Conditioned media from MCF7 and BT474 breast cancer cells induce insulin resistance in skeletal muscle myotubes.

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Abstract.

Background. Metabolic disorders are prevalent in women with breast cancer and breast cancer survivors. Such disorders increase breast cancer mortality and likelihood of relapse 2- and 3-fold, respectively. However, there is a severe lack of research into the physiological sequelae of breast cancer, including the metabolic health consequences. The aim of the present study was to provide novel insights into the causes of metabolic disturbances associated with breast cancer by investigating the effects of breast cancer on insulin sensitivity in skeletal muscle.

Method. L6 myotubes stably expressing GLUT4 were incubated for 72 hours in normal growth medium or medium supplemented with 25% conditioned media (CM) from either MCF7 or BT474 breast cancer cells. Basal and insulin- (100nM) stimulated GLUT4 translocation, 2-deoxyglucose (2DG) uptake, and intracellular insulin signaling was determined in day 7 myotubes.

Results. Basal- and insulin-stimulated GLUT4 translocation was reduced in L6 myotubes incubated with MCF7 (basal: -7%, insulin: -14%, $p<0.01$) or BT474 (basal: -16%, insulin: -8%, $p<0.01$) breast cancer CM. Insulin-stimulated 2DG uptake in L6 myotubes was also reduced by MCF7 (-5%, $p<0.05$) and BT474 (-10%, $p<0.05$) breast cancer CM. Insulin-stimulated p-Akt^{Thr308} (but not p-Akt^{Ser473}) phosphorylation tended to be reduced (-25%, $p<0.1$) in L6 myotubes incubated with MCF7 or BT474 breast cancer CM, while p-TBC1D4^{Thr642} phosphorylation was enhanced (+34%, $p<0.05$) by MCF7 breast cancer CM.

Conclusion. We conclude that breast cancer reduces muscle insulin responsiveness, evidenced as reduced insulin-stimulated GLUT4 translocation, downregulated glucose uptake, and blunted intracellular insulin sigaling in L6 myotubes incubated with breast cancer cell CM. Thus, skeletal muscle insulin resistance might contribute to metabolic disorders prevalent in women with breast cancer and could be a potential treatment target.

Introduction.

Cancer is the second most deadly disease worldwide [1, 2] but fortunately, more people are now surviving their cancer. The majority of cancer survivors constitute women who have survived breast cancer. However, the breast cancer recurs in 10-50% of breast cancer survivors [3]. Recent epidemiological studies have revealed that 60-80% of women with breast cancer develop metabolic disorders [4-6]. Metabolic disorders negatively impact on disease prognosis, as it increases the likelihood of breast cancer recurrence 3-fold [7, 8]. Furthermore, the presence of metabolic disorders, increases mortality among women with breast cancer 2-fold [9]. Thus, it is clearly necessary to treat metabolic disorders in breast cancer patients and survivors, but because the cause is unknown and the problem is underappreciated, current treatment is limited. Thus, there is a severe lack of research into the physiological sequelae of cancer and/or treatment, including the metabolic health consequences of breast cancer [10].

The metabolic dysfunctions observed in breast cancer, include obesity, hyperglycemia, hyperinsulinemia, cardiovascular problems, and glucose intolerance [11]. However, only very few studies have investigated the underlying biological causes hereof [4-6, 11]. Biologically, there is an association between hyperinsulinemia and an increased risk of breast cancer recurrence, as well as an increased breast cancer mortality [12]. The underlying causes of hyperinsulinemia in breast cancer survivors are unknown. However, from advances in diabetes research, it is known that hyperinsulinemia is most often caused by insulin resistance in muscle, fat, and liver [13-15]. In particular, skeletal muscle and adipose tissue are essential for maintaining a normal metabolism as these tissues are responsible for up to 75% of the uptake of glucose from the blood in response to insulin [16, 17]. Insulin-resistant muscle does not respond normally to insulin. In non-cancer studies, skeletal muscle insulin resistance causes severe metabolic disorders, including dyslipidemia, hyperglycemia, and hyperinsulinemia[13, 17-19]; all conditions, which have also been observed in women with breast cancer and breast cancer survivors [4-6, 11].

Cancer cells require an exceptionally high consumption of glucose to sustain their accelerated growth. At the same time, the protein expression of the insulin receptor is 6-fold higher in breast cancer cells than normal breast cells [20], making them very sensitive to the amount of insulin in the body. Insulin promotes the growth of cancer cells [21] and thus, hyperglycemia and hyperinsulinemia could increase breast cancer recurrence in women with metabolic disorders [7, 8]. Since skeletal muscle and adipose tissue play key roles in metabolic regulation by keeping blood

glucose and insulin levels normal [13, 18], it is extremely relevant to clarify the precise involvement of these tissues in breast cancer-related metabolic disorders.

