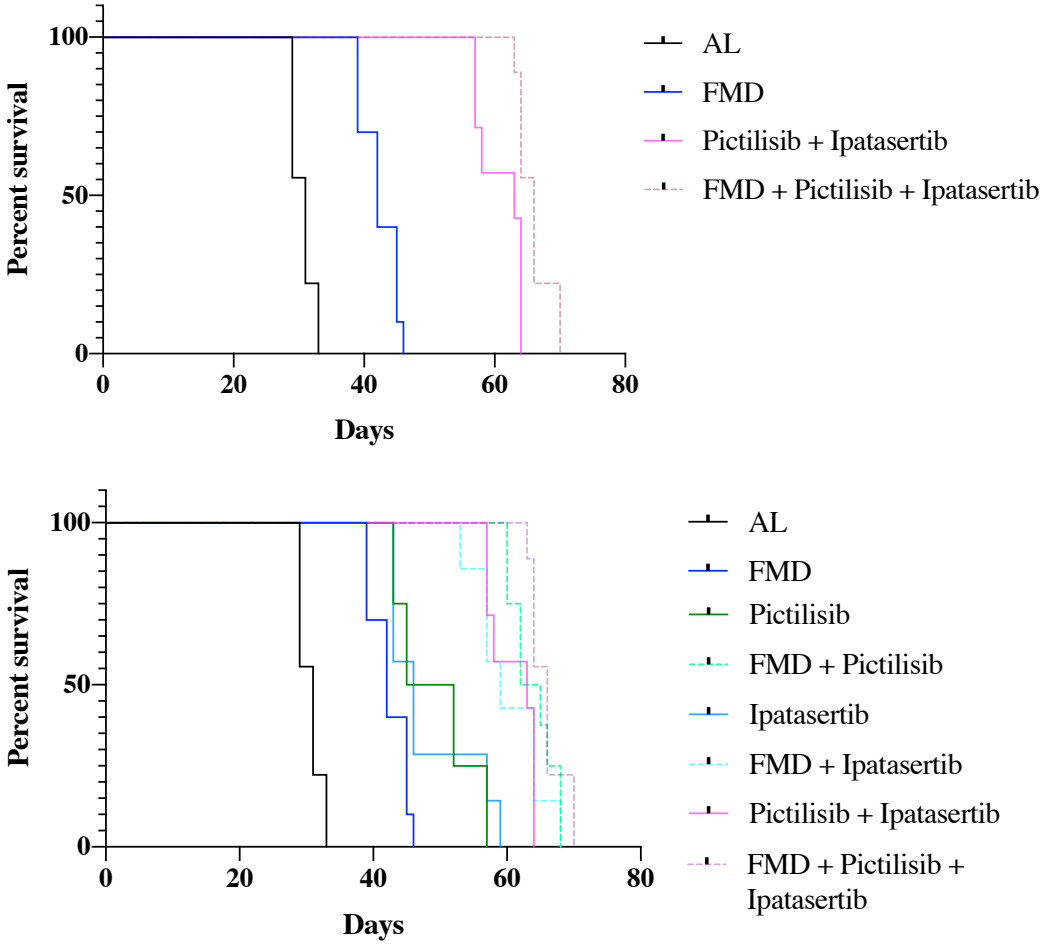



**Figure 45. Pictilisib plus ipatasertib effect, alone or combined to FMD, in SUM159 xenografts.**

8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with pictilisib (100mg/kg) and ipatasertib (75mg/kg) 5 consecutive days a week, by oral gavage. P values were determined by Log-rank (Mantel-Cox) test; Pictilisib vs Pictilisib + Ipatasertib: $p = 0.0009$; Ipatasertib vs Pictilisib + Ipatasertib: $p = 0.0046$; FMD + Ipatasertib vs FMD + Pictilisib + Ipatasertib: $p = 0.0407$; FMD + Pictilisib vs FMD + Pictilisib + Ipatasertib: $p= 0.2294$; Pictilisib + Ipatasertib vs FMD + Pictilisib + Ipatasertib: $p = 0.0066$.

Then, I evaluated the effect of pictilisib in combination with rapamycin. Rapamycin alone greatly delayed tumor progression, compared to other inhibitors, but around 60 days after cells injection tumor masses started to grow very fast in mice fed with standard diet, while FMD cycles combined with rapamycin delayed drug resistance acquisition. Rapamycin, even in term of survival, resulted to be much more effective than pictilisib and ipatasertib, and the addition of FMD slightly enhanced rapamycin effect (Figure 46).

