DOCTORAL PROGRAM IN METABOLISM – CLINIC AND EXPERIMENTAL

2nd YEAR PROGRESS REPORT

UNDERSTANDING THE MECHANISMS OF WARBURG EFFECT INVERSION IN BREAST CANCER UNDER OBESITY CONDITIONS

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INTRODUCTION

Obesity has been receiving exponential attention in the scientific community as a metabolic disease. Adipose tissue is found throughout the body and is considered an endocrine organ capable of producing a wide range of metabolites [1]. Adipose tissue location is decisive in pathological conditions. The accumulation of visceral adipose tissue is associated with increase inflammation and oxidative stress leading to metabolic disturbances. This condition characterizes multiple disease states including metabolic syndrome, diabetes and cancer [2].

Breast cancer is strongly associated with obesity [3]. It is a complex and heterogeneous disease, the most common among women, with high prevalence and mortality rates [4]. The mechanisms linking obesity (and the metabolic disorders associated) and breast cancer is far from being fully understood. The present work intends to unravel the alterations of the central biochemical metabolism in breast cancer under obesity conditions.

Otto Warburg described that tumor cells had certain metabolic characteristics in common: high glucose consumption, low oxygen consumption and high lactate production [5]. Although not fully understood, the Warburg effect is a conceptual principle in clinical application both in diagnosis by detection of radioactive glucose[6] and in therapy [7].

In the last decade, the Warburg effect has been extensively studied and questioned [8]. More and more variations are observed, not only in the exclusive use of aerobic glycolysis but also in alternative and compensatory mechanisms by tumor cells. More recently, it was found that fibroblasts in the tumor microenvironment are able to consume lactate and pyruvate, producing glucose, which in turn is taken up by tumor cells, thus promoting the efficient production of essential energy to meet the proliferative need of these cells [9]. This self-sustainable “Cori cycle” between the microenvironment stroma and the tumor cell was called the “Reverse Warburg Effect”.

In 2019, our group studied the metabolic and hormonal profile of breast tumor cells (MCF-7) under conditions simulating normoglycemia, hyperglycemia and obesity [10]. In this study we observed that tumor cells under obesity conditions consumed lactate giving rise to pyruvate, which leads to the accumulation of glucose. We also observed that, under adipocyte conditioned medium exposure; MCF-7 cells have at their disposal a greater amount of fatty acids that can be oxidized to acetyl-CoA, sustaining an oxidative metabolic pattern as opposed to the anaerobic pattern advocated by the Warburg effect in normoxia. This condition was thus called “Warburg effect inversion”. Additionally, our results demonstrate that this inversion of tumor cell metabolism is concomitant with tumor aggressiveness, as there is a significant increase in tumor cell viability, proliferation and motility.

The understanding of tumor cell metabolism in obese conditions is essential to direct the diagnosis and therapy in obese patients in a personalized way, allowing a more accurate diagnosis and increased treatment effectiveness.

The proposed theme of my thesis is to better understand the Warburg effect inversion and so, decipher the link between adiposity metabolic dysfunction and metabolic plasticity of breast cancer.

AIMS:

1. Identify alterations of glycolytic enzymes expression in obesity in *in vitro* studies;

2. Confirm the glycolytic enzymes expression patterns in human breast cancer tissues from normal-weight and obese patients;

3. Develop a nanobiosensor to monitor possible therapeutic targets in altered enzymes;

4. Validate (through the nanobiosensor) the mechanism in different tumor cell lines;

5. Study the prognostic factors of obese *vs* normal-weight patients.

METHODOLOGY:

The present report describes the methodology done so far in my 2nd year of the doctoral degree for aim 1 (*Identify alterations of glycolytic enzymes expression in obesity in in vitro studies*) and aim 2 (*Confirm the glycolytic enzymes expression patterns in human breast cancer tissues from normal-weight and obese patients*).

The experiments regarding aims 3, 4, and 5 have not yet been carried out.

1. Identify alterations of glycolytic enzymes expression in obesity in *in vitro* studies

The previous results from our group observed the Warburg effect inversion on MCF-7 cell line using the conditioned medium from mouse fibroblasts (3T3-L1) differentiated into adipocytes [10]. In order to achieve more reliable and comparative results, human samples of different origins were used to mimic the obesity condition: blood serum of obese women, secretome of adipose tissue explants (visceral and subcutaneous) and secretome of the differentiation of fibroblasts - preadipocytes isolated from adipose tissue (visceral and subcutaneous) into adipocytes. To compare the results reproducibility, a viability assay was performed alongside with the quantification of LDH activity, glucose and lactate.

1.1. Serum samples

Blood samples of obese women were collected and subsequently centrifuged at 3500 rpm for 10 minutes. Supernatant (serum) was store in the freezer (-20ºC) and defrost for each experiment.

1.2. Adipose tissue explants

Both visceral (VAT) and subcutaneous (SCAT) adipose tissue were collected in the Center for Integrated Responsibility (CRI) of Obesity at the *Centro Hospitalar Universitário de São João* (CHUSJ) and processed independently. Samples were cleaned of blood vessels and connective tissue in PBS with 1% penicillin/streptomycin. Adipose tissue was then dissected into 1-2 cm pieces and weighted. Explants were distributed in 10 cm diameter cell culture Petri dish with different concentration of tissue/medium volume and different incubation periods.