How breast cancer might affect muscle and fat cells is unknown, but we have recently reported that cancer can cause insulin resistance in skeletal muscle and fat in mice [22]. This indicates intriguing endocrine cross-talk between cancer and muscle cells. Thus, breast cancer cells likely secrete substances that rewire the metabolism of muscles and adipose tissues to induce insulin resistance.

Microarray studies have classified breast cancer into at least four biologically and clinically relevant subtypes (Luminal (A/B), triple-negative/basal-like, HER2-enriched, and normal-like) [23-25]. Generally, the luminal subtypes are progesterone (PR)- and estrogen (ER)-receptor positive, HER2-negative or positive (HER2; a tyrosine kinase involved in cell growth and proliferation) and grow slowly. Basal subtypes lack the expression of PR, ER and HER2, and grow rapidly. Based on the classification of breast cancer subtypes, breast cancer is no longer considered a single disease, but rather a collection of several diseases [24]. Importantly, breast cancer cell lines display same abnormalities in genome copy number and gene expression pattern as the primary tumors [26-30]. Thus, the breast cancer cell lines are good representatives of breast carcinoma tumors.

Against this background, we have performed the current study to explore the molecular mechanisms underlying the control of muscle insulin sensitivity by breast cancer-secreted factors. We have utilized *in vitro* models of the two breast cancer cell lines to study their effect on skeletal muscle insulin responsiveness. The first, MCF7-S1 (MCF7) is a highly tumour-necrosis factor (TNF)-α sensitive S1 subclone of MCF7 breast carcinoma cells [31], ER- and PR-positive and categorized within the largest subgroup of luminal breast cancer tumors. The second, BT474 is a luminal B subtype obtained from a stage II localized solid tumor, positive for ER and PR receptor (50 – 60% of all breast cancer cases). We found that insulin responsiveness was significantly reduced in skeletal muscle myotubes incubated with breast cancer-conditioned media.

Methods.

Breast cancer cell lines. The human female breast cancer cell line MCF7 (ER+, PR+, HER2-) was obtained from Marja Jäättela (Cell Death and Metabolism, Danish Cancer Society, Denmark) [31]. BT474 (ER+, PR+, HER2+) was purchased from the American Type Culture Collection (ATCC), Manassas, VA. MCF7 was cultured in Roswell Park Memorial Institute 1640 medium (RPMI, GIBCO #61870-010) supplemented with 6% fetal calf serum (FCS). BT474 was cultured in

Dulbecco's modified Eagle's medium and F12 medium (DMEM/F12, GIBCO #31330-038) supplemented with 10% FCS. 1% penicillin (10,000 U/ml)/streptomycin (10 mg/ml) (GIBCO #15140) was added to all culture media. Upon receipt, cells were expanded and frozen in multiple vials and subsequently used for experiments within the same six passages (P14–P19). The cell lines were authenticated by routinely visual inspection of the morphologic phenotype. For all cell experiments, MCF-7 and BT474 cells were cultured in a humidified incubator at 37 °C and 5% CO_2 in tissue culture T 75-cm2 flasks.

L6 muscle cells. Rat L6 skeletal muscle cells stably over-expressing c-myc epitope-tagged GLUT4 (L6-GLUT4myc, denoted as L6 in the current paper), a kind gift from Amira Klip, were grown in α-MEM media (GIBCO #22571) with 10% fetal bovine serum (GIBCO #23140), 1% penicillin (10,000 U/ml)/streptomycin (10 mg/ml) (GIBCO #15140) (5% CO_2, 37°C).

GLUT4 translocation. L6 myoblasts were plated in 96-well plates and differentiated for 3 days. Meanwhile, breast cancer cells (MCF7 and BT474) were cultured in T-75 cm^2 flasks at 60-90 % confluence for 48 hours. The 48-hr CM was removed from all cell lines, centrifuged and the supernatants were collected, diluted 1:4 in fresh L6 differentiation media (25% CM) and added to the L6 myotubes in the 96-well plates. The CM was refreshed every 24 hr over three days. On the experimental day, day 7 differentiated L6 myotubes were deprived of serum 2 hours prior to insulin-stimulation. For the GLUT4 translocation assay [32], cells were stimulated for 10 min with human insulin (100 nM). At the end of the stimulation period, cells were immediately chilled on ice, the media removed and the cells fixed in 3% paraformaldehyde, blocked in 5% goat serum, incubated with a primary anti-c-Myc antibody (Sigma #C3956). The signal was detected with a HRP-conjugated anti-rabbit secondary antibody, and o-Phenylenediamine (OPD) reagent (Sigma #P5412) was added to each well to initiate a colour reaction with the secondary antibody. The reaction was terminated with 5N HCl and absorbance was measured at 492 nm. Background absorbance (no primary antibody) was subtracted from the measured values of absorbance. Experiments were assayed in n = 6 for each group. The experiments were repeated 3 times.

