Cell Metabolism, Volume *33*

Supplemental information

Fasting-mimicking diet blocks triple-negative breast cancer and cancer stem cell escape

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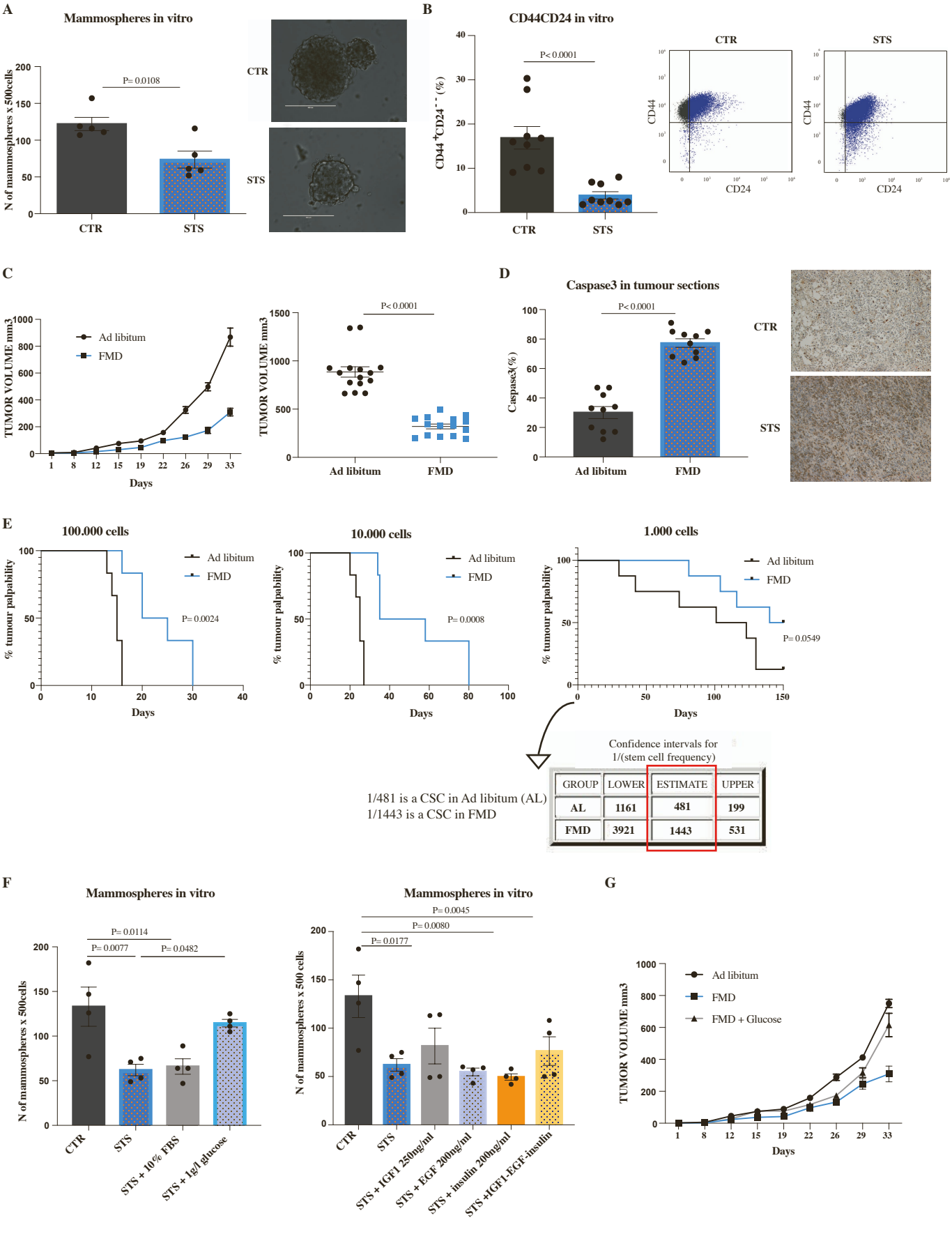

A
Mammospheres in vitro
N of mammospheres x 500cells
P= 0.0108
CTR
STS
B
CD44CD24 in vitro
CD44+CD24- (%)
P< 0.0001
CTR
STS
CD44
CD24
C
TUMOR VOLUME mm3
Ad libitum
FMD
Days
P< 0.0001
D
Caspase3 in tumour sections
Caspase3(%)
P< 0.0001
E
100.000 cells
10.000 cells
1.000 cells
% tumour palpability
P= 0.0024
P= 0.0008
P= 0.0549
Confidence intervals for
1/(stem cell frequency)
GROUP LOWER ESTIMATE UPPER
AL 1161 481 199
FMD 3921 1443 531
1/481 is a CSC in Ad libitum (AL)
1/1443 is a CSC in FMD
F
Mammospheres in vitro
P= 0.0114
P= 0.0077
P= 0.0482
STS + 10% FBS
STS + 1g/l glucose
P= 0.0045
P= 0.0080
P= 0.0177
STS + IGF1 250ng/ml
STS + EGF 200ng/ml
STS + insulin 200ng/ml
STS +IGF1-EGF-insulin
G
FMD + Glucose

Figure S1. FMD effects on CSCs are glucose dependent. Related to Figure 1.

A) 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 to perform the sphere forming assay. The number and the morphology of representative SUM159 spheres, after 8 days of *in vitro* culture (n= 5 biological replicates) are shown.

B) FACS analysis were performed to measure CD44 and CD24 expression in SUM159 breast cancer cell line in vitro (n= 9 biological replicates).