1.3. Adipocyte differentiation

VAT and SCAT were mechanically degraded, and digested using collagenase at 37 °C for 1h30. The stromal vascular fraction (SVF) was washed and filtered to obtain isolated preadipocytes, which were cultured at a 5% CO2 atmosphere at 37 °C on Dulbecco's Modified Eagle Medium/ Nutrient Mixture F12 supplemented with 17.5 mM of glucose, penicillin/streptomycin 1% (v/v), 18 µmM of pantothenic acid, 100 µM of ascorbic acid, 16 µM of biotin and 10% (v/v) and newborn calf serum (NCS). Once in confluence, preadipocytes were differentiated into adipocytes. For this purpose, cells were incubated for 3 days with DMEM/F12 medium supplemented with 17.5 mM of glucose penicillin/streptomycin 1% (v/v), 18 µM of pantothenic acid, 100 µM of ascorbic acid, 16 µM of biotin, 3% (v/v) NCS, 10 µg/mL of insulin, 0.1 µM of dexamethasone, 0.5 mM of 3-isobutyl-methyl-xanthine (IBMX) and 1 µM of rosiglitazone. After this time, cells were incubated with DMEM/F12 medium supplemented with 17.5 mM of glucose penicillin/streptomycin 1% (v/v), 18 µM of pantothenic acid, 100 µM of ascorbic acid, 16 µM of biotin, 3% (v/v) NCS, 10 µg/mL of insulin, 0.1 µM of dexamethasone until the fully differentiated phenotype was reached.

1.4. Cell culture

Human breast carcinoma cells MCF-7 were cultured in DMEM supplemented with 10% Fetal Bovine Serum, 1% penicillin/streptomycin and 3.7 g/L sodium bicarbonate and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were used between passages 20 up to 66. Treatments were performed using DMEM without supplementation as control.

1.5. Viability assay

Breast cancer cell line MCF-7 was cultured in a concentration of 1 x 10^5 cells/ mL and subjected to the different treatments for 24h. Then, culture medium was collected and replaced by PBS and 20 mL of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide] reagent, followed by 2h incubation period. The background absorbance of the medium was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was calculated. The results are given as mean ± SD and are expressed as percentage of control, which was considered to be 100%.

1.6. LDH activity

MCF-7 cells (1 × 10^5 cells/mL) were cultured for 24h with standard treatments. The analysis was performed using the extracellular medium, by examination of the rate of NADH depletion at 340 nm using the Multiskan Ascent spectrophotometer with a Liquick Cor-LDH 30 kit (Cormay, Łomianki, Poland), according to the manufacturer's instructions.

1.7. Glucose and lactate quantification

The medium collected from MCF-7 cells cultured with standard treatments for 24 h was analyzed by the Department of Clinical Pathology at CHUSJ. Analyses of glucose and lactate were determined using an AU5800 Series Clinical Chemistry Analyzers of Beckman Coulter, according to the equipment instructions.

2. Glycolytic enzymes expression patterns in human breast cancer tissues from normal-weight and obese patients;

Human breast cancer tissue was obtained at IPO-Porto, from a previous selection performed by the "Outcomes Research Lab". Fixation and sample processing was performed by IPO-Porto technicians at the Pathology department.

2.1. Hematoxylin and Eosin staining

Paraffin-embedded tissues sections (3μm thick) were deparaffinized and hydrated. Sections were then allowed to react with Hematoxylin. After a brief exposure to ethanol, sections were counterstained with eosin. Afterwards, sections were dehydrated and mounted with Entellan. All stained specimens were visualized under Nikon Eclipse 501.

2.2. Immunohistochemistry assays

Paraffin-embedded tissues sections (3μm thick) were deparaffinized and hydrated. Endogenous peroxidase activity was blocked through incubation with 3% hydrogen peroxide in Phosphate Buffered Saline (PBS). Heat antigenic recovery was used using 10 mM sodium citrate, 0.05% Tween 20, pH = 6, at 98ºC for 10 min. Nonspecific binding was blocked with incubation with 10% human serum and 4% bovine serum albumin (BSA) in PBS. Primary antibody incubation was performed overnight and subsequent incubation with secondary antibody for 30 min (please see table 1 for more information). After secondary antibody, immunoreactivity was visualized with Avidin-Biotin reaction and revelation was performed using DAB (3,3-diaminobenzidine) HRP substrate. All stained specimens were visualized under Nikon Eclipse 501.

Table 1 – Antibody information regarding supplier, reference, dilution, primary and secondary antibody pair and positive control for the immunohistochemistry assays performed. Negative control was performed replacing primary antibody by blocking solution.

Enzyme	Primary antibody			Secondary antibody				Positive control
	Supplier	Reference	Dilution	Description	Supplier	Reference	Dilution	
HK II	Santacruz	sc-374091	1:50	Goat anti mouse (b)	abcam	ab97021	1:600	Human skeletal muscle
PFK L	Santacruz	sc-393713	1:400	Goat anti mouse (b)	abcam	ab97021	1:600	Human liver

Ethical approval

Visceral and subcutaneous adipose tissue was obtained from gastric bypass surgery performed at the CRI of Obesity at CHUSJ, Porto, Portugal. The ethical request for authorization to carry out this research was submitted and approved by the CHUSJ Ethics Committee.