^{3}H-labeled 2-Deoxy-Glucose uptake in L6 cells. Glucose uptake in L6 myotubes was measured using the radio-labeled D-2-deoxy-D-[2,6-^{3}H]glucose (^{3}H-2DG) as described previously [33]. L6 cells were plated in 12-well plates and differentiated in αMEM (2% FBS) for 3 days. 48 hrs CM

from MCF7, BT474 was centrifuged and the supernatants were collected, diluted 1:4 in fresh differentiation media (25% CM) and added to the L6 myotubes in the 12 well plates. L6 myotubes were incubated in serum-free media for 2 hrs, and stimulated with human insulin (100 nM, Actrapid; Novo Nordisk, DK) for 20 min before the end of the starvation-period, followed by 3X wash with HEPES buffered solution (140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM $MgSO_4$, 1.0 mM $CaCl_2$, pH 7.4 at 37°C) and further 5 min incubation with Transport solution (10 µM 2DG and 10 µM ^{3}H-2DG (1.0 µCi/well)) with or without insulin (100 nM). The transport was terminated by 3X wash with Stop solution (0.9% NaCl, 25 mM glucose). Two wells from each run were added cytochalasin B (20 µM) to assess the nonspecific glucose uptake. The experiments were repeated twice.

Cell lysate. Cell lysate proteins were prepared from day 7 L6-GLUT4myc myotubes that were incubated with CM from breast cancer cells (MCF7 and BT474) as described above (see *^{3}H-labeled 2-Deoxy-Glucose uptake in L6 cells*). L6-GLUT4myc day 7 myotubes were deprived of serum 2 hours. After 25 min of insulin stimulation, cells were immediately chilled on ice, washed in ice-cold PBS, and harvested in RIPA buffer supplemented with 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, aprotinin (10 ug/ml), leupeptin (10 ug/ml), 2 mM sodium orthovanadate and 3 mM Benzamidine. Lysates were then clarified by centrifugation and frozen at – 80 C◦. Protein concentrations were determined using the bichinchoninic acid (BCA) method using BSA standards and BCA assay reagents (Pierce).

Immunoblotting. Immunoblotting techniques were used to investigate intracellular signaling in response to insulin. Total protein and phosphorylation levels of relevant proteins were determined by standard immunoblotting technique loading equal amounts of protein. The polyvinylidene difluoride membrane (Immobilon Transfer Membrane; Millipore) was blocked in TBS-Tween 20 containing 2% skim milk for 15 minutes at room temperature, followed by incubation overnight at 4°C with the primary antibody. The primary antibodies used were Akt 2 (#3063, Cell Signaling Technology (CST)), p-Akt^{Ser473} (#4058, CST), Akt^{Thr308} (#4058, CST), P-TBC1D4^{Thr642} (#D27E6, CST) and TBC1D4 (ab189890, abcam). Next, the membrane was incubated with HRP-conjugated secondary antibody (Jackson Immuno Research) for 1 hour at room temperature. Bands were visualized using Bio-Rad ChemiDocTM MP Imaging System and enhanced chemiluminescence

(ECL+; Amersham Biosciences). Coomassie staining was used as a loading control. Amount of protein loaded = 3 µg.

Statistical analyses. Results are shown as individual values with mean ± SEM. Statistical significance was tested using unpaired t-test or two-way ANOVA, as appropriate. If the null hypothesis was rejected, Tukey's post hoc test was used to evaluate main effects and significant interactions in ANOVAs. The significance level was set at p-values <0.05. Statistical analyses were performed using Graph Pad PRISM v.6.01 software (GraphPad Software, Inc., San Diego, CA).

Results

Conditioned media from breast cancer cells reduced GLUT4 translocation in L6 myotubes. Conditioned media (CM) from MCF7 and BT474 cells contain a large accumulation of secreted proteins that is unique to each cell line [34]. We used breast cancer CM to address whether secreted tumorkines in breast cancer might directly affect skeletal muscle insulin responsiveness. L6 myotubes stably expressing a GLUT4myc epitope were incubated with 25% CM from MCF7 or BT474 breast cancer cells for 72 hours, and basal- and insulin-stimulated GLUT4 translocation was measured. CM from MCF7 significantly reduced both basal (-10%) and insulin (-14%) -stimulated GLUT4 translocation in L6 myotubes compared to the control media incubated L6 myotubes (Fig. 1A). The fold-change in response to insulin was also reduced (-13%), indicating a reduced insulin responsiveness of myotubes exposed to CM from MCF7 breast cancer cells (Fig. 1B). BT474 cell CM also reduced basal (-16%) and insulin (-8%) -stimulated GLUT4 translocation in L6 myotubes compared to the control media incubated L6 myotubes (Fig. 1C). However, the fold-change in response to insulin was actually increased by CM from BT474 (+9%), indicative of an increased insulin responsiveness (Fig. 1D), despite the generally lower GLUT4 translocation. These findings show that GLUT4 translocation in skeletal muscle cells are significantly affected by breast cancer derived factors, as both MCF7 and BT474 CM reduced basal- and insulin-stimulated GLUT4 translocation.