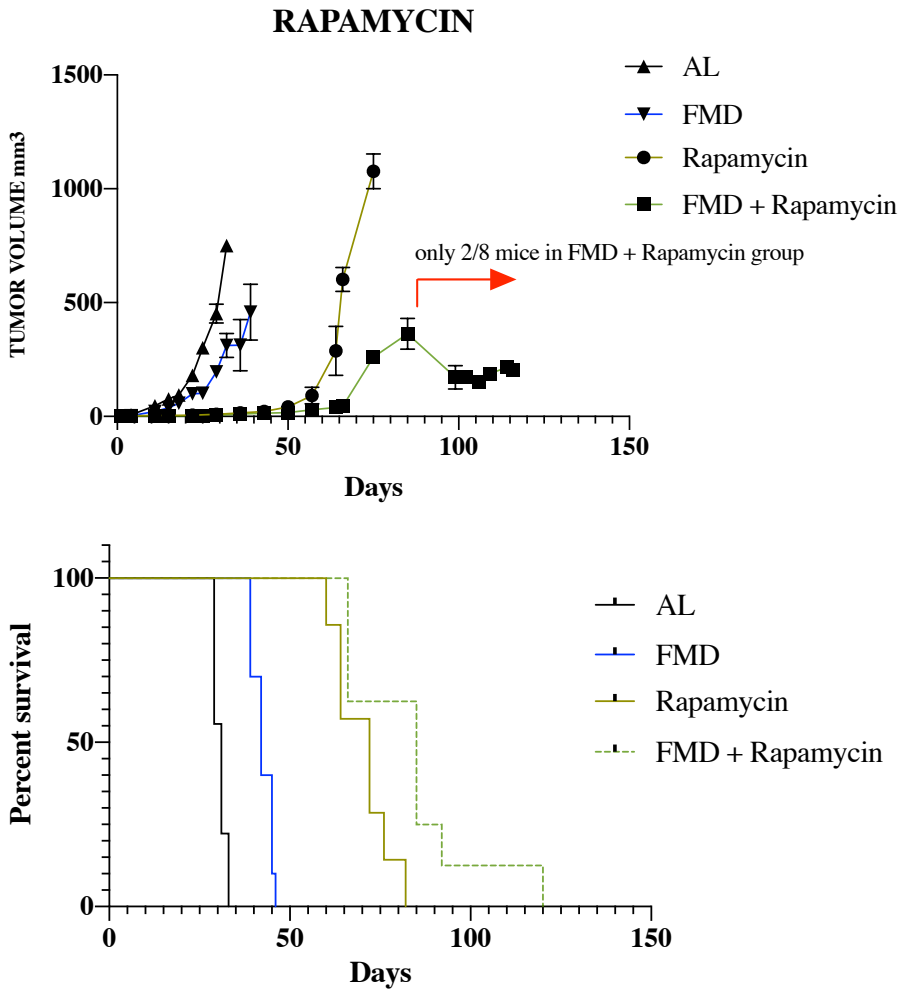



**Figure 46. Rapamycin is more effective than pictilisib plus ipatasertib in increasing survival and delaying tumor progression, both in mice fed AL or subjected to FMD cycles.**
8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with rapamycin (2mg/kg) every other day, i.p. P values were determined by Log-rank (Mantel-Cox) test; AL vs FMD: $p < 0.0001$; AL vs Rapamycin: $p < 0.0001$; AL vs FMD + Rapamycin: $p < 0.0001$; Rapamycin vs FMD: $p < 0.0001$; Rapamycin vs FMD + Rapamycin: $p = 0.0190$; FMD vs FMD + Rapamycin: $p < 0.0001$.

Then, I combined rapamycin with pictilisib; in particular, pictilisib administration was suspended after 9 weeks of treatment (day 70 after cells injection), due to stress caused by daily oral injections. By combing rapamycin with pictilisib, I found that there aren't differences between the effect mediated by the dual treatment and the effect of rapamycin plus FMD, in term of mice survival. However, the addition of FMD to pictilisib-rapamycin significantly increased survival, in comparison both with the dual treatment and with single inhibitors, with and without the addition of FMD, due to its ability to

retard resistance to drugs. Unfortunately, mice were sacrificed following the formation of ulcerations, even though tumor masses were very small (Figure 47).