Biological samples of breast tumor tissue preserved in paraffin were obtained at the Francisco Gentil Portuguese Institute of Oncology of Porto, E. P. E. previously approved by the respective ethics committee.

The study is and will be carried out in accordance with international recommendations for Good Clinical Practice, the Declaration of Helsinki, and applicable international and national legislation. All participants subscribe to an informed and free consent.

RESULTS:

1. Identify alterations of glycolytic enzymes expression in obesity in *in vitro* studies (ongoing)

To validate the reproducibility of previous results regarding the Warburg Effect Inversion, viability assays in MCF7 cells were performed and the quantification of the LDH activity and of the glucose and lactate was obtained to assess the glycolysis and gluconeogenesis rate.

1.1. Serum samples

The results obtained by exposing cells to different concentrations of obese serum (5%, 10%, 15% and 20%) did not reproduce the results obtained in the Warburg effect Inversion.

There were no significant differences in the viability and LDH activity of the MCF-7 cell after incubation with obese serum when compared to control, cells left untreated (Figure 1). Also, there was no evident switch of the glycolytic metabolism to gluconeogenesis (Table 2).

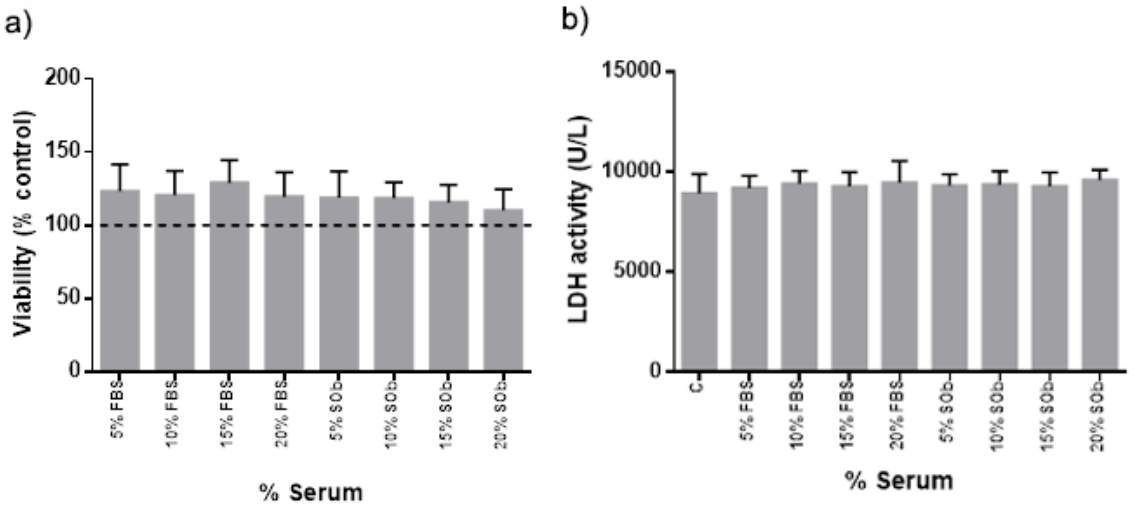


Figure 1 – Results of a) Viability using MTT assay and b) LDH activity; Results were normalized to the control (exposure to medium without serum). C – Control; FBS – Fetal Bovine Serum; Sob – Obese Serum.

Table 2 – Glucose and lactate quantification upon exposure of MCF-7 to obese serum. Quantification was calculated by the difference between glucose/lactate before and after the 24h treatments (T24-T0).

Condition	GLUCOSE (mmol/L)			LACTATO (mg/dL)		
	Hours		DIF.	Hours		DIF.
	0	24		0	24	
Obese serum 5%	479	480	**1**	0,11	0,69	**0,58**
Obese serum 10%	479	451	**-28**	0,21	3,99	**3,78**
Obese serum 20%	450	418	**-32**	0,44	4,38	**3,94**
FBS 10%	469	444	**-25**	1,36	4,69	**3,33**
FBS 20%	419	396	**-23**	2,72	6,25	**3,53**

1.2. Adipose tissue explants

To evaluate the best conditions to mimic obesity, incubation period and concentration of adipose tissue were adjusted. Figure 2 displays the configuration of petri dish with explants of subcutaneous and visceral adipose tissue.

Figure 2 – Image representative of the explant incubation on petri dishes.

Incubation period included 24, 48 and 72 hours of explant exposure to culture medium and concentrations from 0.2 to 0.4 g explant/ml of culture medium in 100% and in 50%. The secretome was collected and used in MCF-7 cell treatments. Contrasting with the results of obese serum, the following conditions of this experiment achieved significant differences in the viability of MCF-7 cells (Figure 3):

- Explant 1 (24h incubation) of VAT at 0,20 g/ml (100%);
- Explant 2 (48h incubation) of VAT at 0,25 g/ml (100% and 50%);
- Explant 2 (48h incubation) of SCAT at 0,30 g/ml (100% and 50%);
- Explant 3 (72h incubation) of VAT at 0,30 g/ml (100%);
- Explant 3 (72h incubation) of SCAT at 0,40 g/ml (100%);

Glucose consumption/ lactate release was proportional to the proliferation of MCF-7 cells (Table 3). None of the conditions showed the Warburg effect Inversion.