C) Growth of SUM159 xenografts in 8-weeks old female NOD scid (NSG) treated with AL diet or 5 cycles of FMD (n=15).

D) 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.

E) SUM159 tumor cells derived from in vivo xenografts were injected in recipient mice at different dilution to perform the limiting dilution assay (n=6-8). The stem cell frequency was calculated using ELDA software. P values were determined by Log-rank (Mantel-Cox) test.

F) 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 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) for a total of 48h. Cells were then plated to perform the in vitro sphere forming assay (n=4 biological replicates).

G) Growth of SUM159 xenografts in 8-weeks old female NOD scid (NSG) mice treated with AL diet or 5 cycles of FMD alone or plus 3% glucose supplementation in drinking water (n=9). Data are represented as mean ± SEM.

P values were determined by two-tailed unpaired t test (Fig. S1A-D) and one-way Anova (Fig. S1F).

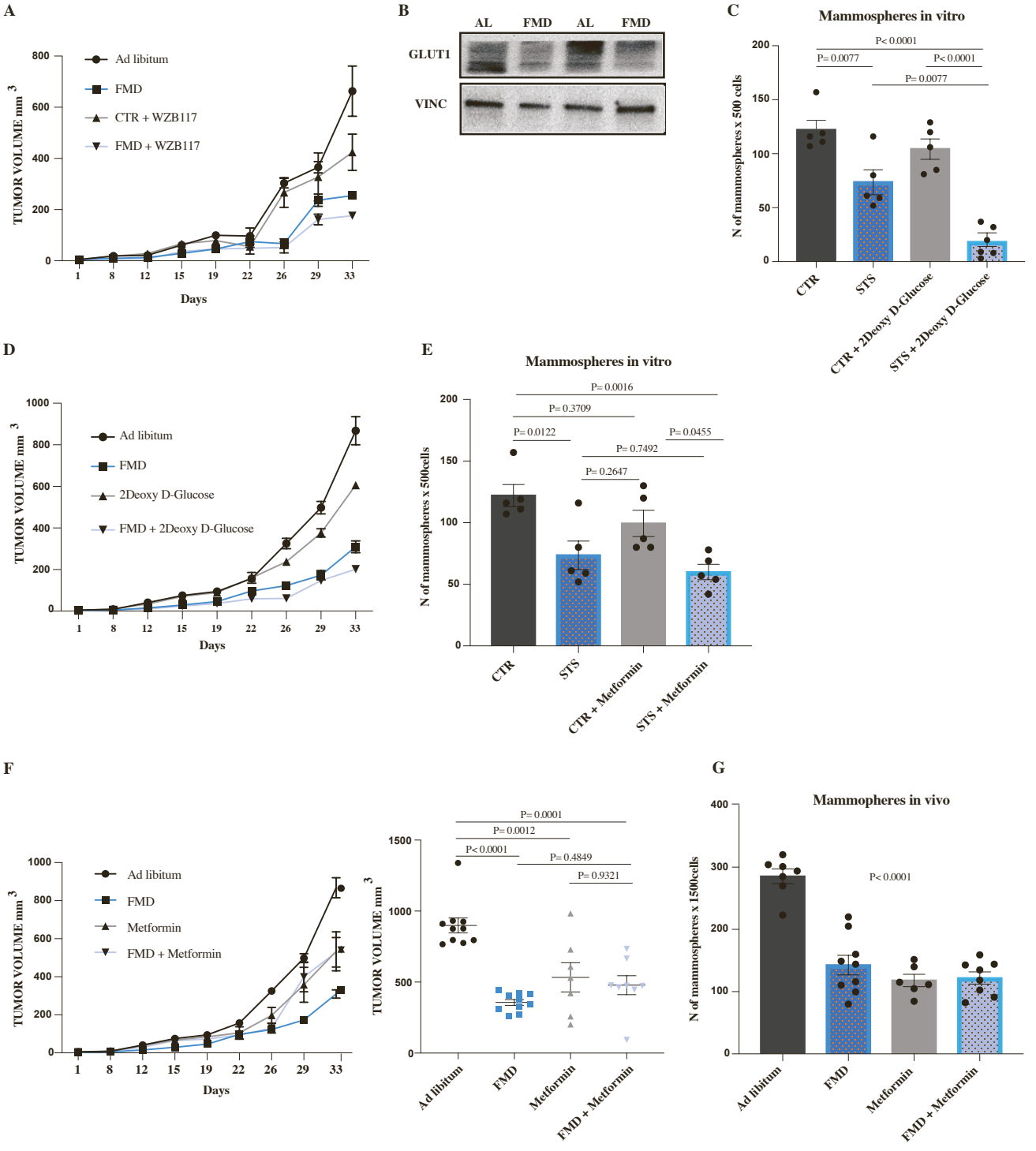

A
TUMOR VOLUME mm 3
Ad libitum
FMD
CTR + WZB117
FMD + WZB117
Days
B
AL
FMD
AL
FMD
GLUT1
VINC
C
Mammospheres in vitro
N of mammospheres x 500 cells
P< 0.0001
P= 0.0077
P< 0.0001
P= 0.0077
CTR
STS
CTR + 2Deoxy D-Glucose
STS + 2Deoxy D-Glucose
D
TUMOR VOLUME mm 3
Ad libitum
FMD
2Deoxy D-Glucose
FMD + 2Deoxy D-Glucose
Days
E
Mammospheres in vitro
N of mammospheres x 500cells
P= 0.0016
P= 0.3709
P= 0.0122
P= 0.0455
P= 0.7492
P= 0.2647
CTR
STS
CTR + Metformin
STS + Metformin
F
TUMOR VOLUME mm 3
Ad libitum
FMD
Metformin
FMD + Metformin
Days
P= 0.0001
P= 0.0012
P< 0.0001
P= 0.4849
P= 0.9321
Ad libitum
FMD
Metformin
FMD + Metformin
G
Mammopheres in vivo
N of mammospheres x 1500cells
P< 0.0001
Ad libitum
FMD
Metformin
FMD + Metformin

Figure S2. FMD effects on CSCs are potentiated by 2-Deoxy-D-Glucose but not by metformin. Related to Figure 1.