Insulin-stimulated 2DG glucose uptake is reduced in L6 myotubes incubated with MCF7 and BT474 conditioned media. Recently, we reported that the presence of cancer in mice significantly reduced insulin-stimulated glucose uptake in mouse skeletal muscle [22]. Therefore, we next

investigated the effect of MCF7 and BT474 CM on insulin-stimulated glucose uptake in L6 myotubes. Because we detected differences in absolute uptake rates between experimental days, we have shown the results for each individual experiment. MCF7 and BT474 CM did not affect L6 myotube glucose uptake during basal state (Fig. 2A+B). We observed a mild, yet significant reduction in insulin-stimulated glucose uptake in L6 myotubes incubated with MCF7 (- 6%) and BT474 (- 15%) CM (Fig. 2C+D) compared to the control condition. These results suggest that cancer can directly reduce insulin sensitivity in L6 myotubes.

Breast cancer conditioned media influences intramyocellular insulin signaling. We next investigated the intracellular insulin signaling in L6 myotubes incubated with MCF7 or BT474 CM to elucidate if intracellular signaling defects were causing the reduced insulin response on GLUT4 translocation and glucose uptake. Thus, we analyzed the phosphorylation (p) of Akt^{Ser473}, Akt^{Thr308}, and $TBC1D4^{Thr642}$ by immunoblotting techniques. p-Akt^{Thr308} tended to be reduced (-34%, p=0.08) with incubation of MCF7 CM compared to the controls (Fig. 3A), while p-Akt^{ser473} was unaltered (Fig. 3B). Surprisingly, given the normal or reduced p-Akt, GLUT4 translocation, and 2DG uptake, insulin-stimulated p-$TBC1D4^{Thr642}$ was increased (+34%) in L6 myotubes incubated with MCF7 CM. L6 myotubes incubated with BT474 CM also tended to have reduced (-37%, p=0.098) insulin-stimulated p-Akt^{Thr308} (Fig. 3D) but normal p-Akt^{ser473} (Fig. 3E) and p-$TBC1D4^{Thr642}$ (Fig. 3F). The protein expression of Akt2 and TBC1D4 were not affected by incubating L6 myotubes with MCF7 or BT474 CM (Fig. 3G+H). Representative blots are shown in Fig. 3I. Taken together, these results suggest that breast cancer cells can directly influence intracellular insulin signaling in muscle without altering protein expression of Akt2 or TBC1D4.

Discussion.

The current study demonstrates that media from breast cancer cells markedly influences insulin responsiveness in skeletal muscle myotubes. This was evidenced on three parameters, i) reduced basal- and insulin-stimulated GLUT4 translocation, ii) impaired insulin-stimulated glucose uptake, and iii) altered intramyocellular insulin signaling. To our knowledge, these findings are the first to suggest that breast cancer might directly rewire skeletal muscle cells rendering them insulin resistant. Our findings are in agreement with a growing number of studies that report that breast cancer survivors experience significant metabolic dysregulations, including hyperglycemia and hyperinsulinemia, which could be caused by skeletal muscle insulin resistance [13, 18].

The physiology of insulin resistance induced by cancer is fascinating; from a cancer point of view designed to limit the host´s glucose uptake and thereby shunt an adequate amount of supply to the growing cancer cells. However, in breast cancer, the underlying mechanisms causing such metabolic perturbations is undescribed. Our results suggest that breast cancer cells might influence skeletal muscle rendering them insulin resistant and we speculate that this could contribute to the metabolic perturbations observed in up to 80% of breast cancer patients and survivors [4-6, 11].
Breast cancer is subdivided into several different subtypes based on their gene expression pattern [23-25]. Each breast cancer subtype develops, metastasizes, and responds to therapy differently and also interacts with the surrounding environment differently [35]. Indeed, CM of breast cancer cell lines from different subtypes have shown to contain a large accumulation of secreted proteins that are unique to every cell line [34, 36]. We used CM of MCF7 (luminal A), BT474 (luminal B) to investigate the insulin responsiveness of L6 muscle cells. CM from both breast cancer cell lines reduced basal and insulin-stimulated GLUT4 translocation and impaired glucose uptake. For both MCF7 and BT474 CM, this was accompanied by a tendency to reduced p-Akt^{Thr308} (but not Ser473), while only MCF7 altered p-TBC1D4^{Thr642}. That only MCF7 influenced the latter, could indicate distinct interaction with its microenvironment compared to the BT474 breast cancer cell line.

We did not identify the factor(s) secreted by breast cancer cells that could induce insulin resistance in muscle. However, other studies have analyzed breast cancer secreted media from BT474 and found more than 1.100 secreted proteins [34, 36]. Another study analyzed the secretomes in CM from breast cancer cells including MCF7 [36]. They identified four biomarkers in the CM that were also found in plasma from breast cancer patients including GM2A and IGFBP using Mass Spectrometry [36]. Elevated levels of GM2A has shown implication in insulin resistant conditions [37], whereas IGFBP overexpressing mice have shown impaired glucose homeostasis and hyperinsulinemia [38]. However, future studies should investigate breast cancer cell-secreted factors involved in induction of muscle insulin resistance.