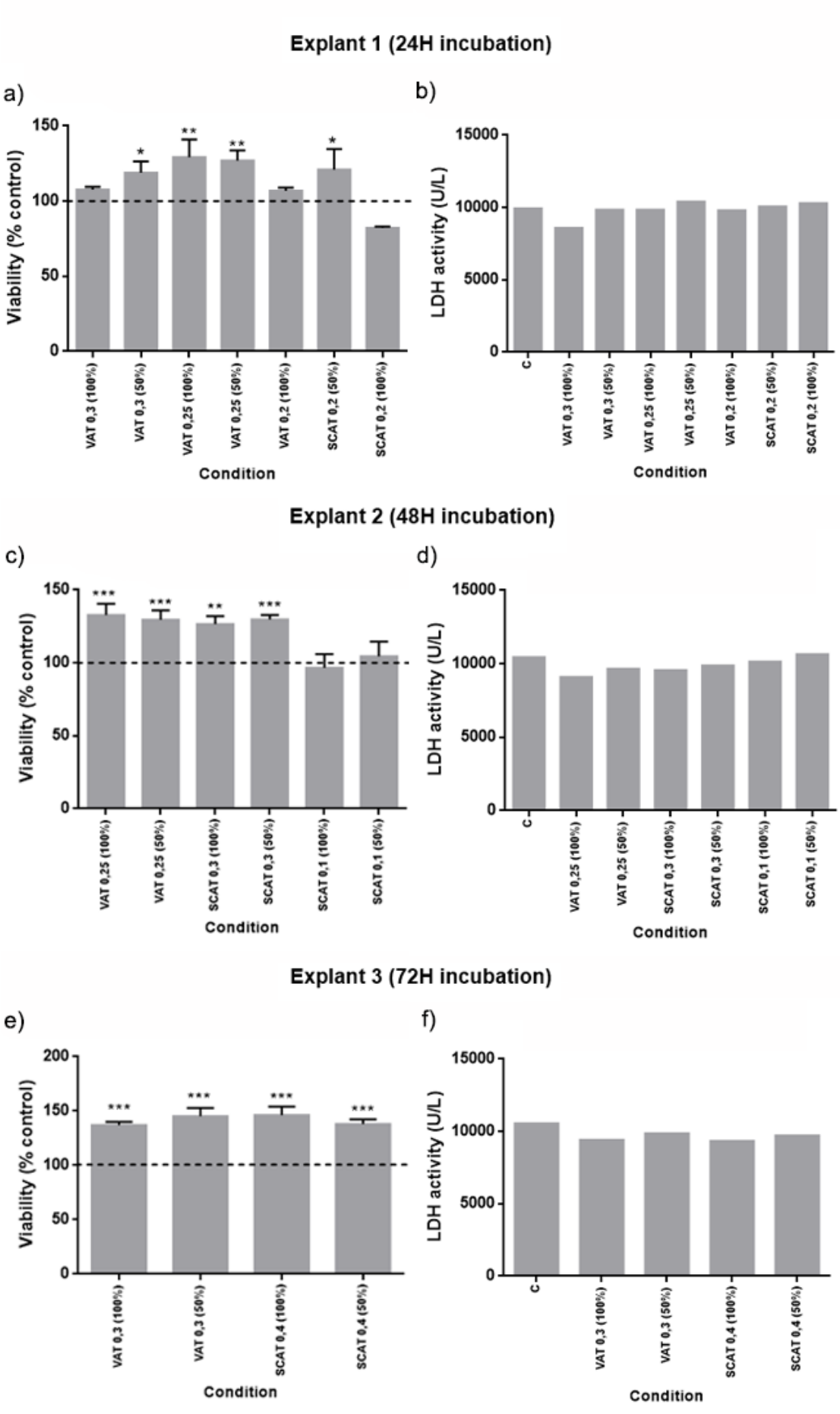

Explant 1 (24H incubation)
a)
b)
Viability (% control)
150
100
50
0
*
**
**
*
VAT 0,3 (100%)
VAT 0,3 (50%)
VAT 0,25 (100%)
VAT 0,25 (50%)
VAT 0,2 (100%)
SCAT 0,2 (50%)
SCAT 0,2 (100%)
Condition
LDH activity (U/L)
15000
10000
5000
0
C-
VAT 0,3 (100%)
VAT 0,3 (50%)
VAT 0,25 (100%)
VAT 0,25 (50%)
VAT 0,2 (100%)
SCAT 0,2 (50%)
SCAT 0,2 (100%)
Condition
Explant 2 (48H incubation)
c)
d)
Viability (% control)
150
100
50
0
***
***
**
***
VAT 0,25 (100%)
VAT 0,25 (50%)
SCAT 0,3 (100%)
SCAT 0,3 (50%)
SCAT 0,1 (100%)
SCAT 0,1 (50%)
Condition
LDH activity (U/L)
15000
10000
5000
0
C-
VAT 0,25 (100%)
VAT 0,25 (50%)
SCAT 0,3 (100%)
SCAT 0,3 (50%)
SCAT 0,1 (100%)
SCAT 0,1 (50%)
Condition
Explant 3 (72H incubation)
e)
f)
Viability (% control)
200
150
100
50
0
***
***
***
***
VAT 0,3 (100%)
VAT 0,3 (50%)
SCAT 0,4 (100%)
SCAT 0,4 (50%)
Condition
LDH activity (U/L)
15000
10000
5000
0
C-
VAT 0,3 (100%)
VAT 0,3 (50%)
SCAT 0,4 (100%)
SCAT 0,4 (50%)
Condition

Figure 3 – Viability and LDH activity in explants of adipose tissue exposed to culture medium in different incubation periods (24, 48 and 72 hours) and different concentration.