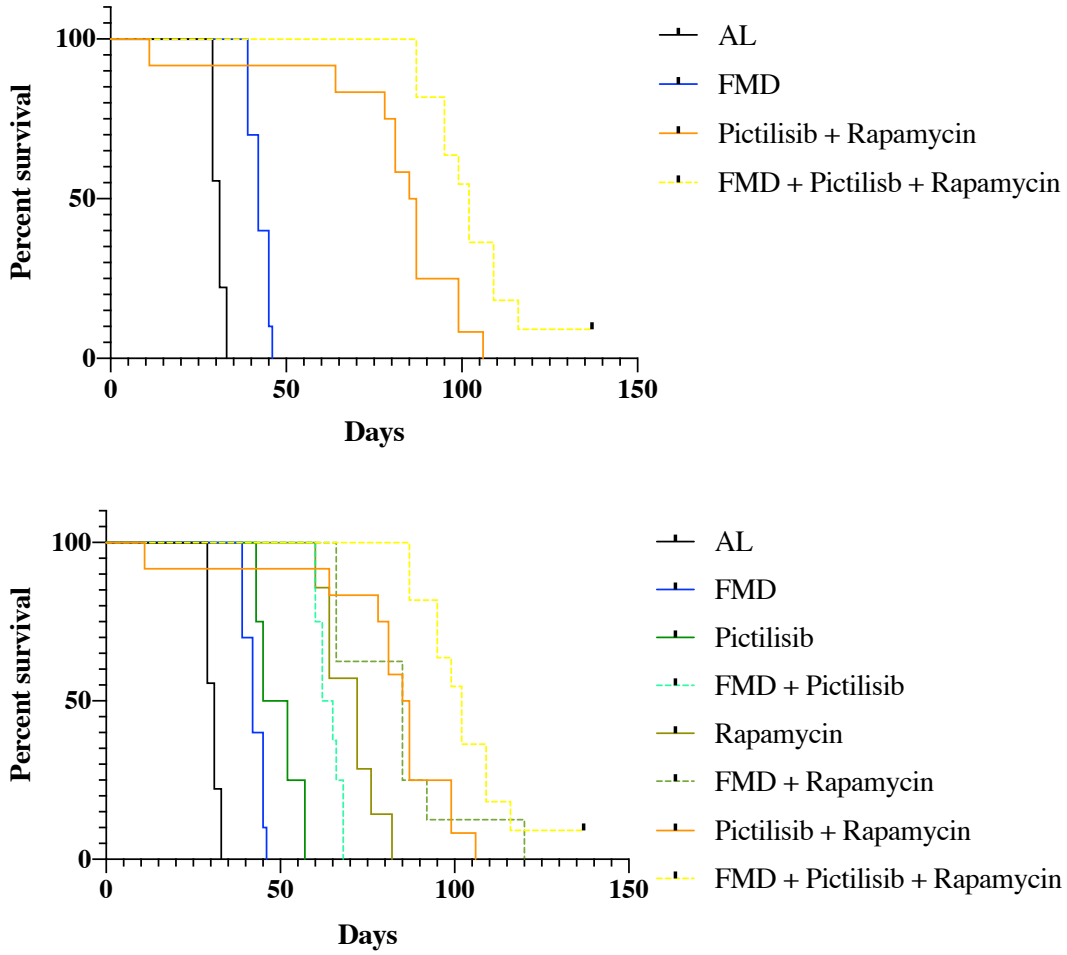


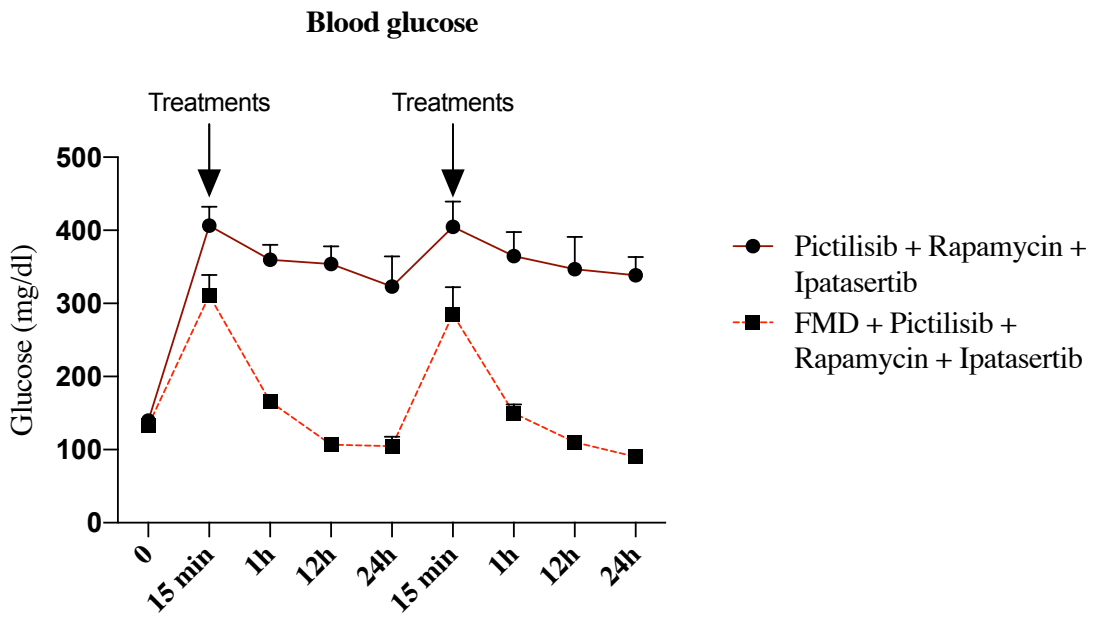

**Figure 47. The addition of FMD to pictilisib-rapamycin dual treatment increases mice survival by retarding drug resistance, in SUM159 xenografts.**

8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with pictilisib (100mg/kg, 5 consecutive days a week, by oral gavage) and rapamycin (2mg/kg, every other day, i.p.). P values were determined by Log-rank (Mantel-Cox) test; Pictilisib vs Rapamycin: p= 0.0002; FMD + Pictilisib vs FMD + Rapamycin: p= 0.0037; Pictilisib vs Pictilisib + Rapamycin: $p < 0.0001$; Rapamycin vs Pictilisib + Rapamycin: $p = 0.0038$; FMD + Rapamycin vs FMD + Pictilisib + Rapamycin: $p = 0.0377$; FMD + Pictilisib vs FMD + Pictilisib + Rapamycin: $p < 0.0001$; Pictilisib + Rapamycin vs FMD + Pictilisib + Rapamycin: $p = 0.0030$; FMD + Rapamycin vs Pictilisib + Rapamycin: p= 0.9922.

Finally, I decided to evaluate the effect of pictilisib, ipatasertib and rapamycin as a triple treatment, both in mice fed with standard diet and subjected to FMD cycles. In particular, mice were treated with three inhibitors until day 70 (post cells injection), when pictilisib and ipatasertib administrations were suspended, and I continued to treat mice with only rapamycin. Treatments with PI3K inhibitors are reported to increase glucose in blood leading, to a persistent hyperglycemia in mice and humans (Bendell JC et al., 2012; Patnaik A et al., 2016; Baselga J et al., 2017; Juric D et al., 2017; Mayer IA et al., 2017). Therefore, I monitored blood glucose levels both after single, double or triple treatments. While I did not observe hyperglycemia after single or double treatments, I noted cases of sickness, due to persistent hyperglycemia, after the administration of the three inhibitors, in mice fed with standard diet. Blood glucose levels, in fact, increased soon after the triple treatment, both in mice fed AL of with FMD, but they turned down at normal levels in those mice subjected to FMD while they remained very high in mice fed with standard diet, leading to persistent hyperglycemia and sometimes to death (Figure 48a).

I evaluated the combined effect of these inhibitors on mice survival and I obtained that pictilisib and rapamycin plus FMD are more effective than the combination of the three drugs in increasing mice survival; in fact, 5 weeks after initiation of therapy, some mice subjected to the triple treatment started to lose weight and died in few days, probably due to persistent hyperglycemia, since blood glucose levels before death were at about 400mg/dl. However, the addition of FMD cycles to triple treatment greatly reduced hyperglycemia and the mortality associated with it, and it resulted in an impressive increase in survival compared to all other groups (Figure 48b).