That breast cancer rewires the muscles and fat, is supported by a preliminary study revealing that skeletal muscles of women with newly diagnosed breast cancer undergo significant molecular changes. Of these, the most significant changes were precisely for proteins involved in metabolic regulation [39], although no functional outcome of these changes were investigated. Thus, our present *in vitro* findings align with *in vivo* results from humans showing significant molecular

rewiring of skeletal muscle in patients with breast cancer. Our study provides mechanistic evidence that might explain why women who develop breast cancer have a higher incidence of metabolic dysfunctions, such as for example diabetes, than women who do not develop breast cancer [39, 40].

Other cancer cell types might also induce muscle insulin resistance, as other cancers have been associated with impaired metabolic regulation. In fact, it has been reported that one-third of cancer patients present diabetic glucose tolerance curves [41]. For example, colorectal cancer is associated with increased risk of developing diabetes within five years after the diagnosis [42]. Additionally, a study from our group has recently shown that Lewis lung cancer implanted into immunocompetent mice caused metabolic perturbations on at least six metabolically essential functions; i) insulin's blood-glucose-lowering effect, ii) glucose tolerance, iii) skeletal muscle and white adipose tissue insulin-stimulated glucose uptake, iv) intramyocellular insulin signaling, v) muscle microvascular perfusion, and vi) basal hepatic glucose production [22]. Such studies suggest that metabolic regulation is disturbed in several cancers.

As 60-80% of women with breast cancer develop metabolic disorders during or following their treatment [4-6], enhancing muscle insulin sensitivity could be a powerful means to improve metabolic regulation for these women. Accordingly, exercise training, which is the most effective means to improve skeletal muscle insulin sensitivity [15], can completely prevent metabolic disturbances in breast cancer survivors [4]. This would have real clinical benefits, as metabolic dysregulation is associated with 3-fold increase likelihood of breast cancer returning [7, 8]. Accordingly, exercise training is associated with reduced risk of breast cancer and breast cancer recurrence [43-46]. Thus, exercise, diet, or other medical intervention to prevent or control insulin resistance should likely be considered as standard breast cancer care.

Conclusion. In conclusion, breast cancer conditioned media significantly impaired the insulin responsiveness in L6 skeletal muscle cells. Thus, our study provide evidence to suggest that breast cancer cells can directly induce skeletal muscle insulin resistance. Given skeletal muscles key role in metabolic regulation, breast cancer-induced skeletal muscle insulin resistance might be an underlying cause of metabolic dysregulation observed in the majority of breast cancer patients and survivors.

Contributions. M.S.A and L.S. conceptualized and designed the study. M.S.A conducted the experiments, performed the laboratory analysis, and analyzed the data. M.S.A and L.S. wrote the

manuscript. M.J., J.L and X.H. all took part in conducting the experiments, performing laboratory analysis and/or interpreting the data. All authors commented on and approved the final version of the manuscript. All authors are guarantor of this work and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends:

Figure 1: Reduced GLUT4 translocation in L6 myotubes incubated with MCF7 or BT474 conditioned media. A) Insulin-stimulated (100nM, 10 minutes stimulation) GLUT4 translocation in L6 myotubes incubated with 25% conditioned media (CM) from MCF7 breast cancer cells for 72 hrs. n = 6. **B)** Insulin/basal fold change, indicating insulin response in L6 myotubes after 72hr incubation with MCF7. **C)** Insulin-stimulated (100nM, 10 minutes stimulation) GLUT4 translocation in L6 myotubes incubated with 25% CM from BT474 breast cancer cells for 72 hrs. n = 6. **D)** Insulin/basal fold change, indicating insulin response in L6 myotubes after 72hr incubation with BT474. In all groups there is a significant effect of insulin ($p<0.001$). Effect of MCF7 or BT474 cell CM: */***: $p<0.05$/$p<0.001$, Interaction between CM and insulin: ## $p<0.01$. Data are presented as mean ± SEM incl. individual values.

Figure 2: Blunted insulin-stimulated glucose uptake in L6 myotubes incubated with MCF7 or BT474 conditioned media. Basal (n=3) 2-deoxyglucose (2DG) uptake in L6 myotubes incubated with 25% conditioned media (CM) from **A)** MCF7 and **B)** BT474 breast cancer for 72 hrs. Insulin-stimulated (100 nM, 25 minutes stimulation) 2DG uptake in L6 myotubes incubated with 25% conditioned media (CM) from **C)** MCF7 and **D)** BT474 breast cancer for 72 hrs (n=3-5). Effect of CM on L6 myotubes insulin-stimulated 2DG uptake is indicated as # $p<0.05$. In all groups there is a significant effect of insulin ($p<0.001$). Results are shown from 2 independent experiments. Data are presented as mean ± SEM incl. individual values.