A) Growth of SUM159 xenografts in 8-weeks old female NOD scid (NSG) mice treated with AL diet or 5 cycles of FMD alone or combined with WZB117 (10mg/kg) once a day, i.p. (n=6).

B) Detection of GLUT1 levels and VINCULIN, as loading control, in SUM159 xenografts.

C) 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 to perform the sphere forming assay (n=5 biological replicates).

D) Growth of SUM159 xenografts in 8-weeks old female NOD scid (NSG) mice treated with AL diet or 5 cycles of FMD alone or combined with 2DG (500mg/kg) once a day, i.p (n=15-16).

E) 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 to perform the sphere forming assay (n=5 biological replicates).

F) Growth of SUM159 xenografts in 8-weeks old female NOD scid (NSG) mice treated with AL diet or 5 cycles of FMD alone or combined with metformin (150mg/kg) once a day, i.p. (n=7-10).

G) Tumor masses were excised and used to perform the *ex vivo* sphere forming assay (n=7-9).

Data are represented as mean ± SEM. P values were determined by one-way Anova.

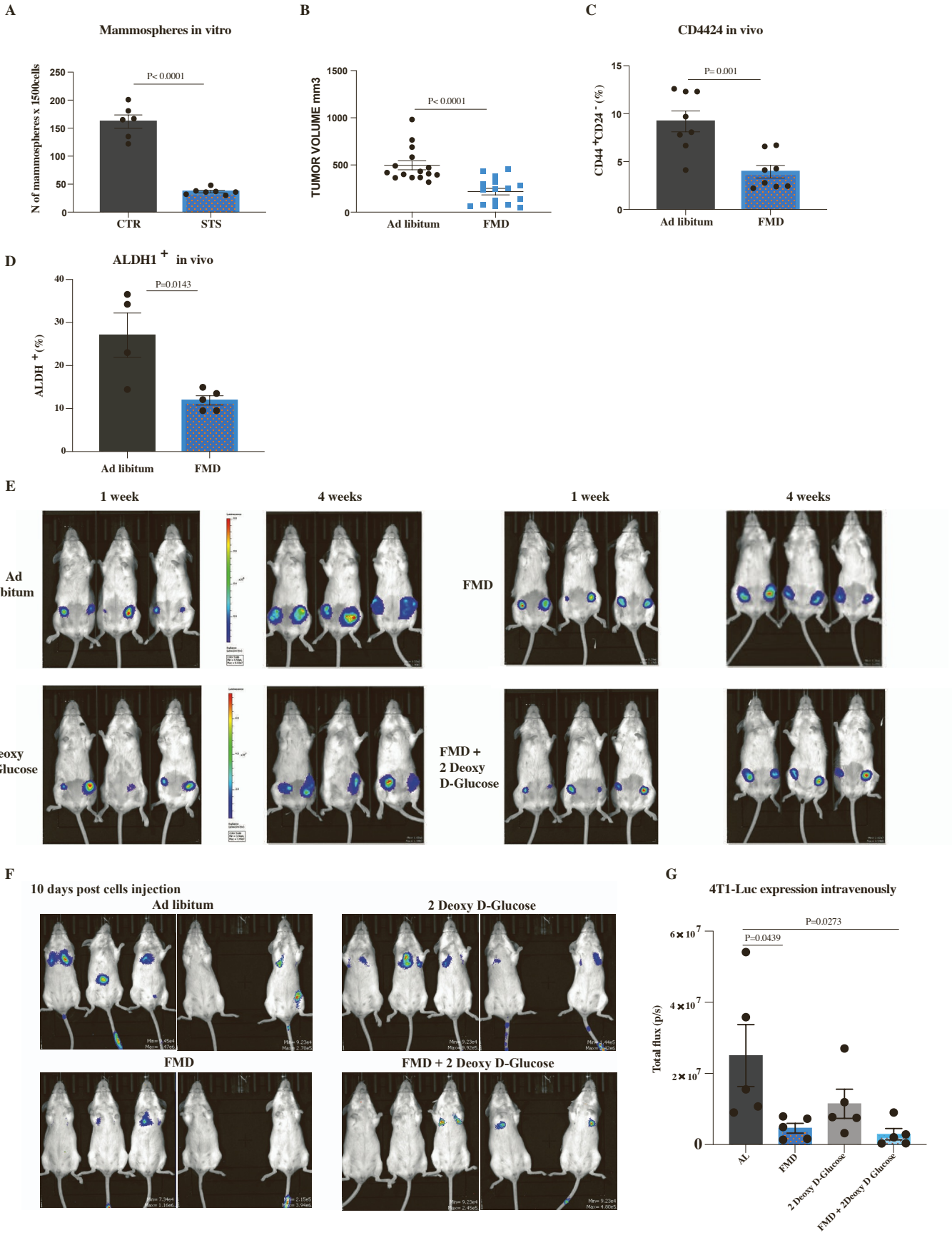

A
Mammospheres in vitro
N of mammospheres x 1500cells
P< 0.0001
CTR
STS
B
TUMOR VOLUME mm3
P< 0.0001
Ad libitum
FMD
C
CD4424 in vivo
CD44 +CD24 - (%)
P= 0.001
Ad libitum
FMD
D
ALDH1 + in vivo
ALDH + (%)
P=0.0143
Ad libitum
FMD
E
1 week
4 weeks
1 week
4 weeks
Ad libitum
FMD
2 Deoxy D-Glucose
FMD + 2 Deoxy D-Glucose
F
10 days post cells injection
Ad libitum
2 Deoxy D-Glucose
FMD
FMD + 2 Deoxy D-Glucose
G
4T1-Luc expression intravenously
Total flux (p/s)
P=0.0273
P=0.0439
AL
FMD
2 Deoxy D-Glucose
FMD + 2Deoxy D Glucose

Figure S3. FMD effects in a syngeneic model of TNBC. Related to Figure 2.

A) 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 to perform the sphere forming assay (n=6-7 biological replicates).