**a**



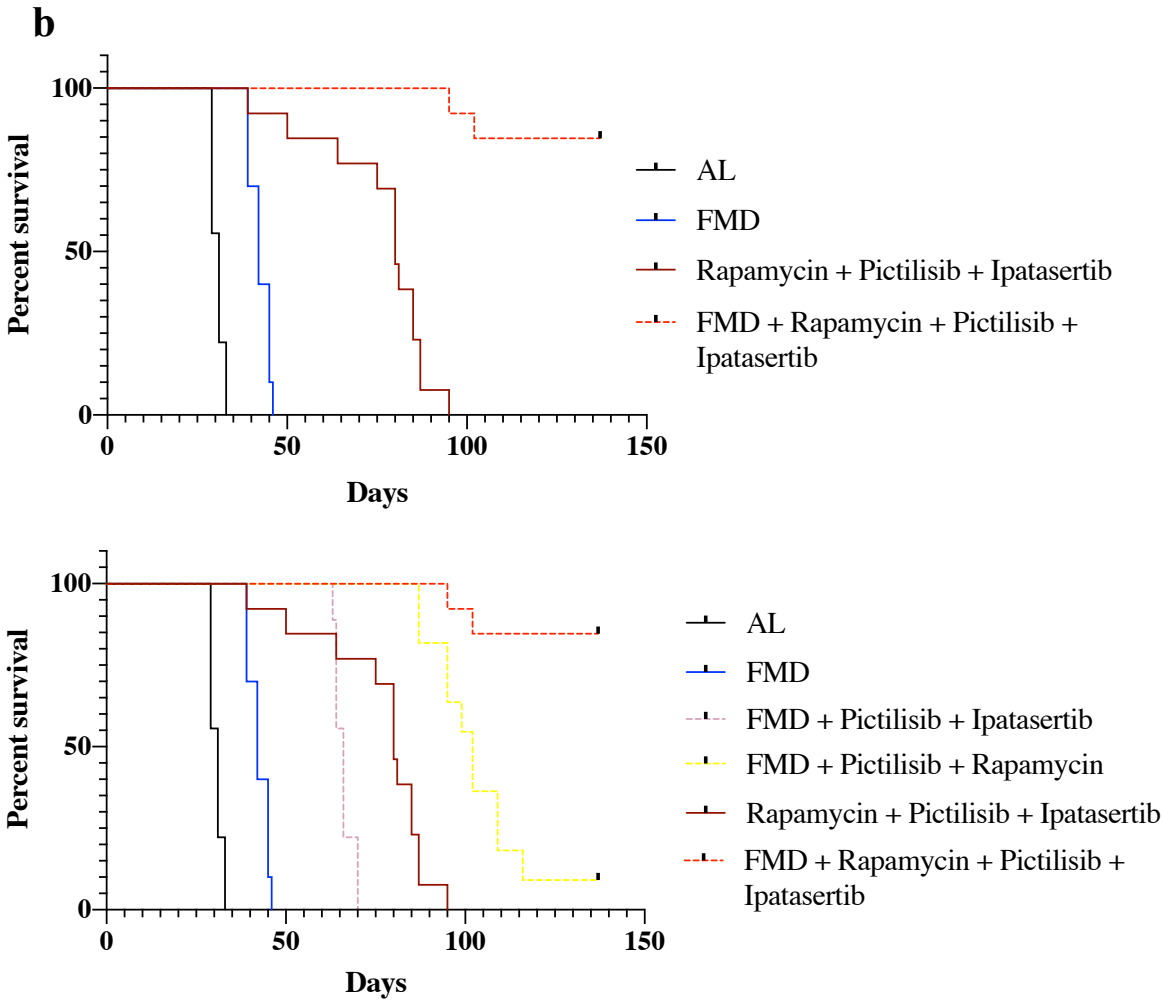



**Figure 48. The addition of FMD to triple treatment prevents tumor growth and protects mice from hyperglycemia side effect, in SUM159 xenografts.**

8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles, alone or in combination with pictilisib (100mg/kg, 5 consecutive days a week, by oral gavage), ipatasertib (75mg/kg, 5 consecutive days a week, by oral gavage) and rapamycin (2mg/kg, every other day, i.p.). a) Blood glucose level was determined through Accu chek guide instrument. b) P values were determined by Log-rank (Mantel-Cox) test; Pictilisib + Ipatasertib + Rapamycin vs FMD + Pictilisib + Rapamycin: $p < 0.0001$; Pictilisib + Ipatasertib + Rapamycin vs FMD + Pictilisib +Ipatasertib = 0.0023; FMD + Pictilisib + Ipatasertib + Rapamycin vs FMD + Pictilisib + Rapamycin: $p = 0.0002$.

Furthermore, I tested whether cyclic FMD combined with PI3K, AKT, mTOR inhibitors can induce the reversal of rapidly growing and advanced-stage tumor progression in human TNBC xenografts. In particular, SUM159-xenograft-bearing mice were subjected to 4 cycles of FMD or fed with standard diet, and 35 days post cells injection, when mice were near to be sacrificed because of tumor dimensions, I started to treat mice with the triple combination of inhibitors, adding also FMD cycles to mice previously fed with standard diet.

This switch caused tumor regression soon after the first week of treatments. In addition, pre-treating mice with FMD cycles prior to the start of treatments improved tolerance to therapy and prevented drugs resistance, leading to complete tumor shrinkage, while tumor masses of mice fed with standard diet before the switch started to acquire drugs resistance effects a few weeks later the start of drugs administration (Figure 49).