Figure 3: MCF7 cell CM altered intracellular insulin signaling in myotubes. Quantifications of basal- or insulin (100nM, 25 minutes stimulation) -stimulated **A)** p-Akt^{Thr308}, **B)** p-Akt^{Ser473}, **C)** p-TBC1D4^{Thr642} in L6 myotubes incubated with 25% conditioned media (CM) from MCF7 breast cancer cells for 72 hrs. Quantifications of basal- or insulin-stimulated **D)** p-Akt^{Thr308}, **E)** p-Akt^{Ser473}, **F)** p-TBC1D4^{Thr642} in L6 myotubes incubated with 25% CM from BT474 breast cancer cells for 72 hrs. Quantification of Akt2 and TBC1D4 in L6 myotubes incubated with 25% CM from **G)** MCF7 or **H)** BT474 breast cancer cells for 72 hr. **I)** Representative western blots. Data are presented as mean ± SEM incl. individual values.

Figure 1

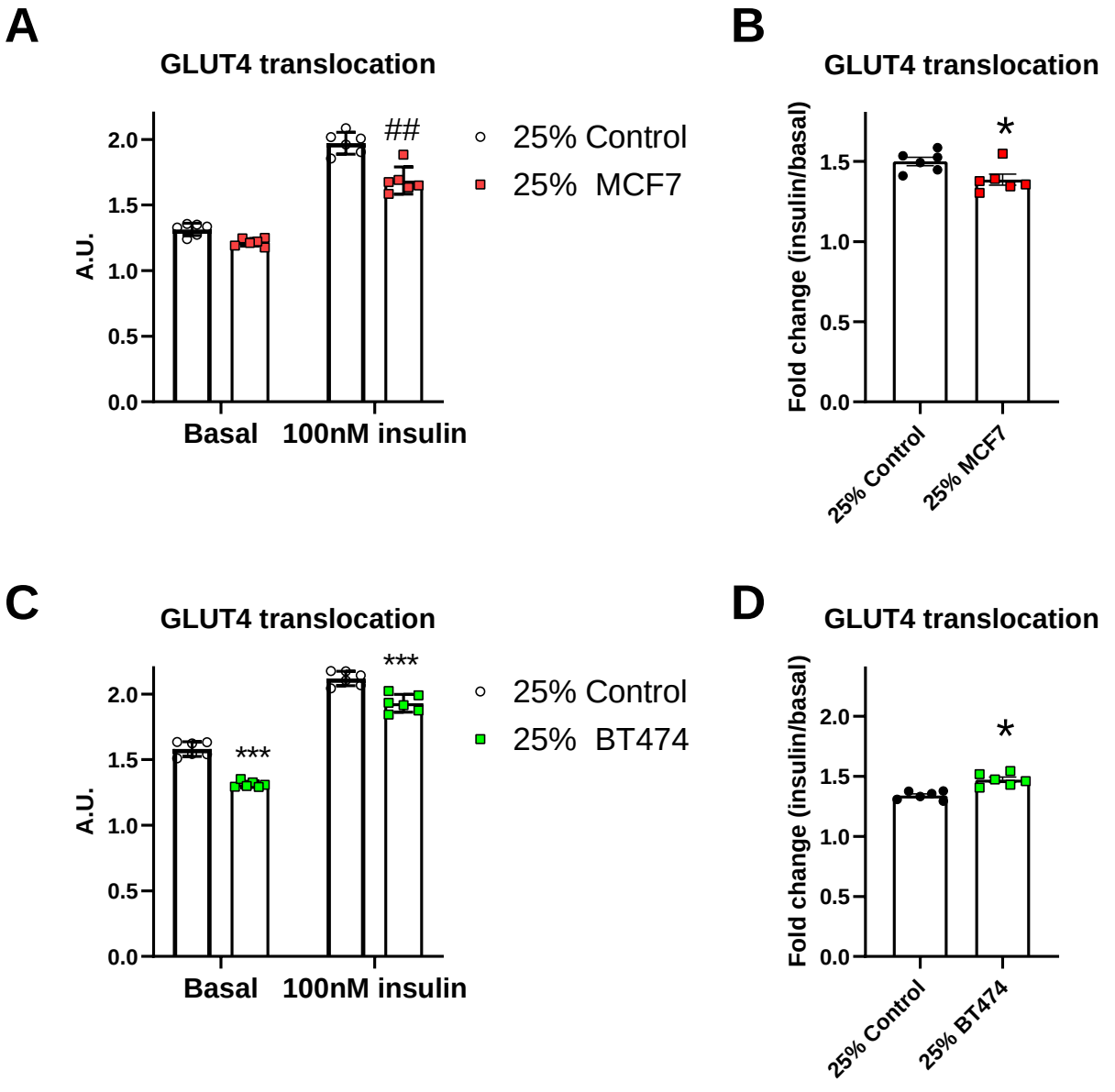

A
GLUT4 translocation
A.U.
2.0
1.5
1.0
0.5
0.0
##
Basal
100nM insulin
25% Control
25% MCF7
B
GLUT4 translocation
Fold change (insulin/basal)
1.5
1.0
0.5
0.0
*
25% Control
25% MCF7
C
GLUT4 translocation
A.U.
2.0
1.5
1.0
0.5
0.0
***
***
Basal
100nM insulin
25% Control
25% BT474
D
GLUT4 translocation
Fold change (insulin/basal)
2.0
1.5
1.0
0.5
0.0
*
25% Control
25% BT474