B) Growth of 4T1 xenografts in 6-weeks old female Balb-c mice treated with AL diet or subjected to 4 cycles of FMD. (n=15-16 per group).

C) FACS analysis were performed to measure CD44 and CD24 expression in 4T1 xenografts (n= 8).

D) Aldefluor analysis were performed by flow cytometry using the ALDEFLUOR kit, to measure ALDH1 expression in 4T1 tumor masses (n=4-5).

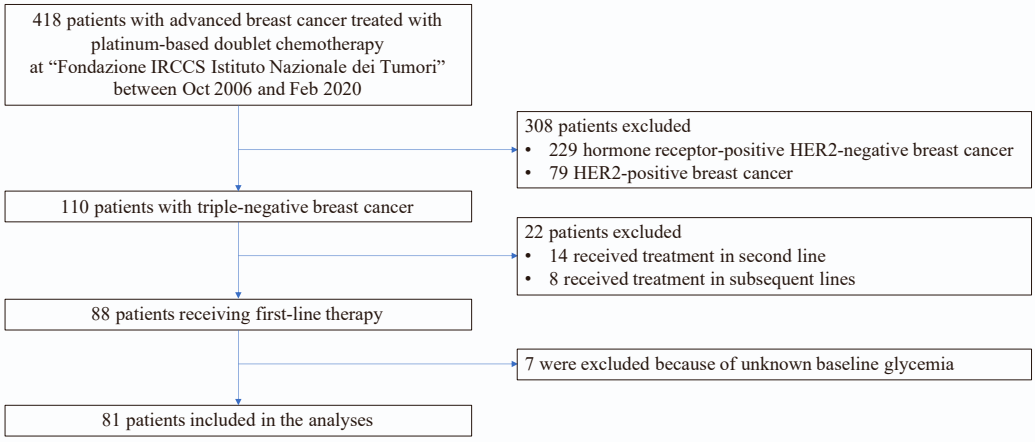
E) Growth of 4T1 xenografts in 6-weeks old female Balb-c mice treated with AL diet or subjected to 4 cycles of FMD, alone or combined with 2DG (500mg/kg) once a day, i.p. Tumor progression was monitored with bioluminescent imaging 1 week and 4 weeks after 4T1-luc cells injection in the mammary fat pad.

F) Growth of 4T1-luc cells intravenously in 6-weeks old female Balb-c mice treated with AL diet or 4 cycles of FMD alone or combined with 2DG (200mg/kg) once a day, i.p. Tumor progression was monitored with bioluminescent imaging 10 days post cells injection.

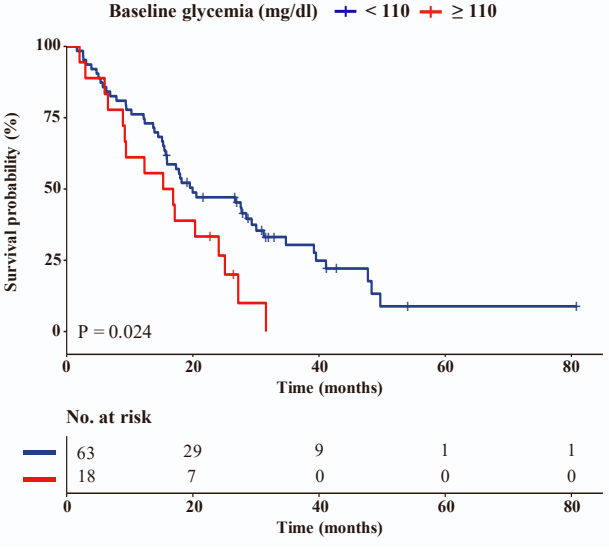
G) Total photon effluxes 17 days post cells injection were measured (n=5 per group).

Data are represented as mean ± SEM. P values were determined by two tailed unpaired t test (Fig. S3 A-D) and one-way Anova (Fig. S3 G).

A



B



C

Multivariable analysis for OS			
Variables	**HR**	**95% CI**	**P value**
Baseline glycemia ≥ 110 mg/dl *vs.* < 110 mg/dl	1.83	0.91 – 3.68	0.092
ECOG PS 1 *vs.* 0	4.72	2.29 – 9.73	**< 0.001**
***De novo* metastatic disease** Yes *vs.* No	0.18	0.02 – 1.45	0.108
Previous taxanes Yes *vs.* No	1.21	0.57 – 2.59	0.620
N° of metastatic sites >3 *vs.* ≤3	0.52	0.18 – 1.46	0.211
Lung metastasis Yes *vs.* No	1.96	0.99 – 3.89	0.054
Liver metastasis Yes *vs.* No	2.21	1.08 – 4.51	**0.029**
Bone metastasis Yes *vs.* No	1.40	0.70 – 2.78	0.339

Figure S4. Low baseline glycemia is associated with increased survival of TNBC patients. Related to Figure 3.