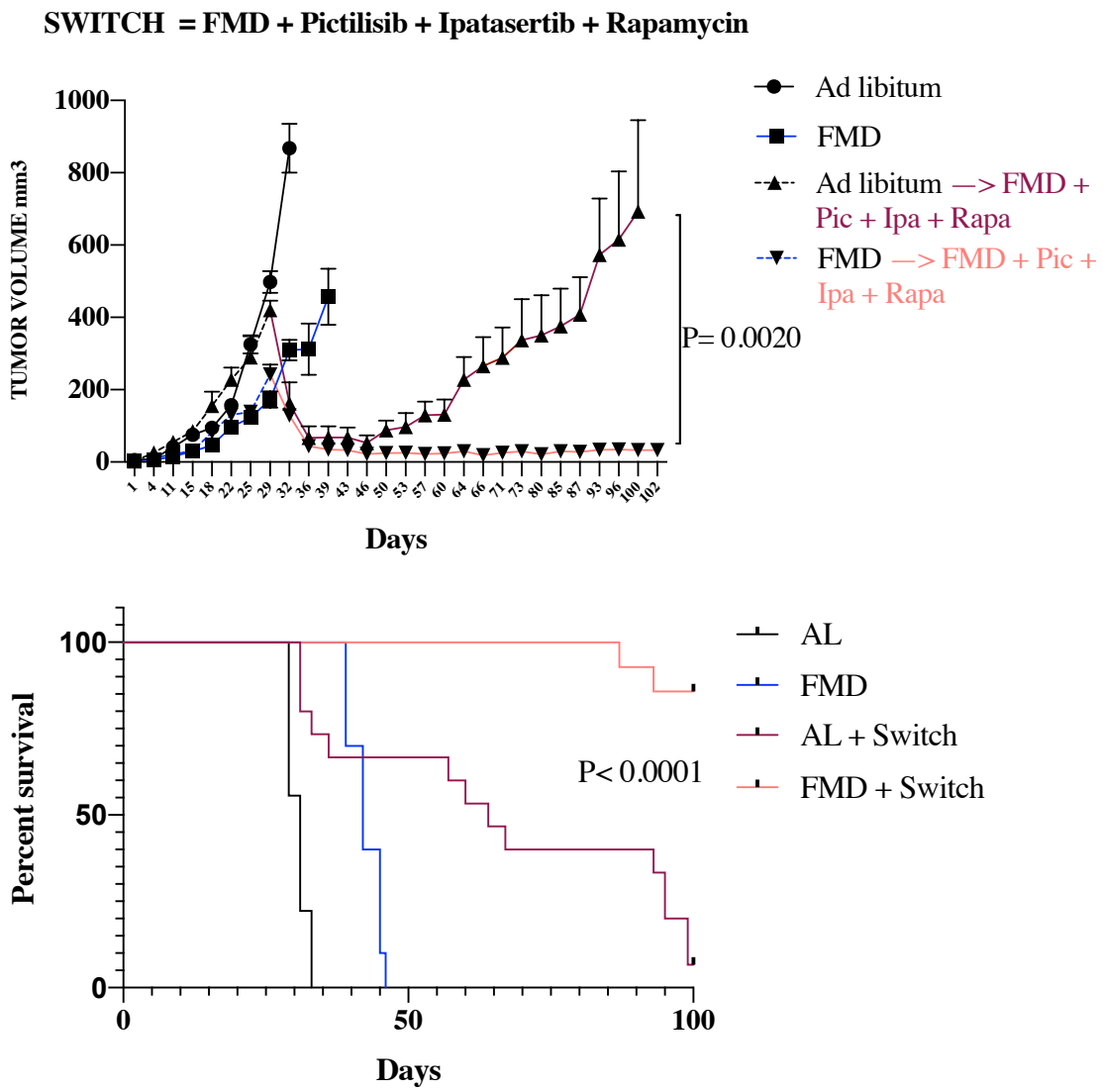


**Figure 49. FMD reverts late stage-tumor progression in human TNBC xenografts.**
8-weeks old female NOD scid (NSG) mice were subcutaneously injected with SUM159 cells and subjected to FMD cycles or fed with standard diet. 35 days post cells injection mice started to be treated with pictilisib (100mg/kg, 5 consecutive days a week, by oral gavage), ipatasertib (75mg/kg, 5 consecutive days a week, by oral gavage) and rapamycin (2mg/kg, every other day, i.p.) plus FMD (n=15). P value was determined by Log-rank (Mantel-Cox) test.

# DISCUSSION

Triple negative breast cancer (TNBC) is an invasive, poorly differentiated, highly proliferative tumor, characterized by high recurrence rates when compared to other breast cancer subtypes. Its aggressiveness is also due to the lack of targeted therapies, since TNBCs do not express either the estrogen or the progesterone receptor. Moreover, this subtype of breast cancer is reported to be enriched in cancer stem cells (CSCs) and growing evidence indicates that treatment failure and cancer recurrence are primarily due to drug resistance and self-renewal, which are specific properties of CSCs (Charafe-Jauffret E et al., 2009).

CSCs are a subset of slow cycling cancer cells with stem cell features. They are also known as tumor initiating cells (TICs), thanks to their capability to self-renew maintaining an undifferentiated population that drives tumorigenesis. CSCs are one of the major causes of therapeutic resistance and allow the tumor to escape from conventional chemotherapies, relapse and metastasize to other organs (Tang C et al., 2007). They are classically identified based on the evaluation of cell surface markers like $CD34^{+}CD38^{-}$ phenotype in leukemia (Bonnet D and Dick JE, 1997) and $ESA^{+}CD44^{+}CD24^{-}$ in breast cancer (Ponti D et al., 2005), and thanks to their increased aldehyde dehydrogenase activity (ALDH) (Rodriguez-Torres M and Allan AL., 2016). In particular, CSCs have a key role in tumorigenesis and biology of TNBC. In fact, TNBC is enriched in $CD44^{+}CD24^{-}$ and $ALDH^{+}$ cells, which contribute to its ability to resist to chemotherapy treatments and to metastasize (O'Conor JC et al., 2018). CSCs are also characterized by a highly active free radical scavenger system which contributes to lower ROS levels, supporting their self-renewal potential and chemo-/radio-therapy resistance. In fact, by activating ROS scavenging machinery, CSCs can reduce radiation-induced ROS formation and, consequently, DNA damage induced by ROS (Skvortsova I et al., 2015). Pharmacologic depletion of ROS scavengers in CSCs significantly decreases their ability to form colonies in vitro and increases radio-therapy sensitivity (Diehn M et al., 2009; Skvortsova I et al., 2015).

Moreover, it has been reported that glycolysis inhibition is also able to reduce the number of CSCs, interfering with their ability to form tumor *in vivo* (Liu PP et al., 2014).

In recent years, our laboratories, have shown that cycles of fasting/fasting mimicking diet (FMD), based on a restriction of 50% of calories, low levels of protein and sugars and relatively high fat content, enhance the efficacy of standard and low toxic therapeutic

agents on different types of cancer, including TNBC, while inducing the protection of normal cells from the toxic side effects (Raffaghello L et al., 2008; Lee C et al., 2012; Di Biase S et al., 2016, Di Tano M et al., 2020; Caffa I et al., 2020). These phenomena are known as "Differential Stress Sensitization" (DSS) and "Differential Stress Resistance" (DSR) respectively. Fasting can induce DSR and DSS partly by reducing PKA activity, insulin growth factor 1 (IGF-1) and glucose levels and by differentially regulating, in normal and cancer cells, genes involved in DNA repair and cell death (Raffaghello L et al., 2008; Lee C et al., 2010; Lee C et al., 2012; Cheng CW et al., 2014).
Fasting/FMD can also promote the switch of cancer cell metabolism from aerobic glycolysis to oxidative phosphorylation (OXPHOS), increasing ROS production (Lee C et al., 2012) and reducing the expression of glucose transporter 1 and 2 (GLUT1, GLUT2) and hexokinase 2 (HK2) enzyme (Bianchi G et al, 2015).

To this purpose, since TNBC progression is reported to be dependent on CSCs and due to the key role of CSCs in tumor initiation, invasion and therapy resistance, I investigated the effect of fasting/FMD on CSCs metabolism and survival and on its differential effects on CSC and differentiated cancer cells.