Figure 2

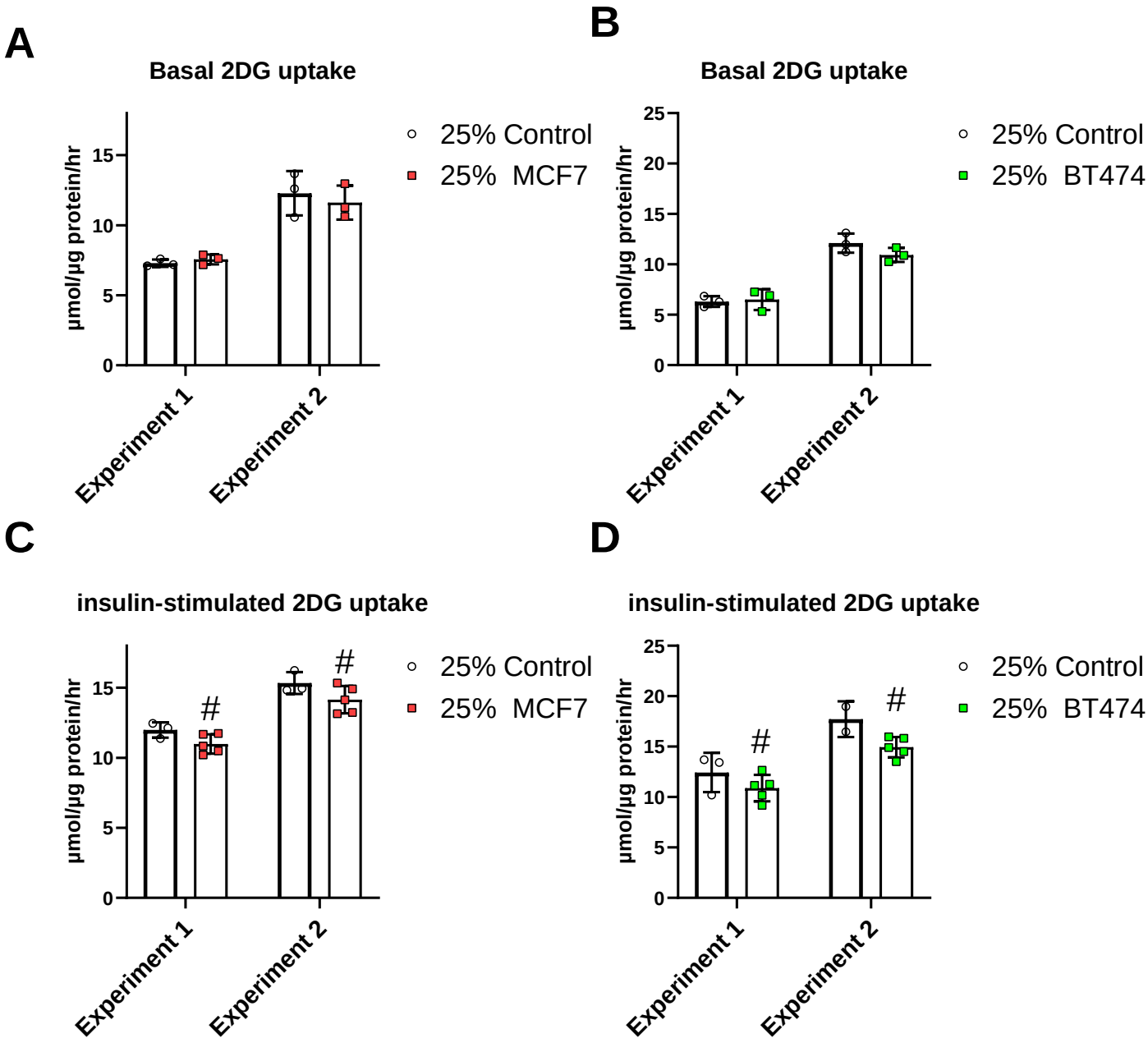
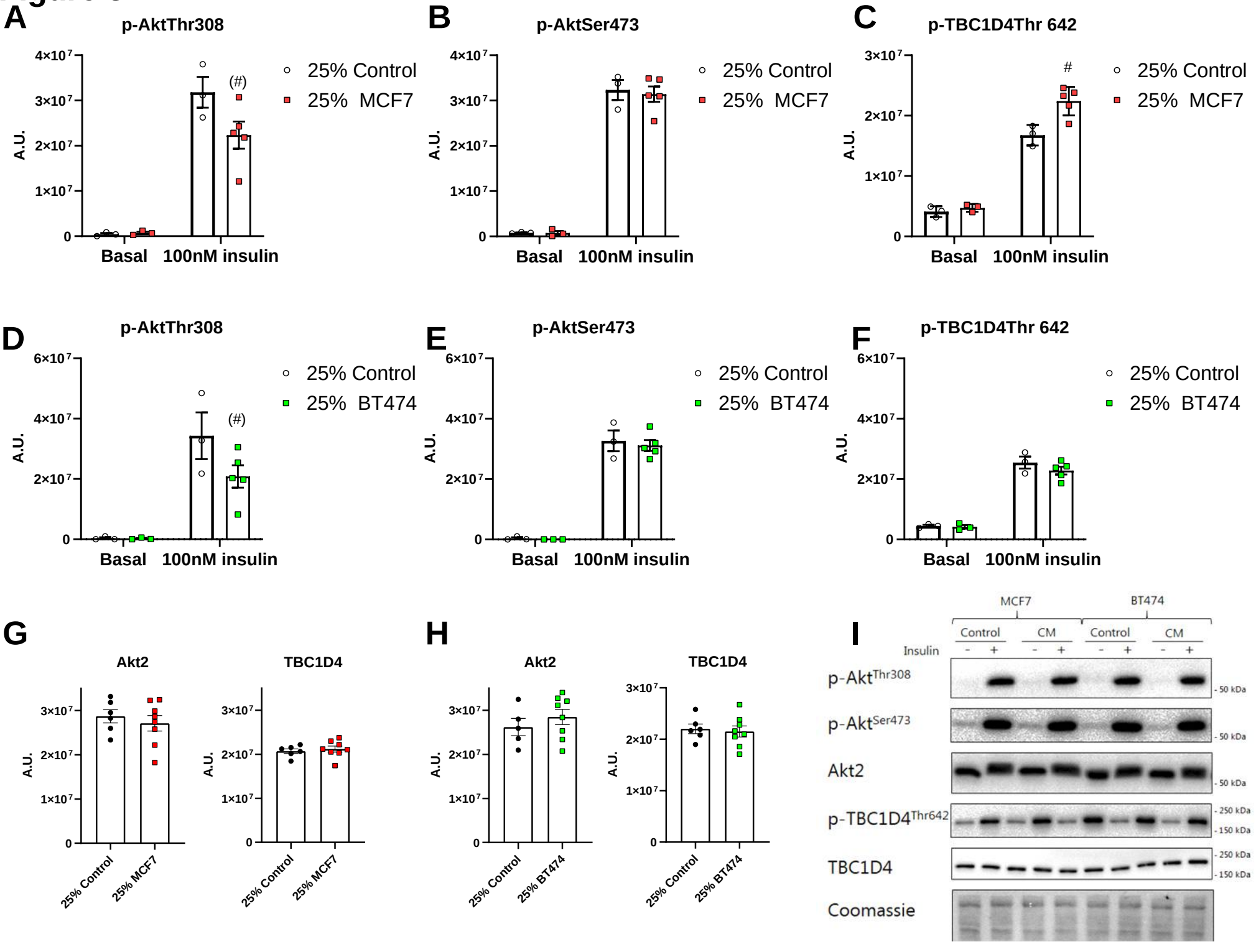