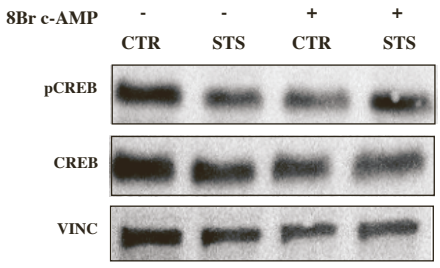
A) CONSORT flow diagram showing the selection of patients with advanced TNBC among a larger dataset of advanced breast cancer patients treated at Fondazione IRCCS Istituto Nazionale dei Tumori.

B) Kaplan-Meier curves for overall survival (OS) according to baseline blood glucose levels in advanced TNBC patients treated with first-line platinum-based doublet chemotherapy. Normoglycemia and hyperglycemia were defined according to 100 mg/dl threshold. The + symbol in Kaplan Meier curves indicates patients who were censored at the time of data cut-off analysis.

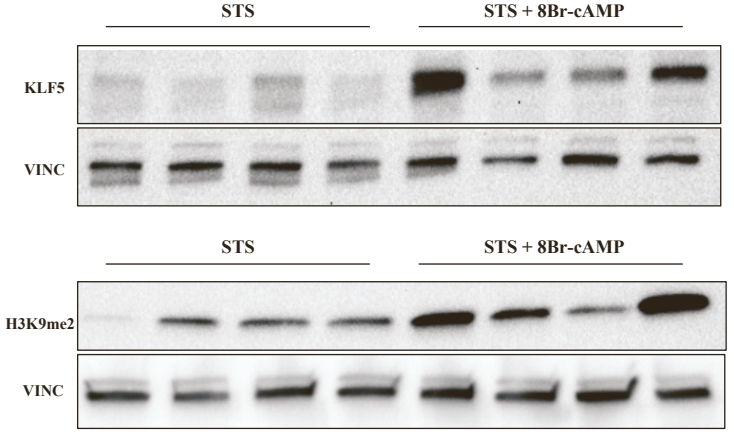
C) Multivariable analysis adjusting the impact of hyperglycemia on OS according to clinical characteristics previously selected on the basis of univariate analysis. For each covariate the hazard ratio (HR), 95% confidence interval (CI) and p value are indicated. CIs not crossing the value of 1 indicates a statistically significant impact of that variable on patient OS.

The p value is indicated in bold numbers when statistically significant.

A


8Br c-AMP
-
-
+
+
CTR
STS
CTR
STS
pCREB
CREB
VINC

B


STS
STS + 8Br-cAMP
KLF5
VINC
STS
STS + 8Br-cAMP
H3K9me2
VINC

C

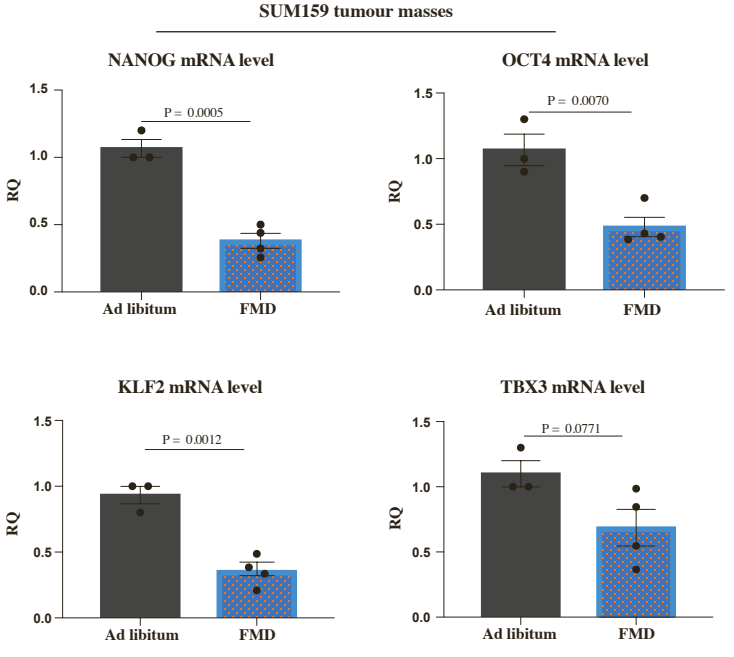

SUM159 tumour masses
NANOG mRNA level
P = 0.0005
RQ
1.5
1.0
0.5
0.0
Ad libitum
FMD
OCT4 mRNA level
P = 0.0070
KLF2 mRNA level
P = 0.0012
TBX3 mRNA level
P = 0.0771

Figure S5. FMD reduces the expression of stemness associated genes. Related to Figure 4.