**Fasting/FMD reduces TNBC CSCs and its effect is mediated by glucose levels lowering**

The cancer stem-like population is reported to present an increased glycolytic activity, accompanied by the up-regulation of glycolytic enzymes, such as GLUT1 and HK2, when compared to more differentiated cancer cells (Shen YA et al., 2015).
Several studies show that CSCs can arise and survive upon extreme environmental conditions thanks to their ability to rearrange their metabolism, taking advantage of glycolysis and elevated glucose uptake, in case of oxygen deprivation. Glucose becomes a fundamental player in the maintenance and spread of CSCs in different type of cancers, including breast carcinoma (Schieber MS and Chandel NS, 2013; Liu PP et al., 2014).
To evaluate the effect of fasting on CSC, *in vitro*, I used low-serum, low-glucose conditions, previously established by our laboratories, referred to as Short-Term Starvation (STS).
Interestingly, I found that STS lowers the generation and volume of mammospheres and reduces the proportion of $CD44^{high}CD24^{low}$ cells, confirming a self-renewal minor

efficiency in TNBC SUM159 cells. Moreover, consistent with my *in vitro* results, I found that cyclic FMD greatly delays tumor progression, partly by increasing the expression of Caspase3, reducing the generation of ex vivo primary mammospheres, the percentage of cells expressing ALDH1 enzyme and TNBC-initiating cell frequency, when compared to control conditions. Notably, I validated STS/FMD effect on CSCs in a syngeneic TNBC model (4T1 allograft) in immune-competent mice, obtaining similar results to those obtained with human SUM159 cells.

These data reveal that STS/FMD is very effective in reducing TNBC stem cells, independently of the immune system, and particularly in combination with drugs enhancing the inhibition of glucose uptake/catabolism.

In fact, I demonstrated that CSCs reduction mediated by STS/FMD is dependent on decrease in glucose levels. In particular, I found that glucose supplementation in mice drinking water only partially rescues FMD-induced delay of tumor progression, further supporting results previously obtained in our laboratory about the involvement of multiple pathways on tumor progression, and it completely reverses STS/FMD effect on mammosphere generation. Similarly, I found that WZB117, specific inhibitor of the glucose transporter GLUT1, mimics the effect of STS/FMD on CSCs, but it does not potentiate FMD efficacy in delaying tumor progression, probably due to the capability of FMD alone to decrease the expression of GLUT1, as shown in my study and in a previously published work (Bianchi G et al, 2015). On the other hand, the HK competitor 2Deoxy-D-Glucose (2DG) greatly potentiated the effect of STS/FMD both in halting TNBCs progression and in reducing tumor initiating cell frequency, in agreement with other studies showing that 2DG affects TNBC cells, impairs cell migration and invasiveness. 2DG toxicity against CSCs is explained not only by the inhibition of glycolysis, but also by indirect effects on different signaling pathways, such as a specific inhibition of mTOR signaling, which is shown to be influenced by the intracellular concentration of ATP (Dennis PB et al, 2001). This evidence suggests that the strong effect of combined STS/FMD and 2DG on CSCs could be due not only to a lowering in glucose levels, but also to a selective downregulation of mTOR signaling in the staminal population.

The antidiabetic compound metformin was reported to reduce CSCs in vitro and in vivo in combination with chemotherapy (Hirsch HA et al., 2009; Rattan R et al., 2012; Shi P et al., 2017). Metformin reduces blood glucose levels resulting in the inhibition of

complex I of the mitochondrial electron-transport chain and consequently in the increase of the intracellular AMP/ADP ratio, which leads to AMPK activation. Recently has been reported that metformin, combined to intermittent fasting, delays tumor progression in different cancer pre-clinical models, while appears to not have any effect in mice fed ad libitum with standard diet (Elgendy et al., 2019). This effect could be partly due to the capability of cancer cells to adapt to metabolic changes; tumor cells, in fact, can switch their metabolism becoming more dependent on glycolysis when OXPHOS is inhibited by metformin. Interestingly, I found that metformin, unlike 2DG, reduces tumor progression and sphere formation in TNBC, when compared to standard conditions, but it does not show any additive or synergistic effect when combined with the FMD, in contrast with results obtained by Elgendy et al about the effect of metformin combined to intermittent fasting.

These data could be explained by the fact that metformin and FMD could be acting both on similar or opposite pathways, at least in CSCs. In fact, FMD, similarly to metformin, is reported to activate AMPK through PKA pathway downregulation (Di Biase S et al., 2017). On the other hand, fasting is reported to affect the enzymatic activity of respiratory complexes and the oxygen consumption rate (OCR) in colon carcinoma cells (Bianchi G et al., 2015). In particular, fasting up-regulates complex I and complex IV of OXPHOS and increases OCR, suggesting an increased oxidative metabolism, while reduces ATP synthesis, indicating a decrease in glucose metabolism. Thereby, FMD could neutralize, at least in part, the effect of metformin, which instead is reported to selectively inhibit the mitochondrial respiratory-chain complex I and decrease OCR (El-Mir MY et al., 2000). Moreover, since TNBCs rely more on glycolysis than mitochondrial respiration, compared to other type of cancers including lung (Ye XQ et al., 2011), glioblastoma (Janiszewska M et al., 2012) and acute myeloid leukemia (Lagadinou ED et al., 2013), metformin resulted to not potentiate FMD effect both in delaying tumor progression and reducing CSCs, differently from what happened when cyclic FMD is combined to 2DG, suggesting that altering OXPHOS doesn't impair TNBC cells metabolism.

However, the advantage provided by FMD conditions is its wide acting effect reaching both CSCs and differentiated cancer cells but also its ability to promote differential effects in normal and cancer cells causing a reduction in side effects. Nonetheless, these results warrant further investigation of the effects of metformin in combination with the drugs investigated in these studies.

Collectively, these data indicate that CSCs are sensitive to glucose deprivation mediated by FMD and that 2DG potentiates its toxicity, confirming the key role of glucose in the maintenance of the staminal population in TNBCs.

**Blood fuel homeostasis maintenance: what happens in humans during fasting**

In two recent works, our laboratory has shown that in humans 5 days of FMD reduce blood glucose levels by ~15%, differently from studies in rodents which report that cyclic FMD leads to a ~40% decrease in blood glucose levels, as also shown in my study (Brandhorst et al., 2015; Wei et al., 2017).

In humans, during fasting, blood glucose levels begin to drop, leading to a decrease in insulin secretion and an increase in glucagon production by the pancreas, as a consequence to low blood-sugar levels. Glucagon stimulates the mobilization of glycogen stores when there is no dietary intake of glucose, stimulating gluconeogenesis in the liver, and blocks glycolysis by lowering the level of fructose 2,6-bisphosphate (F-2,6-BP). Muscle proteolysis even supplies glycogenic amino acids to sustain hepatic gluconeogenesis. The amount of glucose derived from glycogen through glucose 6-phosphate hydrolysis is then released from the liver into the blood. Moreover, during fasting, both liver and muscle use fatty acids instead of glucose in order to maintain blood fuel homeostasis. This interplay among organs maintains the blood-glucose level at or above 80mg/dl, thus allowing humans to sustain cycles of calorie-restricted diet (Owen OE et al., 1979; Longo and Mattson, 2014).