A
Basal 2DG uptake
µmol/µg protein/hr
0
5
10
15
Experiment 1
Experiment 2
25% Control
25% MCF7
B
Basal 2DG uptake
µmol/µg protein/hr
0
5
10
15
20
25
Experiment 1
Experiment 2
25% Control
25% BT474
C
insulin-stimulated 2DG uptake
µmol/µg protein/hr
0
5
10
15
#
#
Experiment 1
Experiment 2
25% Control
25% MCF7
D
insulin-stimulated 2DG uptake
µmol/µg protein/hr
0
5
10
15
20
25
#
#
Experiment 1
Experiment 2
25% Control
25% BT474

Figure 3


A
p-AktThr308
4×10⁷
3×10⁷
2×10⁷
1×10⁷
0
A.U.
(#)
25% Control
25% MCF7
Basal
100nM insulin
B
p-AktSer473
C
p-TBC1D4Thr 642
#
D
p-AktThr308
25% BT474
E
p-AktSer473
F
p-TBC1D4Thr 642
6×10⁷
G
Akt2
TBC1D4
25% Control
25% MCF7
H
25% BT474
I
MCF7
BT474
Control
CM
Insulin
p-Akt Thr308
p-Akt Ser473
Akt2
p-TBC1D4 Thr642
TBC1D4
Coomassie
50 kDa
250 kDa
150 kDa