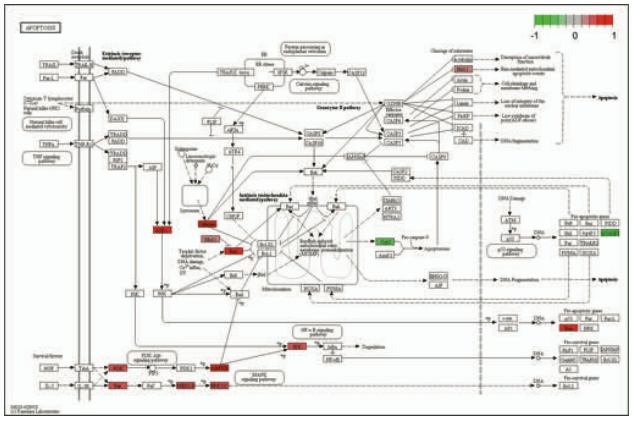
A) Detection of phosphorylated CREB levels, total CREB and VINCULIN, as loading control, in SUM159 cells after 48h in STS medium (0,5 g/L glucose and 1% FBS), alone or in combination with 8-Br-cAMP.

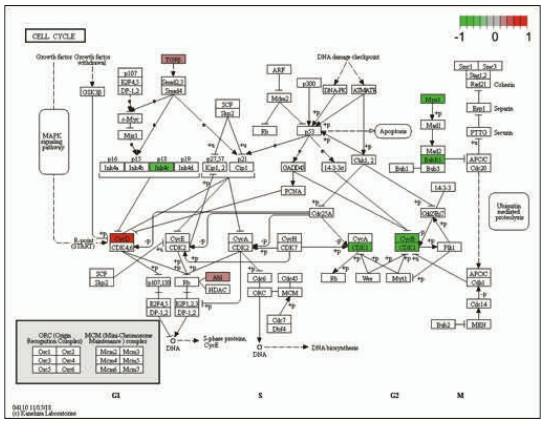
B) Detection of KLF5, H3meK9 levels and VINCULIN, as loading control, in SUM159 cell lysates (n=4 biological replicates).

C) qPCR analysis was performed on SUM159 xenografts bared by mice fed with FMD or standard diet (CTR) (n=3-4).

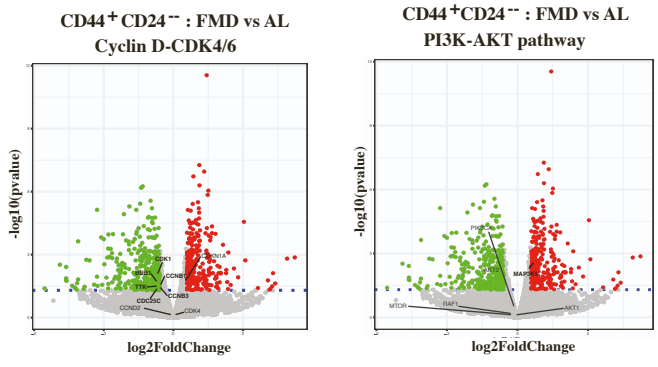
Data are represented as mean ± SEM. P values were determined by two tailed unpaired t test.

A

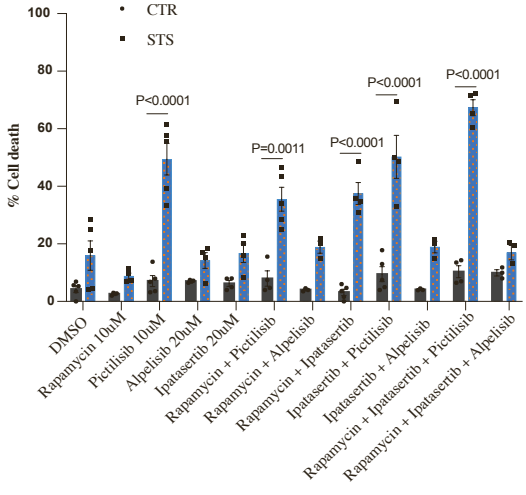



CELL CYCLE
G1
S
G2
M

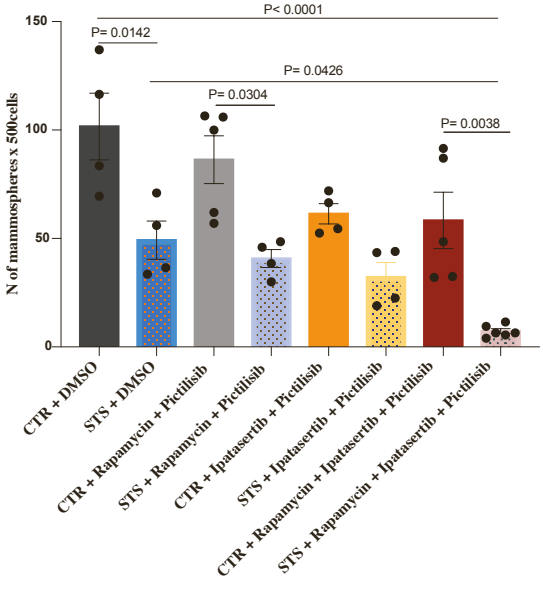
B


CD44+ CD24-- : FMD vs AL
Cyclin D-CDK4/6
CD44+CD24-- : FMD vs AL
PI3K-AKT pathway
-log10(pvalue)
log2FoldChange

C


CTR
STS
% Cell death
P<0.0001
P=0.0011
P<0.0001
P<0.0001
P<0.0001
DMSO
Rapamycin 10uM
Pictilisib 10uM
Alpelisib 20uM
Ipatasertib 20uM
Rapamycin + Pictilisib
Rapamycin + Alpelisib
Rapamycin + Ipatasertib
Ipatasertib + Pictilisib
Ipatasertib + Alpelisib
Rapamycin + Ipatasertib + Pictilisib
Rapamycin + Ipatasertib + Alpelisib