In humans, several pathways are involved in the maintenance of blood fuel homeostasis, while in mice this mechanism is less finely tuned. Therefore, the effect of cyclic FMD on blood glucose levels reduction is not so strong in humans as in mice, because of the different systemic response generated during fasting period.

**PKA activation reverses STS dependent mammosphere reduction**

In our laboratories we have previously shown that fasting/FMD, reducing glucose and glucose consumption, decreases intracellular ATP (Bianchi G et al., 2015), leading to the inhibition of cAMP generation and consequently to PKA pathway downregulation (Cheng CW et al., 2014; Brandhorst S et al., 2015; Di Biase S et al., 2017). In fact, high level of cAMP triggers PKA, which is known to be highly activated in TNBC (Beristain

AG et al., 2015; Shi P et al., 2017). Interestingly, PKA inhibition results in the down-regulation of the stem cell transcription factor Kruppel-like factor 5 (KLF5), a potential target for TNBC (Shi P et al., 2017), through glycogen synthase kinase-3β (GSK3β) phosphorylation. Moreover, PKA, through CREB activity, regulates the H3K9 methyltranferase G9A, which is considered a potential target therapy for several types of cancer (Kondo Y et a., 2008; Li SF et al., 2013; Hua KT et al 2014; Bai K et al., 2016). In fact, H3K9 dimethylation inhibits the expression of several tumor suppressor genes, such as p53 target gene desmocollin 3 (DSC3) and MASPIN in breast cancer (Wozniak RJ et al., 2007), or E-cadherin and p15INK4B in acute myeloid leukemia (Lakshmikuttyamma V et al., 2010), promoting metastasis and cells invasion.

I evaluated the potential involvement of PKA pathway in FMD dependent CSCs reduction and found that FMD inhibits PKA activity in TNBCs, down-regulating CREB phosphorylation. Moreover, my results show that FMD decreases the expression of KLF5 and both G9A and H3K9me2 levels and, accordingly, it reduces the mRNA level of G9A and KL5 downstream target genes involved in pluripotency network, such as Oct4 and Nanog. Interestingly, RNA-seq analysis revealed that FMD-dependent PKA pathway inhibition occurs in the staminal population but not in differentiated cells, suggesting the selective involvement of PKA in affecting CSCs. Consistently, I observed that the PKA activator 8-Bromoadenosine 3',5'-cyclic mono-phosphate (8-Br-cAMP) completely reverses the STS dependent spheres reduction.

Taken together, these results indicate that STS/FMD-induced depletion of TNBC CSCs is mediated, at least in part, by glucose-dependent PKA pathway inhibition.

**FMD reverts TNBC progression and protects from hyperglycemia induced by PI3K pathway inhibitors**

By reducing CSCs, fasting/FMD could prevent drugs resistance, potentiating the effect of several therapies targeting differentiated cancer cells, leading to tumor regression and an increase in survival.

CSCs are reported to be resistant to radiotherapy and standard cytotoxic agents, leading to their enrichment inside the tumor, treatment failure and, consequently, to cancer recurrences. ABC drug transporters are normally overexpressed in CSCs as drug efflux pumps, and are also known as multidrug resistant proteins (MDR) due to their ability to expel toxic agents (Gottesman MM et al., 2002). Preclinical and clinical studies show that

the inhibition of MDR proteins and self-renewal signaling pathways increases the anti-tumor effect of several drugs (O'Connor R et al., 2004; O'Connor R et al., 2007; Britschgi A et al., 2012; Yin S et al., 2013; Wang Y et al., 2016), suggesting that losing CSCs-related properties is fundamental to enhance anti-cancer therapies efficacy.

Although TNBC progression and invasiveness is reported to be CSCs-dependent, differentiated cells' contribution is fundamental.
Starting from this evidence, I performed RNA-seq analysis on SUM159 TNBC tumor masses to identify potential druggable targets that may allow differentiated cancer cells to survive under fasting/FMD conditions. Results revealed that cyclic FMD significantly up-regulates genes involved in PI3K/AKT, mTOR pathways and CCND-CDK4/6. Interestingly, I found that the up-regulation of survival factors mediated by FMD occurs in differentiated cells but not in CSCs, suggesting that cancer cells activate these pathways as escape routes to survive in starvation conditions.
Data obtained from the experiments performed to evaluate the effect of FMD combined to different PI3K/AKT, mTOR and CDK4/6 inhibitors revealed that FMD improves the anti-tumor effect of each inhibitor leading to a significative increase in mice survival. Moreover, when I combined pictilisib, ipatasertib and rapamycin, selective inhibitors for PI3K, AKT and mTOR respectively, the addition of cyclic FMD not only prevented tumor growth for more that 150 days in the 85% of mice, but also protected mice from treatment-induced adverse events, primarily hyperglycemia. In fact, treatments with these drugs strongly increase blood glucose levels leading to constant hyperglycemia in mice fed with standard diet, while FMD restored glycemia to normal levels shortly after the drugs administration preventing toxicity caused by treatments.

A large body of evidence shows that hyperglycemia induced by PI3K inhibitors causes severe side effects in treated patients, leading to a limited use of PI3K and mTOR inhibiting drugs in the clinic (Bendell JC et al., 2012; Patnaik A et al., 2016; Baselga J et al., 2017; Juric D et al., 2017; Mayer IA et al., 2017). Side effects associated to hyperglycemia, which include weight loss, polyuria, polydipsia, diarrhea and renal insufficiency, may cause a progressive decline in quality of life and may lead to dose reductions or treatment discontinuation in patients, resulting in reduced efficacy (Busaidy NL et al., 2012). Hyperglycemia is systemically controlled through the release of insulin, which promote glucose uptake and storage in different organs. Several drugs are used to

prevent hyperglycemia state, including gluconeogenesis inhibitors or insulin production promoters, which lead to decrease in blood glucose levels. However, insulin is a potent stimulator of PI3K signaling pathway in tumors and is associated with cancer progression (Belardi V et al., 2013).