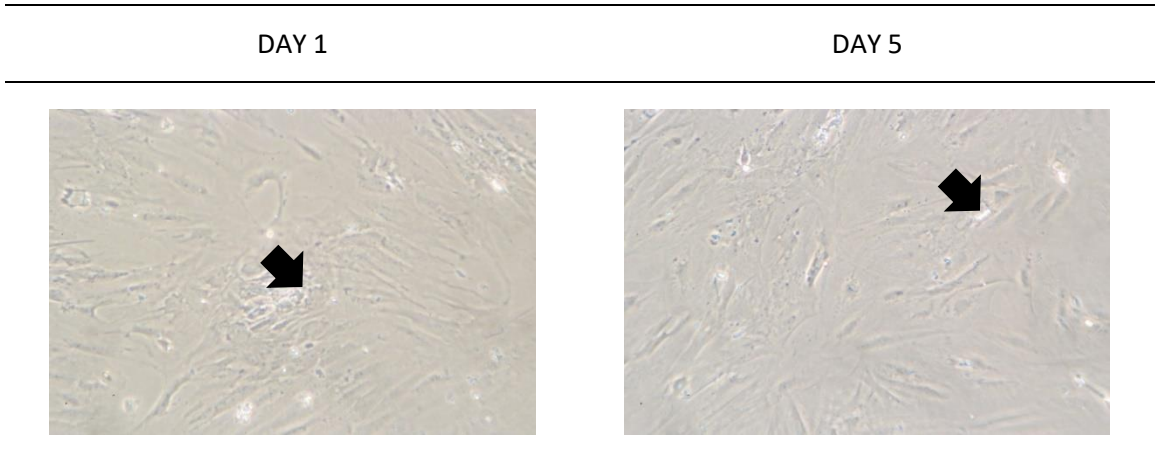
Table 3 – Glucose and lactate quantification in MCF-7 exposed to explant secretome. Quantification was calculated by the difference between glucose/lactate before and after the 24h treatments (T24-T0).

Explant	AT	[CONC.] (g/mL)	%	GLUCOSE (mmol/L)			LACTATE (mg/dL)		
				Hours		DIF.	Hours		DIF.
				0	24		0	24	
1	C	-	-	506	504	**-2**	0	1,81	**1,81**
1	VAT	0,2	100%	310	295	**-15**	1,66	4,62	**2,96**
2	C	-	-	506	499	**-7**	0	2,2	**2,2**
2	VAT	0,25	100%	513	478	**-35**	12,69	16,93	**4,24**
2	VAT	0,25	50%	494	473	**-21**	6,2	9,73	**3,53**
2	SCAT	0,3	100%	523	504	**-19**	11,63	15,59	**3,96**
2	SCAT	0,3	50%	513	482	**-31**	5,81	8,68	**2,87**
3	C	-	-	498	491	**-7**	0	2,76	**2,76**
3	VAT	0,3	100%	404	372	**-32**	21,87	25,77	**3,9**
3	SCAT	0,4	100%	561	526	**-35**	9,02	12,81	**3,79**

1.3. Adipocyte differentiation

Adipocytes were then differentiated from subcutaneous adipose tissue. We were unable to differentiate visceral adipocytes, due to loss of visceral preadipocytes.

Visualization of subcutaneous adipocytes displayed morphological alterations with accumulation of lipids (Figure 4) in 10 days. Differentiation was observed in approximately 30% of the subcutaneous adipocytes. Adipocytes were maintained and secretome was collected for MCF-7 cell assays.



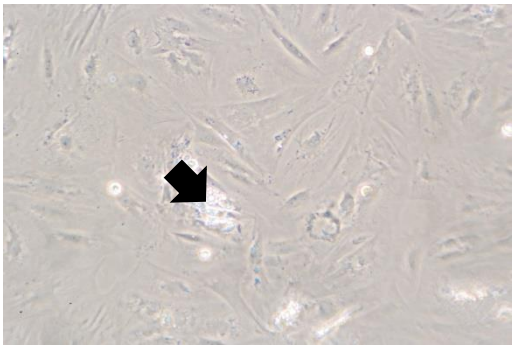
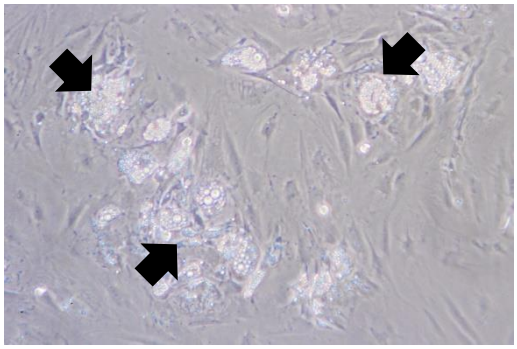
DAY 7	DAY 10
	

Figure 4 – Accumulation of lipids in adipocytes (arrows) in subcutaneous adipose tissue differentiation (Magnification 100x).

MCF-7 viability increased in all conditions (prominent in 50% and 30% secretome diluted in DMEM high glucose without FBS) (Figure 5). Of notice the lack of significant differences in viability in MCF-7 exposed to adipocyte medium (DMEM/F12) when compared to control (data not shown).