D


Mammospheres in vitro
P< 0.0001
P= 0.0142
P= 0.0426
P= 0.0304
P= 0.0038
N of mammospheres x 500cells
CTR + DMSO
STS + DMSO
CTR + Rapamycin + Pictilisib
STS + Rapamycin + Pictilisib
CTR + Ipatasertib + Pictilisib
STS + Ipatasertib + Pictilisib
CTR + Rapamycin + Ipatasertib + Pictilisib
STS + Rapamycin + Ipatasertib + Pictilisib

E

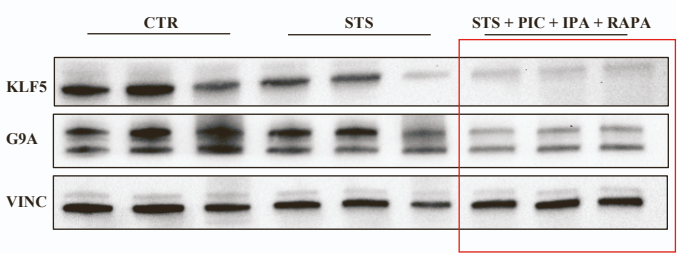

SUM159 cells
CTR
STS
STS + PIC + IPA + RAPA
KLF5
G9A
VINC

Figure S6. PI3K/AKT, mTOR pathways inhibitors improve FMD effects against TNBC cancer cells and CSCs. Related to Figure 6.

A) Graph representation of KEGG apoptosis (hsa04210) and KEGG cell cycle (hsa04110). Significantly up and downregulated genes (by comparing FMD versus AL) are depicted in red and green respectively.

B) Volcano plot showing the significance versus the log2 fold-change in SUM159 tumor masses, in $CD44^{+}CD24^{--}$ population, 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 and CycD-CDK4/6 pathways are highlighted. Significantly deregulated genes are reported in bold.

C) 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, as single, double or triple treatments. Viability was assessed with erythrosine stain (n= 4-5 biological replicates), or

D) cells were plated to perform the sphere forming assay (n=4-6 biological replicates).

E) Detection of KLF5, G9A levels and VINCULIN, as loading control, in SUM159 cell lysates (n=3 biological replicates).

Data are represented as mean ± SEM. P values were determined by multiple t-test (fig S6C) and one-way Anova (fig S6D).