Our previous works show that fasting/FMD lowers blood glucose levels and decreases insulin like growth factor 1 (IGF-1) with beneficial effects on cancer progression and other age-related diseases incidence (Cheng CW et al., 2014; Wei M et al., 2017).

The ability of FMD to protect from hyperglycemia side effect induced by PI3K pathway inhibitors could improve the application of these drugs in clinic, for the treatment of cancer patients.

Furthermore, I found that FMD combined with PI3K, AKT, mTOR inhibitors can induce the reversal of late stage-tumor progression in human TNBC xenografts. In particular, treatment of advanced stage tumors with FMD + the 3 drugs completely reversed tumor progression soon after the first week of treatments, both in mice pre-treated with the FMD or standard diet. Interestingly, I found that pre-treating mice with cyclic FMD prior to the start of drugs administration improves tolerance to therapy and prevents resistance to drugs.

Together with the results previously described in this study, these data suggest that the decrease of CSCs mediated by FMD cycles, prior to the addition of drugs, is fundamental to prevent long-term acquisition of drug resistance, while also reducing side effects therefore increasing progression free survival.

## Conclusions

My findings show that cyclic FMD is sufficient to cause a major reduction in TNBC CSCs, effect partially mediated by glucose-dependent PKA pathway inhibition. Moreover, the use of RNA-seq analysis of tumor masses after FMD cycles allowed the identification of druggable escape pathways, activated by differentiated cells to survive under starvation conditions. I found that the addition of FMD cycles enhances the anti-tumor effect of each combination of treatments used in this study, improves drugs tolerability and retards resistance, underlying the "wild card" property of FMD to increase the effectiveness of a wide range of drugs. In addition, the ability of FMD to protect from hyperglycemia side effect, which is limiting the use of several drugs in the clinic, could provide benefits in the clinical setting in combination with drugs reported to increase glycemia, including PI3K-AKT, mTOR inhibitors.

Overall, these data suggest that FMD has a wide effect on both differentiated and CSCs and provide the rationale for new clinical studies to investigate the potential role of FMD or glucose lowering mimicking drugs as a pre-treatment for TNBC patients, before the administration of standard therapies, to reduce the number of CSCs, and consequently tumor invasiveness, decreasing the possibility of relapses. Moreover, my results pave the way to use cyclic FMD to discover druggable "starvation escape pathways" not only in TNBCs but also in other types of tumors and also to investigate the effect of FMD in combination with inhibitors of the PI3K/AKT/mTORC1 and CCND-CDK4/6 axes in TNBC patients.

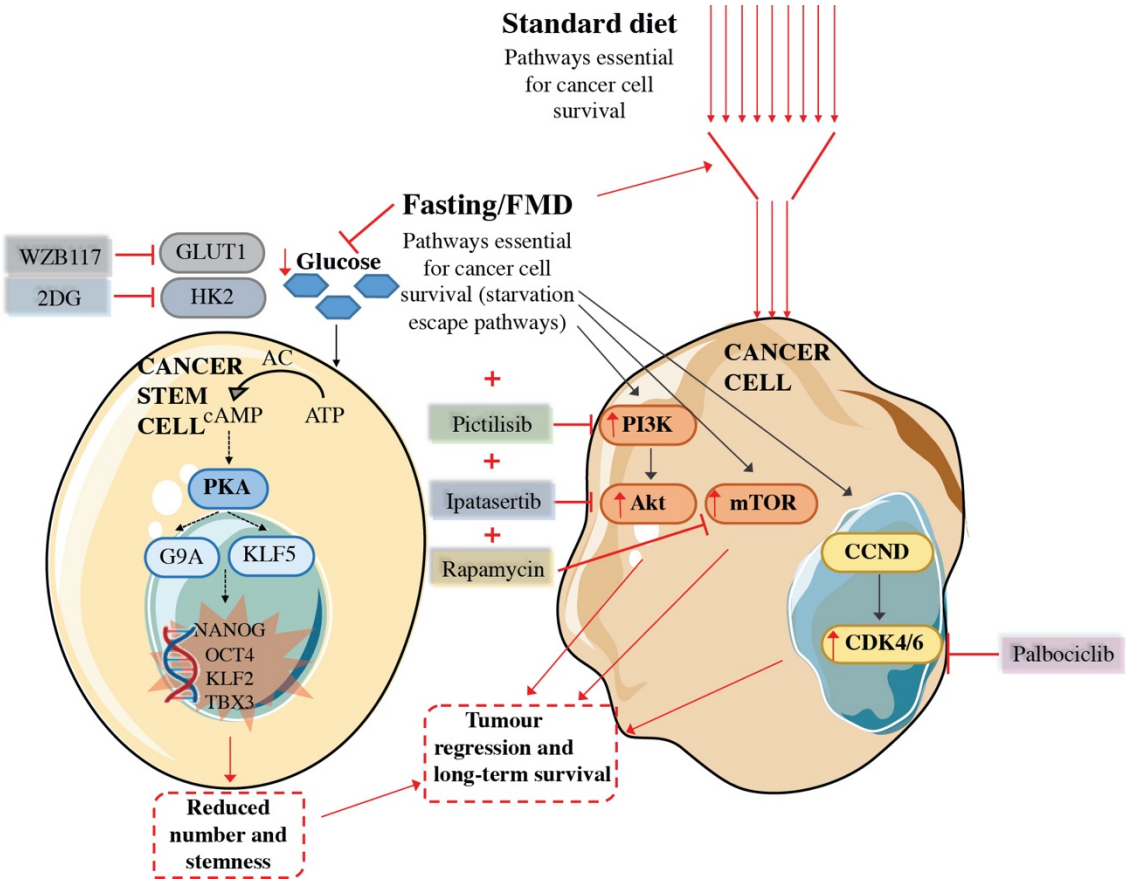



**Figure 1. Putative model for the effect of FMD on SUM159 TNBC cells and CSCs.**

Fasting/FMD reduces blood glucose levels, which reduces the expression of PKA pathway. Downregulation of PKA pathway leads to a decrease of both KLF5 and G9A expression, and then reduces the mRNA levels of stemness associated genes. In differentiated cells, FMD up-regulates PI3K-AKT, mTOR and CCND-CDK4/6 axes. FMD combined with inhibitors of these pathways leads to TNBC regression and long-term survival.

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