Although results in viability were concordant with our previous results of the Warburg effect inversion, it was not observed the expected switch in central biochemistry metabolism of aerobic glycolysis (Table 4).

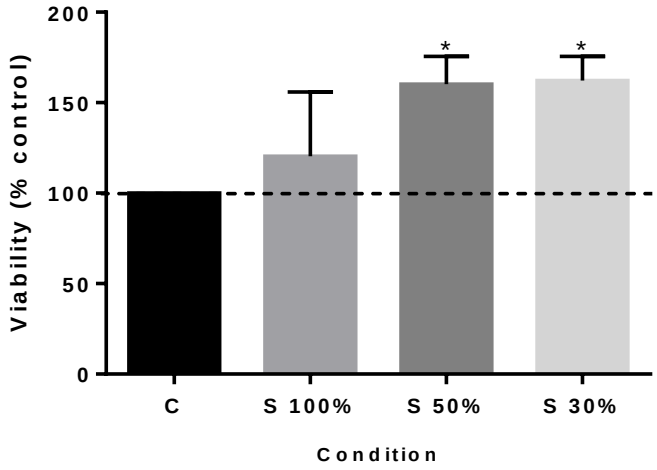


Figure 5 – Increase viability in MCF-7 cell culture when subjected to secretome (S) of adipocyte differentiation 50% and 30% diluted in DMEM High Glucose.

Table 4 - Results of glucose and lactate quantification for exposure of MCF-7 to adipocyte secretome. Quantification was calculated by the difference between glucose/lactate before and after the 24h treatments (T24-T0).

Condition	Glucose (mmol/L)			Lactate (mg/dL)		
	T=0	T=24	Difference	T=0	T=24	Difference
Control	531	515	-16	0	1,93	1,93
1st assay						
S 100%	309	272	-37	1,05	4,29	3,24
S 50%	412	385	-27	0,48	4,16	3,68
S 30%	459	445	-14	0,29	4,17	3,88
2nd assay						
S 100%	307	276	-31	1,07	4,26	3,19
S 50%	407	379	-28	0,51	4,26	3,75
S 30%	448	435	-13	0,29	4,3	4,01
3rd assay						
S 100%	317	288	-29	1,2	4,3	3,1
S 50%	413	377	-36	0,6	4,79	4,19
S 30%	458	427	-31	0,34	4,33	3,99

2. Glycolytic enzymes expression patterns in human breast cancer tissues from normal-weight and obese patients (ongoing);

Sixty-two samples were already included in the study. All samples chosen were invasive ductal carcinomas, the most prevalent type of breast cancer.

Figure 6 displays representative images of Hematoxylin and Eosin staining, a qualitative study of these results is still on progress. Figure 7 and 8 displays representative images of immunohistochemistry of PFL-L and optimization of HK-II, respectively. All images were divided accordingly to molecular classification and BMI. At the moment, immunohistochemistry of Phosphofructokinase isoform L (PFK) is almost finished and will then be quantified by a pathologist. Immunohistochemistry optimization for Hexokinase isoform II (HK-II) is still an ongoing work.

PFK-L and HK-II among the other contemplated enzymes will be quantified and statistically correlated with the following information:

- Diagnostic age;
- BMI;
- Molecular classification;
- Topographic localization;
- Laterality;
- Behavior;
- Pathological Stage;
- *In situ* carcinoma: Size, Extension, Necrosis and micro calcifications;
- Invasive carcinoma: Size, Multifocality, Duct formation, nuclear grade and Mitosis;
- Lymph nodes: Sentinel Nodes, Metastasis nodes and Metastasis Size.