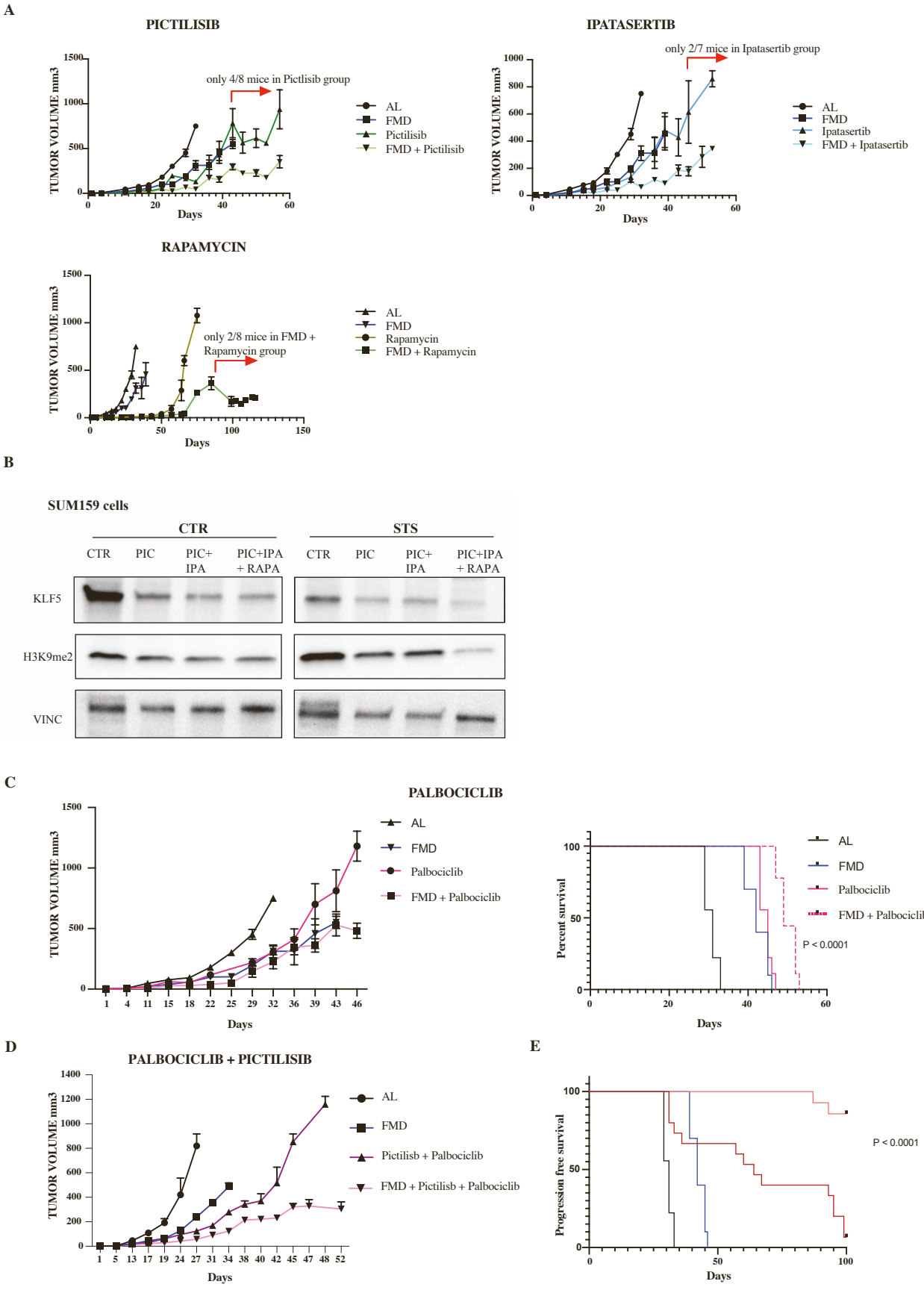

A
PICTILISIB
TUMOR VOLUME mm3
Days
only 4/8 mice in Pictlisib group
AL
FMD
Pictilisib
FMD + Pictilisib
IPATASERTIB
only 2/7 mice in Ipatasertib group
AL
FMD
Ipatasertib
FMD + Ipatasertib
RAPAMYCIN
only 2/8 mice in FMD + Rapamycin group
AL
FMD
Rapamycin
FMD + Rapamycin
B
SUM159 cells
CTR
STS
CTR
PIC
PIC+ IPA
PIC+IPA + RAPA
KLF5
H3K9me2
VINC
C
PALBOCICLIB
AL
FMD
Palbociclib
FMD + Palbociclib
Percent survival
P < 0.0001
D
PALBOCICLIB + PICTILISIB
AL
FMD
Pictilisb + Palbociclib
FMD + Pictilisb + Palbociclib
E
Progression free survival
P < 0.0001

Figure S7. PI3K/AKT, mTOR and CDK4/6 pathways inhibitors improve FMD effects in increasing mice survival. Related to Figure 6, 7.

A) Growth of SUM159 xenografts in 8-weeks old female NOD scid (NSG) mice treated with AL diet FMD, alone or combined with pictilisib (100mg/kg, 5 consecutive days a week, by oral gavage), ipatasertib (75mg/kg, 5 consecutive days a week, by oral gavage) or rapamycin (2mg/kg, every other day, i.p.) (n=8-12).

B) Detection of KLF5, H3K9me2 levels and VINCULIN, as loading control, in SUM159 cell lysates (n=3 biological replicates).

C) Growth of SUM159 xenografts in 8-weeks old female NOD scid (NSG) mice treated with AL diet or FMD, alone or combined with palbociclib (62,5 mg/kg) every other day, by oral gavage. Survival curve is reported.

D) Growth of 4T1 xenografts in 6-weeks old female Balb-c mice treated with AL diet or FMD, alone or combined 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) (n=5).

E) Progression free survival related to experiment in Fig. 7D was monitored over time.

P values were determined by Log-rank (Mantel-Cox) test.

Table S1. Characteristics of triple-negative breast cancer patients treated with platinum-based doublet chemotherapy. Related to Figure 3.

	Overall N. (%)
Age (years): median [range]	56 [34-80]
First line platinum-based chemotherapy	81 (100)
Treatment combination Carboplatin + Paclitaxel Carboplatin + Gemcitabine	 50 (61.7) 31 (38.3)
Baseline glycemia (threshold) Normoglycemic (< 100 mg/dl) Hyperglycemic (≥ 100 mg/dl)	 50 (61.7) 31 (38.3)
Baseline glycemia (threshold) Normoglycemic (< 110 mg/dl) Hyperglycemic (≥ 100 mg/dl)	 63 (77.8) 18 (22.2)
BMI at baseline < 25 kg/m2 ≥ 25 kg/m2	 40 (49.4) 41 (50.6)

Data are presented as N. (%) except where otherwise noted.
Abbreviations: BMI: Body Mass Index; NA: Not Available

Table S2. Univariate analyses for OS according to clinical characteristics of advanced TNBC patients treated with platinum-based doublet chemotherapy. Related to Figure 3.

Variables	HR	95% CI	P value
Baseline glycemia ≥ 100 mg/dl *vs.* < 100 mg/dl	1.72	1.03 – 2.87	0.039*
Baseline glycemia ≥ 110 mg/dl *vs.* < 110 mg/dl	1.95	1.08 – 3.53	0.026*
Age >65 years *vs.* ≤65 years	0.75	0.42 – 1.33	0.318
ECOG PS 1 *vs.* 0	4.11	2.12 – 7.97	< 0.001*
BMI ≥ 25 kg/m2 *vs.* <25 kg/m2	0.88	0.53 – 1.44	0.599
***De novo* metastatic disease** Yes *vs.* No	0.27	0.07 – 1.13	0.073*
Previous taxanes Yes *vs.* No	1.71	0.96 – 3.07	0.071*
Previous anthracyclines Yes *vs.* No	1.57	0.83 – 2.95	0.164
DFI >36 months *vs.* ≤36 months	0.75	0.45 – 1.26	0.274
N° of metastatic sites >3 *vs.* ≤3	2.15	1.13 – 4.09	0.020*
Lung metastasis Yes *vs.* No	1.69	1.02 – 2.79	0.043*
Liver metastasis Yes *vs.* No	1.77	1.01 – 3.09	0.047*
Brain metastasis Yes *vs.* No	1.59	0.72 – 3.50	0.250
Bone metastasis Yes *vs.* No	1.80	1.06 – 3.05	0.030*

*Covariates with P value <0.1 were included in the multivariable model.
Abbreviations: HR: Hazard Ratio; CI: Confidence Interval; ECOG PS: Eastern Cooperative Oncology Group Performance Status; BMI: Body Mass Index; DFI: Disease Free Interval