2.1. HE

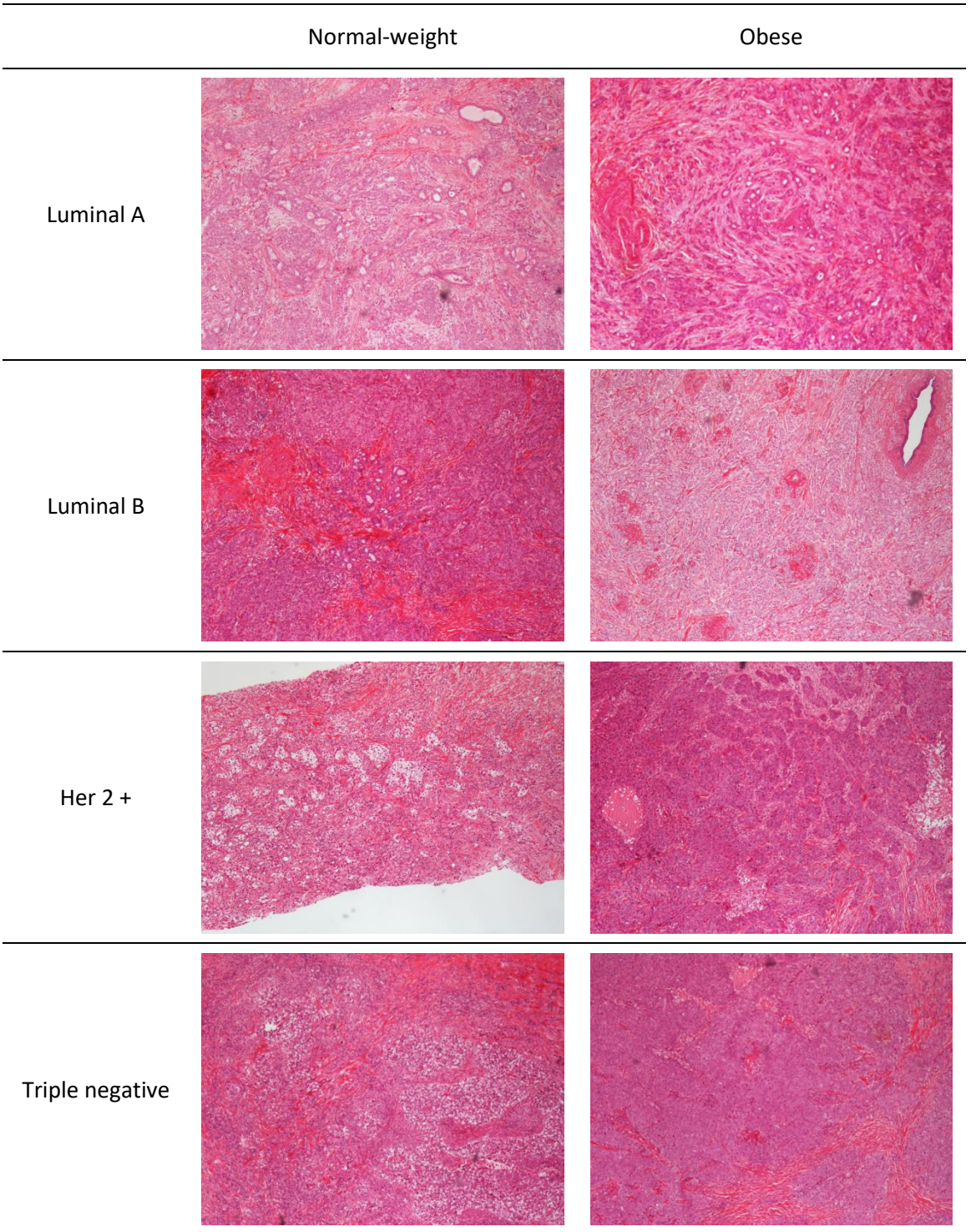


Figure 6 – Representative images of Hematoxylin and Eosin staining in breast cancer tissue divided by molecular classification and BMI (Magnification 100x).

2.2 PFK-L

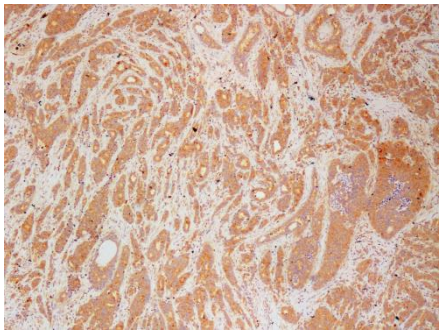
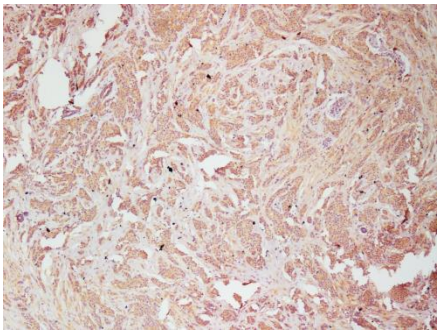
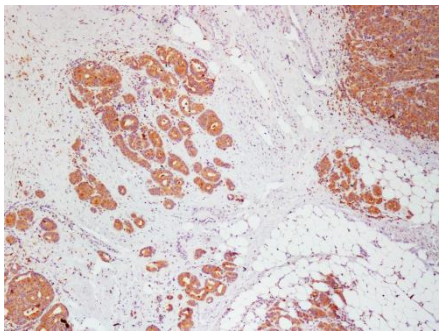
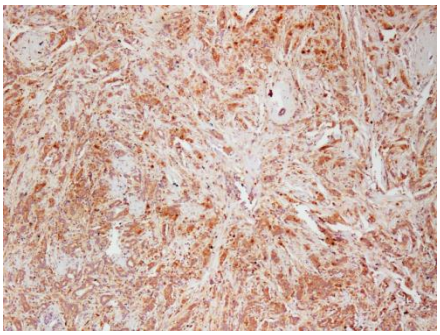
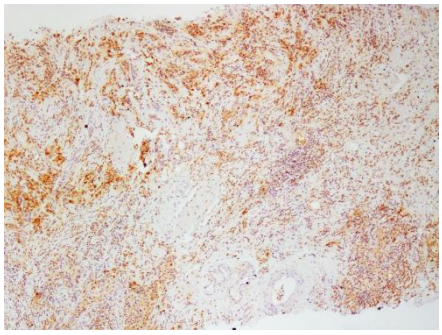
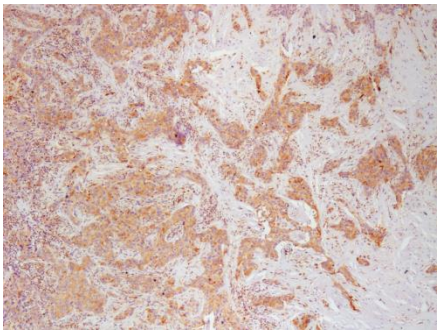
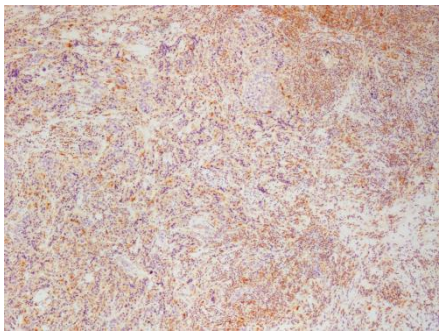
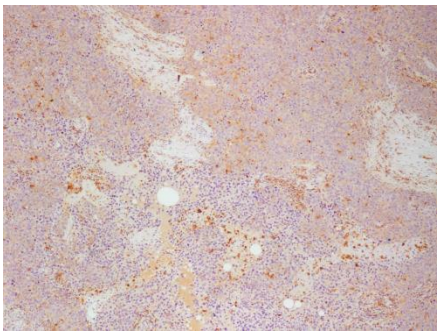
	Normal-weight	Obese
Luminal A		
Luminal B		
Her 2 +		
Triple negative		

Figure 7 – Representative images of immunohistochemistry of Phosphofructokinase (isoform L) divided by molecular classification and BMI (Magnification 100x).

2.3 HK-II

IHQ for HK-II is under development with positive results at the moment. Skeletal muscle was already obtained in Pathological anatomy department from CHUSJ, will be used as positive controls.

Negative control	Positive staining 1	Positive staining 2

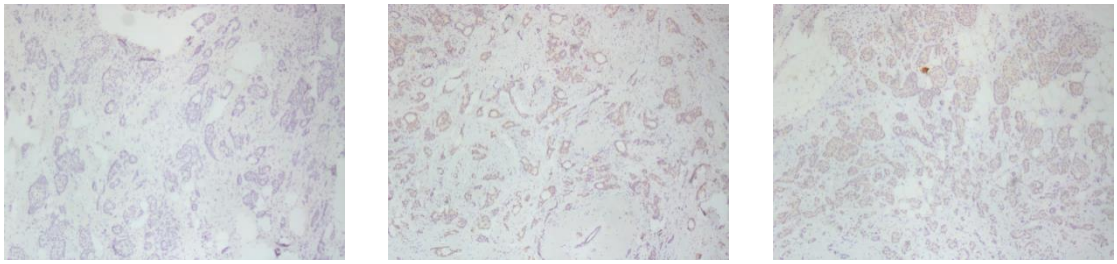


Figure 8 – Representative images of the immunohistochemistry optimization for HK-II (Magnification 100x).

CONCLUSIONS:

Regarding aim 1 (*Identify alterations of glycolytic enzymes expression in obesity in in vitro studies*), the main difficulty encountered so far is the reproducibility of the previous results of mimicking obesity conditions. This problem has delayed the consequent work, which is dependent on these results. So far, we conclude that the best sample to mimic obesity is adipocyte differentiation. I believe that the expected results can be achieved either with visceral adipocytes secretome or with a higher differentiation rate. Therefore, the deadline for this task must be extended by a period of 24 weeks. Due to this delay, aim 3 and 4 must also be postponed accordingly.

In the matter of aim 2 (*Confirm the glycolytic enzymes expression patterns in human breast cancer tis sues from normal-weight and obese patients*), the work is progressing according to plan. Immunohistochemistry for PFK-L is practically finished and HK-II protocol is almost ready to start the assay.

Moreover, regarding aim 5 (*Study the prognostic factors of obese vs normal-weight patients*), we propose to perform a retrospective study regarding oncological obese patients. It is also an ongoing work with a thoughtful discussion to assess the variables that could be relevant to this analysis. This issue is being addressed with both supervisors and the clinicians at IPO, Porto.

RESEARCH DEVIATIONS FROM THE INITIAL PROPOSAL:

The initial proposal aim 2 was to quantify only the enzymes identified in aim 1. Since these were not able to identify, the plan changed to quantify/ analyze exclusively the enzyme expression implicated in glycolysis and gluconeogenesis.

The main alteration observed so far is essentially related to the deadlines that in some cases must be extended (please see UPDATED CHRONOGRAM).

REFERENCES (MAX 10):

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OUTPUTS:

Nothing to report.

UPDATED CHRONOGRAM:

TASKS/ AIMS	INITIAL CHRONOGRAM	UPDATED CHRONOGRAM
1. Identify alterations of glycolytic enzymes expression in obesity in in vitro studies;	Adipocyte differentiation: 01/09/2020 + 6 weeks MCF-7 cell assays: 12/10/2020 + 24 weeks	Adipocyte differentiation: 01/09/2020 – 31/12/2021 MCF-7 cell assays: 01/01/2022 – 31/3/2021
2. Confirm the glycolytic enzymes expression patterns in human breast cancer tissues from normal-weight and obese patients;	01/05/2021 + 24 weeks	01/05/2021 + 24 weeks
3. Develop a nanobiosensor to monitor possible therapeutic targets in altered enzymes;	01/10/2021 + 24 weeks	01/4/2022 – 01/10/2022
4. Validate (through the nanobiosensor) the mechanism in different tumor cell lines;	01/05/2022 + 24 weeks	01/10/2022 – 01/05/2023
5. Study the prognostic factors of obese vs normal-weight patients.	Ethics approval – PhD Conclusion	03/09/2020 – 01/07/2022